The Transmission of Enteric Viruses through the Aquatic Environment in the UK

Volume 1

Submitted by Samantha Treagus to the University of Exeter as a dissertation for the degree of Doctor of Philosophy in Biological Sciences, February 2022.

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College of Life and Environmental Sciences

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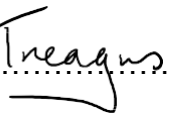
I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

(Signature) 

Abstract

Hepatitis E virus (HEV) and norovirus are known enteric pathogens which can cause a wide range of symptoms. Norovirus is estimated to cause 3 million cases in the UK annually, and whilst HEV cases are reported at much lower levels, it is considered to be an emerging pathogen within more economically developed countries and may be an underestimated health risk. However, the routes of transmission for HEV have not yet been fully elucidated. This PhD endeavours to identify whether the aquatic environment plays a significant role in the transmission of HEV, using norovirus for comparison of prevalence and risk. The studies within this PhD identified HEV and norovirus within sewage and shellfish samples and identified HEV within cetacean liver samples. Sequencing of these samples confirmed norovirus presence within sewage and confirmed HEV presence in sewage and shellfish samples. Additionally, a HEV sequence within a shellfish sample may be classified as a new subtype of genotype 3 and the norovirus genotypes identified within sewage suggest that wastewater monitoring of norovirus may be beneficial for identifying circulating norovirus genotypes. A risk assessment of norovirus and HEV presence in sewage and shellfish samples showed that the risk of norovirus illness from recreational water activities and shellfish consumption may be high, but that risk of HEV illness was very low in comparison. Overall, HEV is present within the aquatic environment in the UK, however the prevalence and levels of HEV in sewage and shellfish suggest that its presence provides little risk to public health. On the other hand, contamination of norovirus within the aquatic environment is a systemic problem in the UK, which is not without public health risk, and must be addressed through limiting release of raw sewage into the environment, standardisation of wastewater treatment practices to make them more effective for removal of viruses.

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Author's Declaration

Chapter 1

The general introduction was written by Samantha Treagus (ST) with editing suggestions from James Lowther, Ben Longdon, William Gaze and Craig Baker-Austin.

Chapter 2

Funding has been provided by Cefas, Seedcorn and the University of Exeter for this work. Ben Longdon is supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (grant no. 109356/Z/15/Z). https://wellcome.ac.uk/funding/sir-henry-dale-fellowships. We would like to thank the anonymous reviewers for their help with submission of this article. ST researched, collated data for, and edited the review article. Conal Wright produced Figure 2.2. Ben Longdon, James Lowther, and Craig Baker-Austin provided editing feedback prior to submission and publication in Food and Environmental Virology.

Chapter 3

The method development chapter was written by ST with editing and structural formatting advice from James Lowther, Ben Longdon, William Gaze and Craig Baker-Austin. Thanks to Christopher Pond for cultivation of mengo virus.

Chapter 4

The studies within this chapter received ethical approval from the university. ST performed all fieldwork, laboratory work and data analysis for the sewage study. Shellfish retailers posted shellfish samples to ST, and these were processed by ST, with help from Charlotte Ford, Tina Stapleton, Basil Fok and Karsan Dhanji. ST performed all subsequent laboratory work and data analysis on the shellfish study. The chapter was written by ST with editing and structural formatting advice from James Lowther, Ben Longdon, William Gaze and Craig Baker-Austin.

Chapter 5

The studies within this chapter received ethical approval from the university. ST performed all fieldwork, laboratory work and data analysis for the sewage study.

The Cetacean Stranding Investigation Program (CSIP) provided cetacean and seal samples which were posted to or collected by ST. ST performed all laboratory work and data analysis for the cetacean and seal study. Training for laboratory work was provided by Tina Stapleton, James Lowther and Frederico Batista. The chapter was written by ST with editing and structural formatting advice from James Lowther, Ben Longdon, William Gaze and Craig Baker-Austin.

Chapter 6

The studies within this chapter received ethical approval from the university. ST and performed all laboratory work, with training from Frederico Batista. David Ryder wrote the Unix code for the sequencing data analysis pipeline, and James Lowther gave advice for data analysis. Further data analysis was performed by ST. The chapter was written by ST with editing and structural formatting advice from Frederico Batista, James Lowther, Ben Longdon, and William Gaze.

Chapter 7

This chapter is purely data-driven and therefore ethical approval was not sought from the university. ST wrote, researched, and edited the chapter, with help editing from James Lowther, Ben Longdon and William Gaze.

Chapter 8

The general discussion was written by ST, with editing suggestions from James Lowther and William Gaze.

List of Abbreviations

APHA – Animal and Plant Health Agency

BLAST – Basic Local Alignment Search Tool

Bp – base pair

cDNA – Complimentary DNA

CEFAS – Centre for Environment Fisheries and Aquaculture Science

CI – 95% Confidence Interval(s)

CL2 – Containment Level 2

CL3 – Containment Level 3

CPU – Central Processing Unit

CSO – Combined Sewer Overflow

CSIP – Cetacean Strandings Investigation Program

C_T – Cycle Threshold

DEFRA – Department for Environment, Food and Rural Affairs

DNA – Deoxyribonucleic acid

E. coli – Escherichia coli

EFSA – European Food Standards Agency ELISA – Enzyme-Linked

Immunosorbent Assay

EMBL-EBI – European Molecular Biology Laboratory - European Bioinformatics

InstituteEU – European Union

FAM – 6-Carboxyfluorescein

gc – genome copies

GIMP - GNU Image Manipulation Program

GI – Genogroup I

GII – Genogroup II

GIII – Genogroup 3

GIV – Genogroup 4

GV – Genogroup 5

GVI – Genogroup 6

GVII – Genogroup 7

GVIII – Genogroup 8

GIX – Genogroup 9

GX – Genogroup 10

G1 – Genotype 1 (Hepatitis E virus)

G2 – Genotype 2 (Hepatitis E virus)

G3 – Genotype 3 (Hepatitis E virus)

G3ra – Genotype 3 HEV, subtype ra (originally identified within rabbits)

G4 – Genotype 4 (Hepatitis E virus)

G5 – Genotype 5 (Hepatitis E virus)

G6 – Genotype 6 (Hepatitis E virus)

G7 – Genotype 7 (Hepatitis E virus)

G8 – Genotype 8 (Hepatitis E virus)

HAV – Hepatitis A virus

HEV – Hepatitis E virus

HG3* – Hazard Group 3*

HS – High Sensitivity

HTST – High Temperature Short Time

IgG – Immunoglobulin G

IgM – Immunoglobulin M

ISO – International Organization for Standardization

MGB – Minor Groove Binder

mRNA – messenger RNA

NCBI – National Centre for Biotechnology Information

NFQ – Non-Fluorescent Quencher

NGS – Next Generation Sequencing

NHS – National Health Service

NoV – Norovirus nPCR – nested PCR

nRT-PCR – nested Reverse Transcription Polymerase Chain Reaction

ONT – Oxford Nanopore Technologies

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PHE – Public Health England

qRT-PCR – Quantitative Reverse Transcription Polymerase Chain Reaction

QMRA – Quantitative Microbial Risk Assessment

RdRp – RNA-dependent RNA polymerase

RIVM – Rijksinstituut voor Volksgezondheid en Milieu (National Institute for Public Health and the Environment the Netherlands)RNA – Ribonucleic acid

rpm – revolutions per minute

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2

SaV – Sapovirus

SDPP – Spray Dried Plasma Powder

SEM – Standard Error of the Mean

SNP – Single Nucleotide Polymorphism

ST – Samantha Treagus

TAMRA – Tetramethylrhodamine

UK – United Kingdom

UKHSA – UK Health Security Agency

USA – United States of America

UV – Ultraviolet light

WGS – Whole Genome Sequencing

WWTP – Wastewater Treatment Plant

Chapter 1

General Introduction

General Overview

Waterborne enteric viruses are a well-established threat to both human and animal health (Fong and Lipp, 2005). Within the many families of human viruses are enteric viruses which cause gastrointestinal illness, amongst other symptoms. Some enteric viruses infect only cells within the gut, leading to symptoms such as vomiting, diarrhoea, and fever. Others are capable of infecting not only the gut, but other organs, such as the liver, causing symptoms such as jaundice and hepatitis, leading to a range of symptoms which are often very diverse even for a single virus and are host dependent. Enteric viruses are generally spread through a faecal-oral route of transmission due to high titre virus shedding within faeces. As such there are many routes for outbreaks, from contaminated water to contaminated foods, or person to person transmission (Tang *et al.*, 1991; Baert *et al.*, 2009). Gastrointestinal pathogens are estimated to have caused 1.8 billion infections and 599,000 associated deaths in 2010, with 677 million of these cases attributed to norovirus alone (Pires *et al.*, 2015). Whilst most cases of gastroenteritis are self-limiting, or even asymptomatic for some viruses (Abutaleb and Kottilil, 2020; Guillois *et al.*, 2015), they can be especially dangerous for people who are immunocompromised or elderly (Chen *et al.*, 2016). Understanding the transmission routes of enteric viruses can be useful to create policies which limit the spread and enable the prevention of viral gastrointestinal illness.

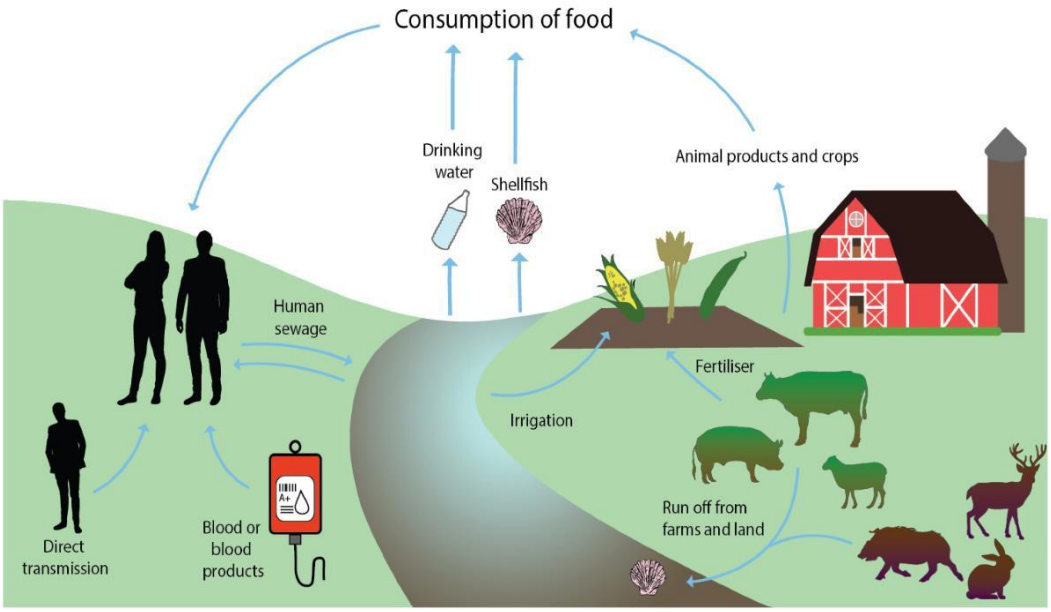


Figure 1.1 Theoretical and confirmed transmission routes of HEV Treagus *et al.*, (2021).

The figure shows confirmed and theoretical routes of HEV transmission to humans. The theoretical routes of transmission include HEV infections contracted from the consumption of shellfish, sheep, and cows, as well as crops, drinking water and recreational water exposure, although no confirmed outbreaks from the latter sources have yet been identified. Illustration created using Adobe Illustrator and edited using GIMP.

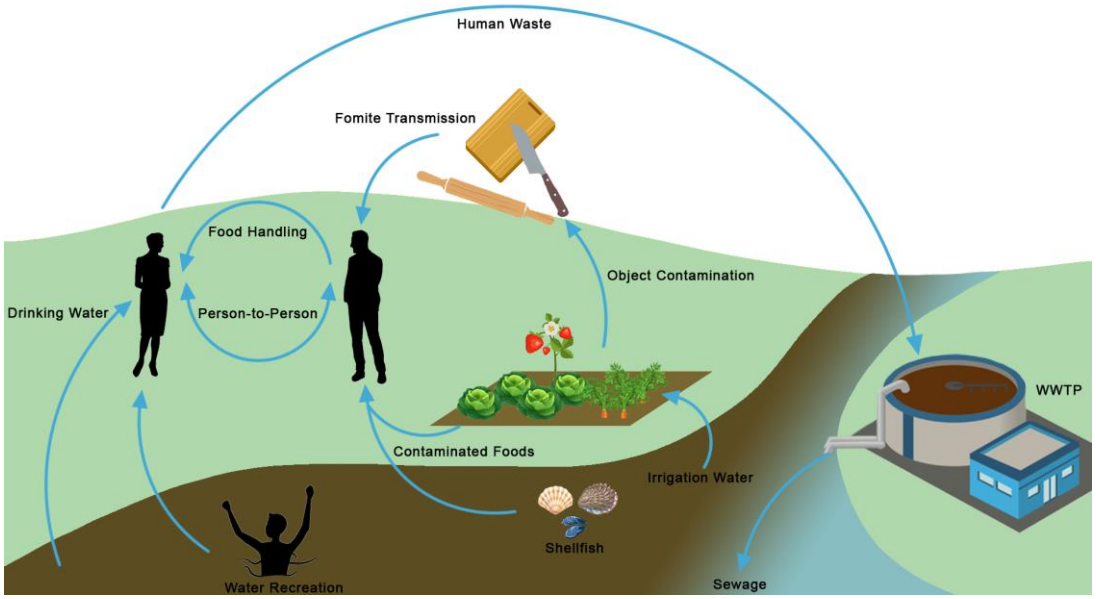


Figure 1.2 Confirmed transmission routes of norovirus

The figure shows the confirmed transmission routes which genogroup I and II norovirus can take to infect humans. Transmission from animal sources has not yet been proven and is not included in the figure, but it is suspected. Illustration created using Adobe Photoshop and edited using GIMP.

Figure 1.1 shows the possible transmission routes for hepatitis E virus (HEV) which causes gastrointestinal and hepatic symptoms and Figure 1.2 shows the known transmission routes for norovirus, which causes enteric symptoms. Enteric virus transmission can follow many pathways (Figures 1.1 and 1.2). One pathway which has received attention in the past two decades, is transmission through the aquatic environment. As most enteric viruses infect cells within the gut, they are commonly shed in the faeces and urine, and sometimes also within vomitus in high concentrations. Sewage is discharged, either as treated effluent or as raw sewage (due to combined sewer overflow (CSO) use), into surface waters such as rivers, seas, and lakes. Wastewater treatment has been shown previously to be effective in reducing numbers of potentially harmful bacteria, with Campos *et al.*, 2016 identifying an average 5.3 $\log_{10}$ reduction of *E. coli* at WWTP A in Southern England . However, it has been shown to be generally less effective for removing viruses, with norovirus RNA still detected in tertiary UV treated effluent (a 2.3 $\log_{10}$ reduction for GI and a 2.6 $\log_{10}$ reduction for GII at WWTP A, see Table 1.1) (Campos *et al.*, 2016). This pattern of lesser reductions for norovirus in comparison to *E. coli* has also been observed by Flannery *et al.*, 2012. However, it is unknown if this remaining norovirus was infectious. In addition to release of treated effluent into water courses, CSOs release raw sewage into surface waters during stormy weather conditions, as rainwater collects within sewer infrastructure. Without CSOs, sewer systems would overflow and flood into homes and businesses. However, in the UK, the Environment Agency released a report stating that across England in 2020, CSOs had been in operation for over 3 million hours collectively, which suggests that contamination of the aquatic environment with raw sewage happens extensively (Environment Agency, 2020a). In addition, farm run-off from farms housing animals or crop farms using manure-based fertilisers can also contaminate the aquatic environment.

There are many viruses which are known to contaminate the aquatic environment, both within the UK and in other countries. Some examples include: norovirus, sapovirus, aichi virus, hepatitis A virus (HAV) and HEV. All of these are non-enveloped single stranded RNA viruses and are able to withstand environmental conditions, such as exposure to UV light and high temperatures, and therefore persist in the environment (aquatic or otherwise)

from days to months (Ngazoa et al., 2008; El-Senousy et al., 2014).

HEV has gained increasing attention in the past decade due to a rapid increase in reported cases globally. HEV causes hepatitis (inflammation of the liver), however this pathogen is just one infectious agent which can cause this condition. Other pathogenic causes include HAV, hepatitis C virus, and *Klebsiella pneumoniae* as examples. HEV typically causes acute hepatitis with an incubation period of 2-6 weeks (Kamar *et al.*, 2014) and is considered to be the most common cause of acute viral hepatitis in the UK (Dalton *et al.*, 2008), as well as worldwide (Wang *et al.*, 2019). There are currently eight classified genotypes (G1-8) of HEV (Smith *et al.*, 2020b). G1, G2 and G4 generally spread via a waterborne route (faecally contaminated water), but G3 and G4 (and potentially G7) can also spread via foodborne routes. **Chapter 2** provides a review of the foodborne transmission of HEV to humans. Only five genotypes have so far been found to cause disease in humans (G1-4 and G7), with G1-4 causing the most diagnosed human cases worldwide. HEV G1 and G2 alone were estimated to cause 2 million cases worldwide in 2005 (World Health Organization, 2019). However, HEV cases are likely to be underestimated due to evidence of asymptomatic cases (Guillois *et al.*, 2015; Yin *et al.*, 2019). HEV causes a mortality rate of approximately 0.5% - 1% within infected people (Peron *et al.*, 2007), but HEV G1 and G2 cause one in five mortalities in infected pregnant women (Kumar *et al.*, 2017; Jin *et al.*, 2016; Kamar *et al.*, 2014), underlining its clinical relevance. It is currently unknown if G3 and G4 HEV also cause this increased mortality rate during pregnancy. Deaths from HEV normally result from the development of fulminant hepatitis (acute liver failure) or from extrahepatic complications of a nephrological, haematological or neurological nature (Santos *et al.*, 2013; Novel *et al.*, 2017; Mallet *et al.*, 2017). HEV is more dangerous in immunocompromised patients, causing chronic hepatitis with potentially fatal outcomes (Nijskens *et al.*, 2016).

In England and Wales, HEV cases have increased since monitoring began in 2003 (Figure 1.3) (Public Health England, 2019), and the majority of these have been identified as autochthonous (originating in the UK) (Ijaz *et al.*, 2013). This indicates that HEV is endemic in the UK, providing an emerging health threat with cases increasing yearly.

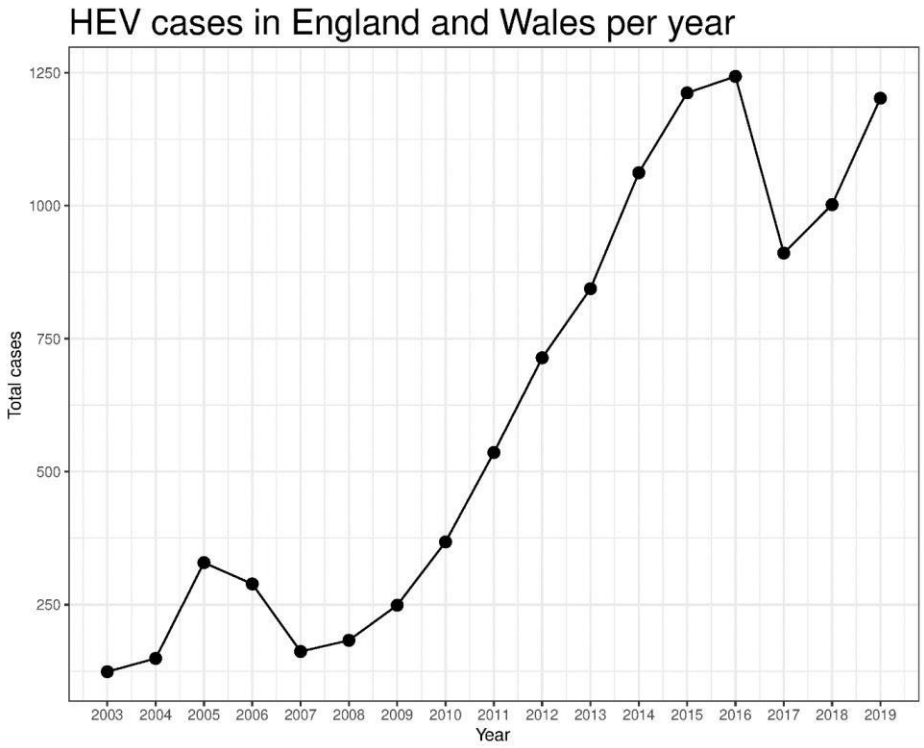


Figure 1.3 Cases of Hepatitis E from 2003 to 2019 (data from The UK Health Security Agency (Public Health England (2019)

Cases of human Hepatitis E virus between 2003 and 2019 have increased steadily; with the only declines seen in in the early 2000s and again in 2017. This could be a cyclical pattern but the dip in 2017 is more likely to be representative of the introduction of blood screening for HEV at the end of 2016, thereby eliminating the possibility of HEV transmission by blood transfusion.

Another enteric virus, HAV, causes gastrointestinal and hepatic illness, and has six genotypes. Genotypes I – III infect humans, and genotypes IV – VI infect non-human primates (Wachtman and Mansfield, 2012). HAV is spread via faecal-oral routes, like HEV, however, unlike HEV, it is not zoonotic. HAV has also been found to spread via foodborne and waterborne routes, through faecal contamination (Tallon *et al.*, 2008; Tang *et al.*, 1991). HAV is considered to be endemic worldwide, though some countries appear to have a lower endemicity than others, with some countries in South America, Africa and Asia generally showing higher levels of endemicity (Kroneman *et al.*, 2018). The main symptoms are nausea and sickness, jaundice, and fever, similar to HEV. It is a self-limiting disease with a case-fatality rate of around 0.1-0.5% (Chen *et al.*, 2016; Jung *et al.*, 2010), and higher age and underlying health disease are known to be risk factors of more severe disease (Chen *et al.*, 2016). HAV was once considered the most common form of acute viral hepatitis, although HEV

is now considered to be more prevalent (Wang *et al.*, 2019) perhaps due to the introduction of HAV vaccines. Regardless, there are still an approximate 1.4 million infections worldwide, and there were 503 cases in 2019 in England and Wales (Lapp and Rochling, 2013; Public Health England, 2021b); however cases may be underestimated due to asymptomatic infections which occur most commonly within children, and go unreported (Abutaleb and Kottilil, 2020).

Sapovirus is an enteric virus within the family Caliciviridae which infects cells within the gastrointestinal tract, causing symptoms such as nausea, vomiting and diarrhoea. The number of sapovirus cases worldwide yearly is unknown, as it is often not classed as a notifiable disease, which is true of the UK. There are 19 genogroups of sapoviruses currently classified, with genogroups GI, GII, GIV and GV capable of human infection (Oka *et al.*, 2015), and the other genogroups infecting other animals, such as pigs (Sunaga *et al.*, 2019). There has been no evidence of zoonotic or foodborne transmission of sapoviruses, and they are currently considered to spread through waterborne transmission (faecally contaminated water) (Kauppinen *et al.*, 2019). The geographical distribution of sapovirus is unknown due to lack of data, however outbreaks of Sapovirus have occurred previously in countries such as Finland (Kauppinen *et al.*, 2017), China (Li *et al.*, 2020a), Japan (Iizuka *et al.*, 2010), and the USA (Lee *et al.*, 2012); which suggests a worldwide distribution. Sapovirus has been reported to infect children and the elderly more commonly, though infection in other age groups is possible (Svraka *et al.*, 2010; Lee *et al.*, 2012).

Globally, norovirus, also part of the Caliciviridae family, is the single most significant cause of gastroenteritis, and was estimated to have caused 213,515 deaths globally in 2010 alone (Pires *et al.*, 2015). In the UK, from June 2018 to June 2019, 6172 norovirus cases were confirmed just in England, with 252 hospital ward closures as a result of norovirus outbreaks (Public Health England, 2021a). However, due to under-reporting of cases and possible asymptomatic infection, it is probable that the actual number of norovirus infections is higher. Indeed, the European Food Standards Agency (EFSA) estimates that there are 3 million cases of norovirus in the UK annually (Gherman *et al.*); and Tam *et al.*, (2012) estimated that only one in 288 norovirus cases is reported to the UK national surveillance system. Norovirus

has ten classified genogroups (GI-GX), containing 48 genotypes; the majority of which are within genogroups I and II (Chhabra *et al.*, 2019). GI, GII and GIV infect humans, whilst the other genogroups infect animals, and the majority of norovirus outbreaks are caused by GI and GII (Bruggink et al., 2017; Gao *et al.*, 2019). The virus causes nausea, vomiting, diarrhoea and fever within infected symptomatic individuals, however it can also be an asymptomatic infection (Qi *et al.*, 2018). Norovirus is not reported to be a zoonotic virus, and there are currently no known animal reservoirs as a source of infection. Generally, norovirus causes outbreaks through faecally contaminated water, person-to-person transmission, and food contamination as it is highly infectious. Large foodborne outbreaks have frequently occurred where infected food handlers have contaminated the foods of patrons. In addition, contaminated water used to wash fruits can also lead to outbreaks. Another source of outbreaks is the consumption of raw or minimally cooked shellfish which have been contaminated with faecal pollution from surface waters.

Enteric Viruses within Wastewater

As stated previously, enteric viruses are normally shed in the faeces and urine of infected individuals. Wastewater can act as a good surveillance target for quantifying viral disease burden within a population, as it will include viruses shed from both symptomatic and asymptomatic individuals. Indeed, surveillance of wastewater for the presence of SARS-CoV-2 has been used during the COVID-19 pandemic, and this has also been used to identify variants within the population, and document increases in population infections as new cases arise, or before new cases become symptomatic (Medema *et al.*, 2020b). Wastewater surveillance can also give an idea of the potential pollutants and pathogens entering water courses, either through release of raw sewage from CSOs, or after treatment.

Norovirus has been detected in wastewater in many countries, for example in Spain, GI noroviruses were detected in 32/46 (70%) of influent samples, and GII noroviruses were found in 35/46 (76%) (Santiso-Bellón *et al.*, 2020). In Japan, norovirus GI and GII were detected year round, in 12/12 (100%) of samples (Thongprachum *et al.*, 2018). In the UK, norovirus has been detected in wastewater at various stages of treatment, from influent through to tertiary

UV treated effluent. Four WWTPs with different treatment practices were investigated for their ability to remove E. coli and norovirus by Campos *et al.*, 2016, the results of which are summarised in Table 1.1. The lack of standard treatment practices between WWTPs leads to very variable results. The frequent occurrence of norovirus in raw and treated wastewater leads to contamination of the aquatic environment, and this surface water contamination has been detected in many countries, including Italy, France, Japan and the UK (La Rosa *et al.*, 2017b; Sedji *et al.*, 2018; Haramoto *et al.*, 2005; Wyn-Jones *et al.*, 2011).

Table 1.1 Summary of Campos et al., 2016 showing the total log reductions of *E. coli* and norovirus after different wastewater treatment practices

Treatment level	**WWTP A**	**WWTP B**	**WWTP C**	**WWTP D**
Preliminary treatment	Screens and grit removal	Screens and grit removal	Screens and grit removal	Screens and grit removal
Primary treatment	Primary settlement	Primary settlement	Primary settlement	Primary settlement
Secondary (biological) treatment	Optimised activated sludge (modified Ludzack-Ettinger)	Trickling filters; humus tanks	Trickling filters; humus tanks	Trickling filters (biotower); biological-aerated filters; humus tanks
Tertiary treatment	UV disinfection	UV disinfection	None	None
Log Norovirus removal	GI 2.31 $\log_{10}$; GII 2.6 $\log_{10}$	GI ND*; GII 2.08 $\log_{10}$	GI 1.8 $\log_{10}$; GII 2.4 $\log_{10}$	GI 1.71 $\log_{10}$; GII 1.93 $\log_{10}$
***Log E. coli* removal**	5.3 $\log_{10}$	4.7 $\log_{10}$	2.1 $\log_{10}$	2.4 $\log_{10}$

* ND = Not Detected

In addition, HEV has been detected in wastewater and surface waters in many countries. In Germany, HEV was detected in 114/134 (85%) of influent samples and 30/88 (34%) of effluent samples from four wastewater treatment works (Beyer *et al.*, 2020). In 2020, a systematic study was published which identified HEV RNA in 74/1374 (5.4%) influent samples from wastewater treatment plants across Italy (Iaconelli *et al.*, 2020). In Scotland, HEV was found in 14/15 (93%) of influent samples in Edinburgh (Smith et al., 2016). In Portugal, HEV was detected in 21/27 (78%) surface water samples (from a river and a dam) and in 24/36 (67%) of drinking water samples (Salvador *et*

al., 2020). This study also identified viable HEV from surface water and drinking water samples, using cell culture methods (Salvador *et al.*, 2020). HEV was also detected in 2/12 (17%) of river samples from the Netherlands (Rutjes *et al.*, 2009). These studies, plus several others, indicate a problem with wastewater treatment and surface water contamination. However, no UK-wide systematic study has been performed to assess HEV presence in wastewater or surface waters. **Chapter 4** describes novel research into the presence of HEV and norovirus within influent and effluent from Southern England.

Viruses in the Aquatic Environment

Contamination of the aquatic environment with raw sewage, treated effluent and animal waste can lead to contamination of bivalve shellfish that bioaccumulate chemicals and pathogens during filter feeding. This leads to an inherent human health risk, as most bivalve shellfish are consumed raw or minimally cooked.

Bioaccumulation of pathogenic viruses within shellfish is perhaps best demonstrated by the widespread discoveries of norovirus presence in shellfish. In Italy, norovirus was detected in 36/253 (14%) of shellfish samples from harvesting areas (La Bella *et al.*, 2017), whilst in France 35/387 (9.0%) samples of ready to eat shellfish were determined to be positive for norovirus (Schaeffer *et al.*, 2013). In contrast, a study in the UK observed that 643/844 (76%) of shellfish samples from harvesting areas were positive for norovirus (Lowther *et al.*, 2012); and a later study then identified 433/630 (68%) of ready to eat shellfish samples to be positive for norovirus RNA (Lowther *et al.*, 2018). The difference in the levels of norovirus between France and Italy and the UK is quite large and indicates a systemic problem in the treatment and release of sewage into surface waters in the UK. In addition to the prevalence of norovirus in shellfish worldwide, outbreaks have also been conclusively linked to shellfish. Bivalves were identified as the source of seven outbreaks between 2000 and 2007 (Baert *et al.*, 2009). One study in France identified shellfish to be directly implicated in several norovirus outbreaks (Polo *et al.*, 2016). Another study in Canada identified two outbreaks to be linked to shellfish produce, leading to the closure of 12 oyster farms (Meghnath *et al.*, 2019). Other outbreaks from shellfish have also been reported in Italy, the UK,

and the USA (Prato *et al.*, 2004; Smith *et al.*, 2012; Alfano-Sobsey *et al.*, 2012), amongst other countries. Consumption of shellfish is estimated to cause 11,800 norovirus cases a year in the UK (Hassard *et al.*, 2017), however, from June 2017 to June 2018, norovirus cases reached 6719 in England and Wales (Public Health England, 2018), indicating that many norovirus cases go unreported. As such, the number of observed and reported cases captured in national surveillance programmes for foodborne pathogens is often called the "tip of the iceberg" because the vast majority of infections are missed, through a variety of factors (Figure 1.4).

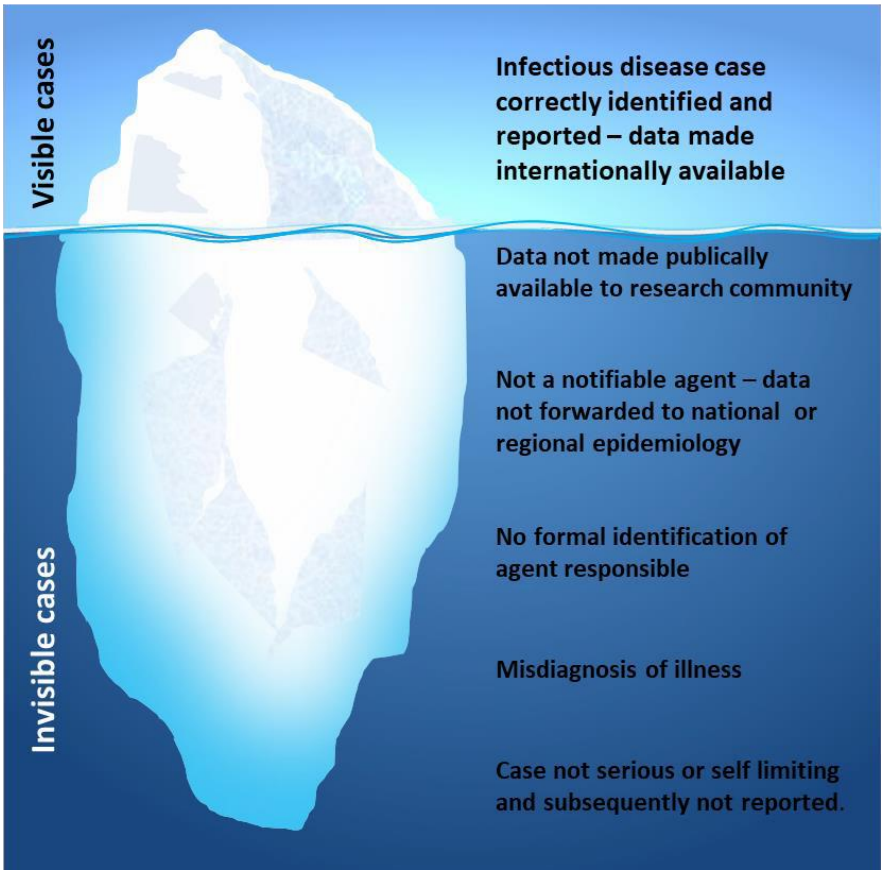


Figure 1.4 Factors which can affect the reporting of gastroenteritis cases (provided by Craig Baker-Austin)

Gastroenteritis cases are vastly under reported and this figure shows some of the factors through which they can be missed by health surveillance systems. A small amount of cases in the UK are correctly identified and reported, with the data made available, whilst a large proportion of cases may go unreported for a number of reasons, including the illness being short or self limiting, because it's misdiagnosed as another illness, because testing of samples to identify the pathogen is not possible or too late, or even because the pathogen is not a notifiable agent which requires reporting to public health agencies. This leads to under-reporting of case numbers for gastrointestinal pathogens, such as norovirus.

However, though norovirus is the most widely reported human pathogenic virus

in shellfish, there are others which have also been detected in shellfish, and some which have also caused outbreaks from shellfish consumption. HEV has been detected in shellfish in many countries. The countries which have detected HEV are summarised in Table 1.2.

Table 1.2 HEV in shellfish in different countries

Location	Study	Percentage of shellfish HEV-positive
China	Gao *et al.*, (2015)	18% (22/126) of shellfish samples* of various species from production areas
Italy	La Rosa *et al.*, (2018)	2.6% (10/384) of shellfish samples* of various species from production areas
Japan	Li et al., (2007)	6.3% (2/32) of Yamato-Shijimi clam samples*
Scotland	Crossan *et al.*, (2012)	85% (41/48) of individual wild mussels
	O'Hara *et al.*, (2018)	2.9% (9/310) of retail shellfish samples* (mussels and oysters)
Spain	Mesquita *et al.*, (2016)	15% (12/81) of mussel samples* from a production area
	Rivadulla *et al.*, (2019)	24% (41/164) of mussel, clam, and cockle samples*

*It was either stated or assumed that each "sample" was formed by ten or more shellfish individuals and is therefore technically a pooled sample.

Though there have been no conclusive outbreaks of HEV from shellfish consumption, a retrospective study of a HEV outbreak on a cruise ship (a world cruise returning to the UK) identified that the most likely source was shellfish consumption (Said *et al.*, 2009). However, the UK has not investigated HEV presence in shellfish in a systematic UK-wide study.

HAV is another virus which has been associated with foodborne outbreaks, not only from shellfish but also other foods. Between 2012 and 2018, 12 foodborne outbreaks of HAV, causing 2,114 cases, occurred worldwide, and these outbreaks were sourced from frozen foods such as berries (Nasheri et al., 2019). Shellfish (specifically clams) were also involved in a large outbreak of HAV in China, which affected around 290,000 people (Tang *et al.*, 1991), and

have been reported to have caused other HAV outbreaks subsequently (Sánchez *et al.*, 2002). HAV has been detected in shellfish in several countries. In France, 10/73 (14%) French mussel samples were identified to contain HAV RNA (Le Guyader *et al.*, 2000). In Italy, two recent studies have identified HAV in bivalve shellfish samples, with Fusco *et al.*, (2019) identifying 26/289 (8.9%) contained HAV RNA, and La Rosa *et al.*, (2021) detecting HAV in 102/746 (14%). In Spain, 29/68 (43%) cultured mussel samples and 40/92 (44%) wild shellfish samples (various species) were identified to be HAV positive (Manso *et al.*, 2010). HAV has also been detected in shellfish in other countries.

Sapovirus has also been detected in shellfish in multiple countries and has caused several outbreaks associated with shellfish consumption in Japan. One outbreak of gastroenteritis in Japan was linked to noroviruses and sapoviruses within clams, where a sequence similarity of 99 – 100% was observed between sapoviruses isolated from clam packaging liquid and stool samples of people who had consumed the clams (Iizuka *et al.*, 2010). Sapovirus RNA has been detected in shellfish in Italy, where 54/289 (19%) shellfish samples were sapovirus positive (Fusco *et al.*, 2019). It has also been detected in the packaging of Japanese clams, specifically in 4/57 (7.0%) packages (Hansman *et al.*, 2007). Spanish shellfish have also been shown to be contaminated, with detection in 30/80 (38%) mussel samples from Galicia (Varela et al., 2016a); as well as 30/168 (18%) of a range of shellfish species from Galicia (Varela et al., 2016b). Sapovirus RNA has also been detected within surface waters in Spain (Sano *et al.*, 2011). The presence of sapovirus in shellfish and surface waters indicates a possible emerging or underestimated threat.

Whilst it is important to investigate the presence of enteric viruses in the aquatic environment for the sake of human health, little consideration is made for how human faecal pollution may affect aquatic life. In Cuba, a study of captive dolphins (*Tursiops truncatus*) identified clinical HEV infections, and these were sequenced as G3 HEV, being most similar to sequences from humans in Germany and the Netherlands. The results were identified through qRT-PCR, serology and sequencing techniques (Villalba *et al.*, 2017). It was theorised that perhaps the dolphins had contracted HEV infections from their food sources, and this study opens up the possibility that human viruses, shed

faecally, could potentially have harmful effects for aquatic life.

Despite the global emergence of these viruses and the ability for them to potentially cause foodborne human outbreaks and illness in marine mammals, investigations into their presence in UK shellfish have been limited to studies focussing solely on norovirus, and no UK-wide systematic studies have been performed to assess HEV, HAV or Sapovirus presence in shellfish. In addition, no studies have investigated whether wild marine mammals may be infected by HEV. **Chapter 5** investigates the presence of norovirus, sapovirus, HAV and HEV in shellfish from England and Scotland to provide an assessment of the presence of these enteric viruses in shellfish. **Chapter 5** also investigates the presence of HEV in seal and cetacean liver samples donated by the Cetacean Strandings Investigation Program, which collects and performs post-mortem examinations on beached cetaceans and seals to identify the causes of their deaths and the causes of mass stranding events.

Sources of Viral Contamination

There are many sources of faecal contamination of the aquatic environment. Norovirus, Sapovirus and HAV, which can cause outbreaks from consumption of shellfish, originate purely from human faecal pollution as the genotypes or genogroups which infect humans are not zoonotic. However, assessing where in the world strains of these viruses have originated from can enable the tracking of pandemic strains and new variants, and this technology has been used most recently in tracking new variants of SARS-CoV-2 (Tyson *et al.*, 2020). However, HEV is an established zoonotic pathogen. The most common genotype of HEV in the UK is G3 (Ijaz *et al.*, 2013), and the main host reservoir for this genotype is swine. HEV G3 is thought to spread mostly through a foodborne route of transmission, by consuming raw or undercooked pork products. Indeed, outbreaks of HEV have been directly linked to consumption of pork products (Rivero-Juarez *et al.*, 2017; Guillois *et al.*, 2015). One study in the UK assessed the seroprevalence of HEV antibodies in swine at slaughter, and found that 584/629 (93%) of blood samples were seroprevalent (Grierson *et al.*, 2015). They also found that 129 animals (21%) had current HEV infection at slaughter, defined by detection of HEV RNA in either blood or faecal samples. However, other animals are also capable of carrying the virus,

including deer, goats, cattle and rabbits (Kenney, 2019), and these animals may also act as foodborne transmission routes for the virus.

With such a significant number of enteric virus cases and deaths annually, research must be done to better understand the pathogens which cause these illnesses. The aquatic environment has been identified as an important reservoir for enteric viruses, and as an indirect transmission route for some viruses to humans. Subsequently, research is needed to identify the sources of viral contamination, to better prevent human illness. Critically, the sources of contamination of the aquatic environment are numerous and can vary in both space and time. Human faecal pollution containing viruses can contaminate the aquatic environment when released as treated effluent, or when released as raw sewage during storm overflow events. In addition, where some viruses (such as HEV) are zoonotic, animal farm run off containing animal waste could also be a source of viral contamination. Wild animals which defecate near or in water sources could also contaminate surface waters, as could manure based fertiliser run-off from farms used for crop production. To identify the sources of contamination, sequencing can be utilised to compare sample sequences from various environmental and clinical sources. Traditionally, sequencing of virus genomes from food and environmental samples has been difficult due to factors such as low viral abundance of target sequences and RNA degradation. Whole genome sequencing (WGS) is especially difficult from such samples. However, in recent years, the development of whole genome amplicon sequencing (also known as tiling) has become a technique used not only for low levels of Zika virus in clinical samples such as plasma and urine (Quick *et al.*, 2017), but also has become widely used during the COVID-19 pandemic for WGS of SARS-CoV-2 by the Artic Network, which is a part of the COVID-19 Genomics UK Consortium (COG-UK) (Tyson *et al.*, 2020).

Where whole genome data is not expected to be possible to obtain, next generation amplicon sequencing is becoming recognised as a useful tool. Next generation sequencing (NGS) can not only be more economical than traditional Sanger sequencing, but it can also be useful for obtaining more sequencing data. NGS can be used to sequence whole genomes within relatively short periods of time, or for sequencing of amplicons from many samples at once.

Two of the most used NGS sequencing technologies in the UK are Oxford Nanopore Technologies (ONT) and Illumina. Whilst the Illumina platform boasts high accuracy, it is only capable of short read lengths, and so genomes have to be fragmented prior to sequencing, and it is also expensive (Petersen *et al.*, 2019). In comparison, the ONT platform provides sequencing data with lower accuracy, but has the capability of sequencing a whole genome from a single DNA molecule (Batista *et al.*, 2020), and the potential for surveillance in resource-limited settings due to its portability (Petersen *et al.*, 2019). It can also be established rapidly to monitor outbreaks, and this has been the case for sequencing of SARS-CoV-2 during the COVID-19 pandemic, being used frequently by COG-UK (Tyson *et al.*, 2020). It may also be the better platform for picking up sequences of lower abundance within samples (Petersen *et al.*, 2019).

NGS amplicon sequencing has been used for sequencing of Ilarviruses from trees in Australia (Kinoti *et al.*, 2017), and on the Illumina platform for the sequencing of HEV from wastewater (Iaconelli *et al.*, 2020). However, to our knowledge, ONT amplicon sequencing of HEV and norovirus has not been attempted on wastewater and shellfish samples previously, and this may be beneficial for resource limited settings, as well as in outbreak situations. This technique could allow an assessment of the main sources of faecal pollution of the aquatic environment (source apportionment) which may be critical to enable policies to be introduced to prevent this contamination. It may also provide a more robust framework on which to assess whether the aquatic environment is directly involved in the transmission of HEV back to humans, perhaps through consumption of shellfish or through recreational water activities. Such approaches can also provide knowledge on the origins of HEV RNA within marine mammals. Therefore, **Chapter 6** used NGS in order to trace the sources of HEV and norovirus contamination, using a metabarcoding amplicon sequencing approach on the ONT MinION platform.

Assessment of the Risks to Human Health by Viruses in the Aquatic Environment

Many studies worldwide have investigated the presence of enteric viruses within sewage and surface waters. For example, Beyer *et al.*, (2020) identified HEV in 114/134 (85%) sewage samples, and in 48/161 (30%) of river water samples from Germany. In the Netherlands, 10/10 (100%) treated and untreated sewage samples and 8/8 (100%) river water samples contained enterovirus, norovirus, reovirus and rotavirus RNA (Lodder and de Roda Husman, 2005). Some studies have also attempted to provide an estimate of the risk to human health by contamination of the aquatic environment with faecally derived pathogens. Prüss, (1998) reviewed 22 studies to identify that the relative risk between swimming in polluted vs clean water was often significant. In addition, Leonard *et al.*, (2018) found that there is a higher chance of contracting gastrointestinal illnesses in persons who undertake recreational water activities (odds ratio 1.29). However, Arnold *et al.*, (2013) identified that there was no association between bacterial indicators of water quality and increased risk of illness, and with the identification that bacterial faecal indicators are not always adequate indicators of virus presence in sewage (Kitajima *et al.*, 2014), water (Gibson *et al.*, 2011), and bivalve shellfish (Sharp *et al.*, 2021), this suggests that microbial risk assessments which focus on bacteria only may be underestimating the risk which viruses may pose to human health from recreational water activities, especially as many of the symptoms of gastrointestinal illness reported suggest viruses as possible causes. Risk assessments which have focussed on viruses within recreational waters have often identified an increased risk to human health from recreational water activities, with Vergara *et al.*, (2016) identifying the probable risk of illness from norovirus and Bortagaray *et al.*, (2020) identifying the daily risk of infection from rotaviruses in recreational waters.

In the UK, Leonard *et al.*, (2015) conducted a review in order to calculate the volumes of water ingested during water related activities, and by extension, calculate the amount of antibiotic resistant *Escherichia coli* ingested. No studies, as far as we are aware, have yet investigated the risk of illness from aquatic environment contamination of HEV, using quantitative data. Using risk assessments which have investigated the infectious dose response of

norovirus in human challenge and outbreak settings (Teunis *et al.*, 2008b; Thebault *et al.*, 2013), we aimed to assess the risks of aquatic environment contamination from norovirus and HEV. **Chapter 7** uses data derived from previous chapters, and formulae from published papers to calculate the likely levels of virus particles ingested during recreational water activities, and the likelihood of contracting illness from these activities, and the consumption of shellfish.

This PhD has examined whether the enteric viruses mentioned are present in the aquatic environment in the UK, and if so, looked to identify their sources and whether they may have wider impacts on marine mammals. It also aimed to assess the risk that contamination of the aquatic environment may have for causing human illness.

References

Abutaleb, A. and Kottilil, S. (2020) 'Hepatitis A: Epidemiology, natural history, unusual clinical manifestations, and prevention', *Gastroenterology Clinics,* 49(2), pp. 191-199.

Alfano-Sobsey, E., Sweat, D., Hall, A., Breedlove, F., Rodriguez, R., Greene, S., Pierce, A., Sobsey, M., Davies, M. and Ledford, S. L. (2012) 'Norovirus outbreak associated with undercooked oysters and secondary household transmission', *Epidemiol Infect,* 140(2), pp. 276-82.

Arnold, B. F., Schiff, K. C., Griffith, J. F., Gruber, J. S., Yau, V., Wright, C. C., Wade, T. J., Burns, S., Hayes, J. M., McGee, C., Gold, M., Cao, Y., Weisberg, S. B. and Colford, J. M. J. (2013) 'Swimmer Illness Associated with Marine Water Exposure and Water Quality Indicators: Impact of Widely Used Assumptions', *Epidemiology,* 24(6), pp. 845-853.

Baert, L., Uyttendaele, M., Stals, A., Van Coillie, E., Dierick, K., Debevere, J. and Botteldoorn, N. (2009) 'Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context', *Epidemiology & Infection,* 137(3), pp. 316-325.

Batista, F. M., Stapleton, T., Lowther, J. A., Fonseca, V. G., Shaw, R., Pond, C., Walker, D. I., van Aerle, R. and Martinez-Urtaza, J. (2020) 'Whole Genome Sequencing of Hepatitis A Virus Using a PCR-Free Single-Molecule Nanopore Sequencing Approach', *Front Microbiol,* 11, pp. 874.

Beyer, S., Szewzyk, R., Gnirss, R., Johne, R. and Selinka, H.-C. (2020) 'Detection and characterization of hepatitis E Virus genotype 3 in wastewater and urban surface waters in Germany', *Food and environmental virology*, pp. 1-11.

Bortagaray, V., Girardi, V., Pou, S., Lizasoain, A., Tort, L. F. L., Spilki, F. R., Colina, R. and Victoria, M. (2020) 'Detection, Quantification, and Microbial Risk Assessment of Group A Rotavirus in Rivers from Uruguay', *Food Environ Virol,* 12(2), pp. 89-98.

Bruggink, L. D., Moselen, J. M. and Marshall, J. A. (2017) 'The molecular epidemiology of norovirus outbreaks in Victoria, 2014 to 2015', *Communicable diseases intelligence quarterly report,* 41(1), pp. E21-E32.

Campos, C. J., Avant, J., Lowther, J., Till, D. and Lees, D. N. (2016) 'Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes', *Water research,* 103, pp. 224-232.

Chen, C. M., Chen, S. C., Yang, H. Y., Yang, S. T. and Wang, C. M. (2016) 'Hospitalization and mortality due to hepatitis A in Taiwan: a 15-year nationwide cohort study', *Journal of viral hepatitis,* 23(11), pp. 940-945.

Chhabra, P., de Graaf, M., Parra, G. I., Chan, M. C.-W., Green, K., Martella, V., Wang, Q., White, P. A., Katayama, K. and Vennema, H. (2019) 'Updated classification of norovirus genogroups and genotypes', *The Journal of general*

virology, 100(10), pp. 1393.

Crossan, C., Baker, P. J., Craft, J., Takeuchi, Y., Dalton, H. R. and Scobie, L. (2012) 'Hepatitis E virus genotype 3 in shellfish, United Kingdom', *Emerging infectious diseases,* 18(12), pp. 2085.

Dalton, H., Stableforth, W., Hazeldine, S., Thurairajah, P., Ramnarace, R., Warshow, U., Ijaz, S., Ellis, V. and Bendall, R. (2008) 'Autochthonous hepatitis E in Southwest England: a comparison with hepatitis A', *European Journal of Clinical Microbiology & Infectious Diseases,* 27(7), pp. 579-585.

El-Senousy, W. M., Osman, G. A. and Melegy, A. A. (2014) 'Survival of adenovirus, rotavirus, hepatitis A virus, pathogenic bacteria and bacterial indicators in ground water', *World Applied Sciences Journal,* 29(3), pp. 337-348.

Environment Agency. (2020) 'Event Duration Monitoring - Storm Overflows - 2020' [Database of CSO use throughout UK in 2020]. Available at: https://environment.data.gov.uk/dataset/21e15f12-0df8-4bfc-b763-45226c16a8ac (Accessed.

Flannery J, Keaveney S, Rajko-Nenow P, O'Flaherty V, Doré W. Concentration of norovirus during wastewater treatment and its impact on oyster contamination. Appl Environ Microbiol. 2012 May;78(9):3400-6. doi: 10.1128/AEM.07569-11. Epub 2012 Feb 24. PMID: 22367079; PMCID: PMC3346491.

Fong, T.-T. and Lipp, E. K. (2005) 'Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools', *Microbiology and molecular biology reviews : MMBR,* 69(2), pp. 357-371.

Fusco, G., Anastasio, A., Kingsley, D. H., Amoroso, M. G., Pepe, T., Fratamico, P. M., Cioffi, B., Rossi, R., La Rosa, G. and Boccia, F. (2019) 'Detection of hepatitis A virus and other enteric viruses in shellfish collected in the Gulf of Naples, Italy', *International journal of environmental research and public health,* 16(14), pp. 2588.

Gao, S., Li, D., Zha, E., Zhou, T., Wang, S. and Yue, X. (2015) 'Surveillance of hepatitis E virus contamination in shellfish in China', *International journal of environmental research and public health,* 12(2), pp. 2026-2036.

Gao, Z., Liu, B., Yan, H., Li, W., Jia, L., Tian, Y., Chen, Y., Wang, Q. and Pang, X. (2019) 'Norovirus outbreaks in Beijing, China, from 2014 to 2017', *Journal of Infection,* 79(2), pp. 159-166.

Gherman, I., Holland, D., Clifford, R., Mahmoudzadeh, N., Uy, F., Adkin, A., Kainth, B., Cook, P., Day, A. and Wilson, A. 'Technical Report: Review of Quantitative Risk Assessment of foodborne norovirus transmission', *Science,* 1, pp. 1.

Gibson, K. E., Opryszko, M. C., Schissler, J. T., Guo, Y. and Schwab, K. J. (2011) 'Evaluation of human enteric viruses in surface water and drinking water

resources in southern Ghana', *The American journal of tropical medicine and hygiene,* 84(1), pp. 20-29.

Grierson, S., Heaney, J., Cheney, T., Morgan, D., Wyllie, S., Powell, L., Smith, D., Ijaz, S., Steinbach, F. and Choudhury, B. (2015) 'Prevalence of hepatitis E virus infection in pigs at the time of slaughter, United Kingdom, 2013', *Emerging infectious diseases,* 21(8), pp. 1396.

Guillois, Y., Abravanel, F., Miura, T., Pavio, N., Vaillant, V., Lhomme, S., Le Guyader, F. S., Rose, N., Le Saux, J.-C. and King, L. A. (2015) 'High proportion of asymptomatic infections in an outbreak of hepatitis E associated with a spit-roasted piglet, France, 2013', *Clinical Infectious Diseases,* 62(3), pp. 351-357.

Hansman, G. S., Oka, T., Okamoto, R., Nishida, T., Toda, S., Noda, M., Sano, D., Ueki, Y., Imai, T. and Omura, T. (2007) 'Human sapovirus in clams, Japan', *Emerging infectious diseases,* 13(4), pp. 620.

Haramoto, E., Katayama, H., Oguma, K. and Ohgaki, S. (2005) 'Application of cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan', *Applied and Environmental Microbiology,* 71(5), pp. 2403-2411.

Hassard, F., Sharp, J. H., Taft, H., LeVay, L., Harris, J. P., McDonald, J. E., Tuson, K., Wilson, J., Jones, D. L. and Malham, S. K. (2017) 'Critical review on the public health impact of norovirus contamination in shellfish and the environment: a UK perspective', *Food and environmental virology,* 9(2), pp. 123-141.

Iaconelli, M., Bonanno Ferraro, G., Mancini, P., Suffredini, E., Veneri, C., Ciccaglione, A. R., Bruni, R., Della Libera, S., Bignami, F. and Brambilla, M. (2020) 'Nine-year nationwide environmental surveillance of hepatitis E virus in urban wastewaters in Italy (2011–2019)', *International journal of environmental research and public health,* 17(6), pp. 2059.

Iizuka, S., Oka, T., Tabara, K., Omura, T., Katayama, K., Takeda, N. and Noda, M. (2010) 'Detection of sapoviruses and noroviruses in an outbreak of gastroenteritis linked genetically to shellfish', *Journal of medical virology,* 82(7), pp. 1247-1254.

Ijaz, S., Said, B., Boxall, E., Smit, E., Morgan, D. and Tedder, R. S. (2013) 'Indigenous hepatitis E in England and Wales from 2003 to 2012: evidence of an emerging novel phylotype of viruses', *The Journal of infectious diseases,* 209(8), pp. 1212-1218.

Jin, H., Zhao, Y., Zhang, X., Wang, B. and Liu, P. (2016) 'Case-fatality risk of pregnant women with acute viral hepatitis type E: a systematic review and meta-analysis', *Epidemiology & Infection,* 144(10), pp. 2098-2106.

Jung, Y. M., Park, S. J., Kim, J. S., Jang, J. H., Lee, S. H., Kim, J. W., Park, Y. M., Hwang, S. G., Rim, K. S. and Kang, S. K. (2010) 'Atypical manifestations of hepatitis A infection: a prospective, multicenter study in Korea', *Journal of medical virology,* 82(8), pp. 1318-1326.

Kamar, N., Dalton, H. R., Abravanel, F. and Izopet, J. (2014) 'Hepatitis E virus infection', *Clinical microbiology reviews,* 27(1), pp. 116-138.

Kauppinen, A., Al-Hello, H., Zacheus, O., Kilponen, J., Maunula, L., Huusko, S., Lappalainen, M., Miettinen, I., Blomqvist, S. and Rimhanen-Finne, R. (2017) ‘Increase in outbreaks of gastroenteritis linked to bathing water in Finland in summer 2014', *Eurosurveillance,* 22(8), pp. 30470.

Kauppinen, A., Pitkänen, T., Al-Hello, H., Maunula, L., Hokajärvi, A. M., Rimhanen-Finne, R. and Miettinen, I. T. (2019) 'Two Drinking Water Outbreaks Caused by Wastewater Intrusion Including Sapovirus in Finland', *Int J Environ Res Public Health,* 16(22).

Kenney, S. P. (2019) 'The current host range of hepatitis E viruses', *Viruses,* 11(5), pp. 452.

Kinoti, W. M., Constable, F. E., Nancarrow, N., Plummer, K. M. and Rodoni, B. (2017) 'Generic amplicon deep sequencing to determine Ilarvirus species diversity in Australian Prunus', *Frontiers in microbiology,* 8, pp. 1219.

Kitajima, M., Iker, B. C., Pepper, I. L. and Gerba, C. P. (2014) 'Relative abundance and treatment reduction of viruses during wastewater treatment processes — Identification of potential viral indicators', *Science of The Total Environment,* 488-489, pp. 290-296.

Kroneman, A., de Sousa, R., Verhoef, L., Koopmans, M. P. and Vennema, H. (2018) 'Usability of the international HAVNet hepatitis A virus database for geographical annotation, backtracing and outbreak detection', *Eurosurveillance,* 23(37), pp. 1700802.

Kumar, N., Das, V., Agarwal, A., Pandey, A. and Agrawal, S. (2017) 'Fetomaternal outcomes in pregnant women with hepatitis E infection; still an important fetomaternal killer with an unresolved mystery of increased virulence in pregnancy', *Turkish Journal of Obstetrics and Gynecology,* 14(2), pp. 106.

La Bella, G., Martella, V., Basanisi, M., Nobili, G., Terio, V. and La Salandra, G. (2017) 'Food-borne viruses in shellfish: investigation on norovirus and HAV presence in Apulia (SE Italy)', *Food and environmental virology,* 9(2), pp. 179-186.

La Rosa, G., Della Libera, S., Iaconelli, M., Proroga, Y., De Medici, D., Martella, V. and Suffredini, E. (2017) 'Detection of norovirus GII. 17 Kawasaki 2014 in shellfish, marine water and underwater sewage discharges in Italy', *Food and environmental virology,* 9(3), pp. 326-333.

La Rosa, G., Mancini, P., Bonanno Ferraro, G., Iaconelli, M., Veneri, C., Paradiso, R., De Medici, D., Vicenza, T., Proroga, Y. T. R. and Di Maro, O. (2021) 'Hepatitis A Virus Strains Circulating in the Campania Region (2015–2018) Assessed through Bivalve Biomonitoring and Environmental Surveillance', *Viruses,* 13(1), pp. 16.

La Rosa, G., Proroga, Y., De Medici, D., Capuano, F., Iaconelli, M., Della

Libera, S. and Suffredini, E. (2018) 'First detection of hepatitis E virus in shellfish and in seawater from production areas in Southern Italy', *Food and environmental virology,* 10(1), pp. 127-131.

Lapp, R. T. and Rochling, F. (2013) 'Acute cholestatic hepatitis a virus infection presenting with hemolytic anemia and renal failure: a case report', *Case reports in hepatology,* 2013.

Le Guyader, F., Haugarreau, L., Miossec, L., Dubois, E. and Pommepuy, M. (2000) 'Three-year study to assess human enteric viruses in shellfish', *Applied and environmental microbiology,* 66(8), pp. 3241-3248.

Lee, L. E., Cebelinski, E. A., Fuller, C., Keene, W. E., Smith, K., Vinjé, J. and Besser, J. M. (2012) 'Sapovirus outbreaks in long-term care facilities, Oregon and Minnesota, USA, 2002-2009', *Emerg Infect Dis,* 18(5), pp. 873-6.

Leonard, A. F., Zhang, L., Balfour, A. J., Garside, R. and Gaze, W. H. (2015) 'Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters', *Environment international,* 82, pp. 92-100.

Leonard, A. F. C., Singer, A., Ukoumunne, O. C., Gaze, W. H. and Garside, R. (2018) 'Is it safe to go back into the water? A systematic review and meta-analysis of the risk of acquiring infections from recreational exposure to seawater', *International journal of epidemiology,* 47(2), pp. 572-586.

Li, J. Y., Mai, W., Tan, H. Q., Jian, M. T., Deng, H., Chen, Z. P. and Chen, H. H. (2020) '[An outbreak of acute gastroenteritis caused by Sapovirus in a community of Guangdong province]', *Zhonghua Liu Xing Bing Xue Za Zhi,* 41(2), pp. 226-230.

Li, T.-C., Miyamura, T. and Takeda, N. (2007) 'Detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (Corbicula japonica) in Japan', *The American journal of tropical medicine and hygiene,* 76(1), pp. 170-172.

Lodder, W. J. and de Roda Husman, A. M. (2005) 'Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands', *Applied and environmental microbiology,* 71(3), pp. 1453-1461.

Lowther, J., Gustar, N., Powell, A., O'Brien, S. and Lees, D. (2018) 'A one-year survey of norovirus in UK oysters collected at the point of sale', *Food and environmental virology,* 10(3), pp. 278-287.

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E. and Lees, D. N. (2012) 'Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom', *Applied and environmental microbiology,* 78(16), pp. 5812-5817.

Mallet, V., Bruneau, J., Zuber, J., Alanio, C., Leclerc-Mercier, S., Roque-Afonso, A.-M., Kraft, A. R., Couronné, L., Roulot, D. and Wedemeyer, H. (2017) 'Hepatitis E virus-induced primary cutaneous CD30 (+) T cell lymphoproliferative disorder', *Journal of Hepatology*.

Manso, C. F., Polo, D., Vilariño, M. L. and Romalde, J. L. (2010) 'Genotyping of

hepatitis A virus detected in bivalve shellfish in Galicia (NW Spain)', *Water Sci Technol,* 61(1), pp. 15-24.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R. and Brouwer, A. (2020) 'Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands', *Environmental Science & Technology Letters,* 7(7), pp. 511-516.

Meghnath, K., Hasselback, P., McCormick, R., Prystajecky, N., Taylor, M., McIntyre, L., Man, S., Whitfield, Y., Warshawsky, B., McKinley, M., Bitzikos, O., Hexemer, A. and Galanis, E. (2019) 'Outbreaks of Norovirus and Acute Gastroenteritis Associated with British Columbia Oysters, 2016-2017', *Food Environ Virol,* 11(2), pp. 138-148.

Mesquita, J. R., Oliveira, D., Rivadulla, E., Abreu-Silva, J., Varela, M. F., Romalde, J. L. and Nascimento, M. S. (2016) 'Hepatitis E virus genotype 3 in mussels (Mytilus galloprovinciallis), Spain', *Food microbiology,* 58, pp. 13-15.

Nasheri, N., Vester, A. and Petronella, N. (2019) 'Foodborne viral outbreaks associated with frozen produce', *Epidemiology & Infection,* 147.

Ngazoa, E., Fliss, I. and Jean, J. (2008) 'Quantitative study of persistence of human norovirus genome in water using TaqMan real-time RT-PCR', *Journal of Applied Microbiology,* 104(3), pp. 707-715.

Nijskens, C. M., Pas, S. D., Cornelissen, J., Caliskan, K., Hoek, R. A., Hesselink, D. A., van der Eijk, A. A. and Robert, A. (2016) 'Hepatitis E virus genotype 3 infection in a tertiary referral center in the Netherlands: Clinical relevance and impact on patient morbidity', *Journal of Clinical Virology,* 74, pp. 82-87.

Novel, C. E., Durupt, S., de Laforcade, L., Lecoq, M., Durieu, I. and Reynaud, Q. (2017) 'Acute kidney injury, a rare complication of acute hepatitis E infection', *Medecine et maladies infectieuses,* 47(7), pp. 502.

Oka, T., Wang, Q., Katayama, K. and Saif, L. J. (2015) 'Comprehensive review of human sapoviruses', *Clinical microbiology reviews,* 28(1), pp. 32-53.

O'Hara, Z., Crossan, C., Craft, J. and Scobie, L. (2018) 'First Report of the Presence of Hepatitis E Virus in Scottish-Harvested Shellfish Purchased at Retail Level', *Food and environmental virology*, pp. 1-5.

Peron, J., Bureau, C., Poirson, H., Mansuy, J., Alric, L., Selves, J., Dupuis, E., Izopet, J. and Vinel, J. (2007) 'Fulminant liver failure from acute autochthonous hepatitis E in France: description of seven patients with acute hepatitis E and encephalopathy', *Journal of viral hepatitis,* 14(5), pp. 298-303.

Petersen, L. M., Martin, I. W., Moschetti, W. E., Kershaw, C. M. and Tsongalis, G. J. (2019) 'Third-Generation Sequencing in the Clinical Laboratory: Exploring the Advantages and Challenges of Nanopore Sequencing', *Journal of clinical microbiology,* 58(1), pp. e01315-19.

Pires, S. M., Fischer-Walker, C. L., Lanata, C. F., Devleesschauwer, B., Hall, A.

J., Kirk, M. D., Duarte, A. S., Black, R. E. and Angulo, F. J. (2015) 'Aetiology-specific estimates of the global and regional incidence and mortality of diarrhoeal diseases commonly transmitted through food', *PloS one,* 10(12), pp. e0142927.

Polo, D., Schaeffer, J., Fournet, N., Le Saux, J.-C., Parnaudeau, S., McLeod, C. and Le Guyader, F. S. (2016) 'Digital PCR for quantifying norovirus in oysters implicated in outbreaks, France', *Emerging infectious diseases,* 22(12), pp. 2189.

Prato, R., Lopalco, P. L., Chironna, M., Barbuti, G., Germinario, C. and Quarto, M. (2004) 'Norovirus gastroenteritis general outbreak associated with raw shellfish consumption in south Italy', *BMC infectious diseases,* 4, pp. 37-37.

Prüss, A. (1998) 'Review of epidemiological studies on health effects from exposure to recreational water', *International Journal of Epidemiology,* 27(1), pp. 1-9.

Public Health England. (2019) *Hepatitis E: symptoms, transmission, treatment and prevention*. gov.uk: Public Health England. Available at: https://www.gov.uk/government/publications/hepatitis-e-symptoms-transmission-prevention-treatment/hepatitis-e-symptoms-transmission-treatment-and-prevention (Accessed: 27 August 2019).

Public Health England. (2021a) *2018 to 2019 Season Norovirus Report National norovirus laboratory and outbreak data in England*, gov.uk: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/966775/2021-01-29-Norovirus_annual_report_2018_to_2019.pdf.

Public Health England. (2021b) *Laboratory reports of hepatitis A infections in England and Wales, 2019*, UK: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/982962/hpr0715_HAV19_v2.pdf.

Qi, R., Huang, Y.-t., Liu, J.-w., Sun, Y., Sun, X.-f., Han, H.-J., Qin, X.-R., Zhao, M., Wang, L.-j. and Li, W. (2018) 'Global prevalence of asymptomatic norovirus infection: a meta-analysis', *EClinicalMedicine,* 2, pp. 50-58.

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T. F. and Beutler, N. A. (2017) 'Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples', *nature protocols,* 12(6), pp. 1261.

Rivadulla, E., Varela, M. F., Mesquita, J. R., Nascimento, M. S. and Romalde, J. L. (2019) 'Detection of hepatitis E virus in shellfish harvesting areas from Galicia (Northwestern Spain)', *Viruses,* 11(7), pp. 618.

Rivero-Juarez, A., Frias, M., Martinez-Peinado, A., Risalde, M., Rodriguez-Cano, D., Camacho, A., García-Bocanegra, I., Cuenca-Lopez, F., Gomez-

Villamandos, J. and Rivero, A. (2017) 'Familial hepatitis E outbreak linked to wild boar meat consumption', *Zoonoses and public health*.

Rutjes, S. A., Lodder, W. J., Lodder-Verschoor, F., van den Berg, H. H. J. L., Vennema, H., Duizer, E., Koopmans, M. and de Roda Husman, A. M. (2009) 'Sources of hepatitis E virus genotype 3 in The Netherlands', *Emerging infectious diseases,* 15(3), pp. 381-387.

Said, B., Ijaz, S., Kafatos, G., Booth, L., Thomas, H. L., Walsh, A., Ramsay, M. and Morgan, D. (2009) 'Hepatitis E outbreak on cruise ship', *Emerging infectious diseases,* 15(11), pp. 1738.

Salvador, D., Neto, C., Benoliel, M. J. and Caeiro, M. F. (2020) 'Assessment of the Presence of Hepatitis E virus in Surface Water and Drinking Water in Portugal', *Microorganisms,* 8(5), pp. 761.

Sano, D., Pérez-Sautu, U., Guix, S., Pintó, R. M., Miura, T., Okabe, S. and Bosch, A. (2011) 'Quantification and genotyping of human sapoviruses in the Llobregat river catchment, Spain', *Applied and environmental microbiology,* 77(3), pp. 1111-1114.

Santiso-Bellón, C., Randazzo, W., Pérez-Cataluña, A., Vila-Vicent, S., Gozalbo-Rovira, R., Muñoz, C., Buesa, J., Sanchez, G. and Rodríguez Díaz, J. (2020) 'Epidemiological surveillance of norovirus and rotavirus in sewage (2016–2017) in Valencia (Spain)', *Microorganisms,* 8(3), pp. 458.

Santos, L., Mesquita, J., Pereira, N. R., Lima-Alves, C., Serrão, R., Figueiredo, P., Reis, J., Simões, J., Nascimento, M. and Sarmento, A. (2013) 'Acute hepatitis E complicated by Guillain-Barre syndrome in Portugal, December 2012–a case report', *Platelets (x 109/L),* 328(308), pp. 286.

Schaeffer, J., Le Saux, J.-C., Lora, M., Atmar, R. L. and Le Guyader, F. S. (2013) 'Norovirus contamination on French marketed oysters', *International journal of food microbiology,* 166(2), pp. 244-248.

Sedji, M. I., Varbanov, M., Meo, M., Colin, M., Mathieu, L. and Bertrand, I. (2018) 'Quantification of human adenovirus and norovirus in river water in the north-east of France', *Environmental science and pollution research,* 25(30), pp. 30497-30507.

Sharp, J. H., Clements, K., Diggens, M., McDonald, J. E., Malham, S. K. and Jones, D. L. (2021) 'E. coli Is a Poor End-Product Criterion for Assessing the General Microbial Risk Posed From Consuming Norovirus Contaminated Shellfish', *Front Microbiol,* 12, pp. 608888.

Smith, A. J., McCarthy, N., Saldana, L., Ihekweazu, C., McPhedran, K., Adak, G. K., Iturriza-Gómara, M., Bickler, G. and O'Moore, É. (2012) 'A large foodborne outbreak of norovirus in diners at a restaurant in England between January and February 2009', *Epidemiol Infect,* 140(9), pp. 1695-701.

Smith, D. B., Izopet, J., Nicot, F., Simmonds, P., Jameel, S., Meng, X.-J., Norder, H., Okamoto, H., van der Poel, W. H. and Reuter, G. (2020) 'Update: proposed reference sequences for subtypes of hepatitis E virus (species

Orthohepevirus A)', *Journal of General Virology*, pp. jgv001435.

Smith, D. B., Paddy, J. O. and Simmonds, P. (2016) 'The use of human sewage screening for community surveillance of hepatitis E virus in the UK', *Journal of medical virology,* 88(5), pp. 915-918.

Sunaga, F., Masuda, T., Aoki, H., Ito, M., Sano, K., Naoi, Y., Katayama, Y., Omatsu, T., Oba, M. and Furuya, T. (2019) 'Complete genome sequencing and genetic characterization of porcine sapovirus genogroup (G) X and GXI: GVI, GVII, GX, and GXI sapoviruses share common genomic features and form a unique porcine SaV clade', *Infection, Genetics and Evolution,* 75, pp. 103959.

Svraka, S., Vennema, H., van der Veer, B., Hedlund, K.-O., Thorhagen, M., Siebenga, J., Duizer, E. and Koopmans, M. (2010) 'Epidemiology and genotype analysis of emerging sapovirus-associated infections across Europe', *Journal of clinical microbiology,* 48(6), pp. 2191-2198.

Sánchez, G., Pintó, R. M., Vanaclocha, H. and Bosch, A. (2002) 'Molecular characterization of hepatitis A virus isolates from a transcontinental shellfish-borne outbreak', *Journal of Clinical Microbiology,* 40(11), pp. 4148-4155.

Tallon, L. A., Love, D. C., Moore, Z. S. and Sobsey, M. D. (2008) 'Recovery and sequence analysis of hepatitis a virus from springwater implicated in an outbreak of acute viral hepatitis', *Applied and environmental microbiology,* 74(19), pp. 6158-6160.

Tam, C., Viviani, L., Adak, B., Bolton, E., Dodds, J., Cowden, J., Evans, M., Gray, J., Hunter, P. and Jackson, K. (2012) 'The second study of infectious intestinal disease in the community (IID2 study)', *UK Food Standards Agency*.

Tang, Y., Wang, J., Xu, Z., Guo, Y., Qian, W. and Xu, J. (1991) 'A serologically confirmed, case-control study, of a large outbreak of hepatitis A in China, associated with consumption of clams', *Epidemiology & Infection,* 107(3), pp. 651-657.

Teunis, P. F., Moe, C. L., Liu, P., Miller, S. E., Lindesmith, L., Baric, R. S., Le Pendu, J. and Calderon, R. L. (2008) 'Norwalk virus: how infectious is it?', *J Med Virol,* 80(8), pp. 1468-76.

Thebault, A., Teunis, P. F. M., Le Pendu, J., Le Guyader, F. S. and Denis, J.-B. (2013) 'Infectivity of GI and GII noroviruses established from oyster related outbreaks', *Epidemics,* 5(2), pp. 98-110.

Thongprachum, A., Fujimoto, T., Takanashi, S., Saito, H., Okitsu, S., Shimizu, H., Khamrin, P., Maneekarn, N., Hayakawa, S. and Ushijima, H. (2018) 'Detection of nineteen enteric viruses in raw sewage in Japan', *Infection, Genetics and Evolution,* 63, pp. 17-23.

Treagus, S., Wright, C., Baker-Austin, C., Longdon, B. and Lowther, J. (2021) 'The Foodborne Transmission of Hepatitis E Virus to Humans', *Food and Environmental Virology*, pp. 1-19.

Tyson, J. R., James, P., Stoddart, D., Sparks, N., Wickenhagen, A., Hall, G.,

Choi, J. H., Lapointe, H., Kamelian, K., Smith, A. D., Prystajecky, N., Goodfellow, I., Wilson, S. J., Harrigan, R., Snutch, T. P., Loman, N. J. and Quick, J. (2020) 'Improvements to the ARTIC multiplex PCR method for SARS-CoV-2 genome sequencing using nanopore', *bioRxiv*, pp. 2020.09.04.283077.

Varela, M. F., Polo, D. and Romalde, J. L. (2016) 'Prevalence and genetic diversity of human sapoviruses in shellfish from commercial production areas in Galicia, Spain', *Applied and environmental microbiology,* 82(4), pp. 1167-1172.

Vergara, G. G. R. V., Rose, J. B. and Gin, K. Y. H. (2016) 'Risk assessment of noroviruses and human adenoviruses in recreational surface waters', *Water Research,* 103, pp. 276-282.

Villalba, M. C. M., Martínez, D. C., Ahmad, I., Lay, L. A. R., Corredor, M. B., March, C. G., Martínez, L. S., Martínez-Campo, L. S. and Jameel, S. (2017) 'Hepatitis E virus in bottlenose dolphins Tursiops truncatus', *Diseases of aquatic organisms,* 123(1), pp. 13-18.

Wachtman, L. and Mansfield, K. (2012) 'Viral diseases of nonhuman primates', *Nonhuman primates in biomedical research*, pp. 1.

Wang, L., Teng, J. L., Lau, S. K., Sridhar, S., Fu, H., Gong, W., Li, M., Xu, Q., He, Y. and Zhuang, H. (2019) 'Transmission of a novel genotype of hepatitis E virus from Bactrian camels to cynomolgus macaques', *Journal of Virology,* 93(7), pp. e02014-18.

World Health Organisation. (2019) *Hepatitis E*: World Health Organization. Available at: https://www.who.int/news-room/fact-sheets/detail/hepatitis-e (Accessed: 08 September 2021).

Wyn-Jones, A. P., Carducci, A., Cook, N., D'agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R. and Höller, C. (2011) 'Surveillance of adenoviruses and noroviruses in European recreational waters', *Water research,* 45(3), pp. 1025-1038.

Yin, W., Han, Y., Xin, H., Liu, W., Song, Q., Li, Z., Gao, S., Jiang, F., Cao, J., Bi, S. and Liu, H. (2019) 'Hepatitis E Outbreak in a Mechanical Factory in Qingdao City, China', *Int J Infect Dis*.

Chapter 2

The Foodborne Transmission of Hepatitis E Virus to Humans

Abstract

Globally, Hepatitis E virus (HEV) causes over 20 million cases worldwide. HEV is an emerging and endemic pathogen within economically developed countries, chiefly resulting from infections with genotype 3 (G3) HEV. G3 HEV is known to be a zoonotic pathogen, with a broad host range. The primary source of HEV within more economically developed countries is considered to be pigs, and consumption of pork products is a significant risk factor and known transmission route for the virus to humans. However, other foods have also been implicated in the transmission of HEV to humans. This review consolidates the information available regarding transmission of HEV and looks to identify gaps where further research is required to better understand how HEV is transmitted to humans through food.

Introduction

Globally, hepatitis E virus (HEV), of the family *Hepeviridae*, is considered the most common cause of acute viral hepatitis. There were an estimated 20 million infections worldwide annually in 2005 from genotype 1 (G1) and 2 (G2) HEV combined (Rein *et al.*, 2012), and 44,000 recorded fatalities due to the virus in 2015 (World Health Organization, 2019). Generally, HEV causes an acute, self-limiting infection which resolves within a few weeks; however, in some persons (such as the immunocompromised) it can cause chronic infections, fulminant hepatitis (acute liver failure) and extrahepatic manifestations (infections in other organs), which can be fatal. Table 2.1 shows a summary of the pattern of infection of the different HEV genotypes, demonstrating the variable clinical manifestations and factors such as average age of infection and gender, where these are known. There is limited data to be able to estimate the number of infections worldwide, but with a high level of asymptomatic infections seen in numerous outbreaks it is probable that more infections occur worldwide than estimated in 2005 (Guillois *et al.*, 2015; Yin *et al.*, 2019); especially considering that this estimate was made only considering G1 and G2.

Table 2.1 Pattern of infection for the different genotypes of HEV, adapted from (Centers for Disease Control and Prevention, 2020)

Genotype	Transmission in humans?	Transmission Routes	Geographical distribution pattern	Extrahepatic manifestations	Age groups at higher risk	Sex more commonly affected	Lethality
1	Yes	Faecal-oral; Waterborne; Blood transfusion; Organ donation	Economically developed and developing countries	Pancreatic	Differs by country [a,b]	Differs by country [a,b]	0.5-1%[c]; 20% in pregnant women [d,e,f]
2	Yes	Faecal-oral; Waterborne; Blood transfusion; Organ donation	Economically developing countries	Unknown	Young adults	Unknown	0.5-1%[c]
3	Yes	Foodborne; Blood transfusion; Organ donation	Economically developed and developing countries	Chronic infections in immunocompromised patients. Neurological, haematological, immunological, and renal manifestations[g]	Older adults (>40 years)	Males	0.5-1%[c]
4	Yes	Foodborne; Blood transfusion; Organ donation	Economically developed and developing countries	Unknown	Young adults	Possibly males (limited data)[h]	0.5-1%[c]
5	No	Faecal-oral	Unknown	Unknown	Unknown	Unknown	Unknown
6	No	Faecal-oral	Unknown	Unknown	Unknown	Unknown	Unknown
7	Yes	Foodborne; Faecal-oral; Blood transfusion?	Unknown	Unknown	Unknown	Unknown	Unknown
8	No	Faecal-oral	Unknown	Unknown	Unknown	Unknown	Unknown

[a]Pathak and Barde, (2017), [b]Spina *et al.*, (2017), [c]Peron *et al.*, (2007), [d]Kumar *et al.*, (2017), [e]Jin *et al.*, (2016), [f]Kamar *et al.*, (2014), [g]Horvatits *et al.*, (2019), [h]Mizuo *et al.*, (2005)

HEV was once thought only to be endemic to certain economically developing countries within Asia and Africa, but research over the past decade has highlighted the emergence of HEV within higher income countries. The virus spreads through a faecal oral route, making it easily transmissible through faecally contaminated water. Indeed, this is thought to be the main transmission route within China, where G1 and genotype 4 (G4) HEV are dominant (Wang *et al.*, 2001). However, it is also possible for the virus to be transmitted through foodstuffs such as pork, due to the ability for some HEV genotypes to infect non-human animals. Currently, the virus is classified into eight genotypes, with G1-4 and genotype 7 (G7) capable of infecting humans. There is, however, a diverse host range for the different genotypes, with G1 and G2 generally only infecting humans and non-human primates, but genotypes 3-8 infecting many other animals, such as pigs, deer, camels, rabbits, and dolphins. A summary of the different host species of HEV was published recently (Kenney, 2019), and is summarised briefly in Table 2.2. Genotype 3 (G3) HEV has been found to be the most geographically diverse of the viruses thus far (Pérez-Gracia *et al.*, 2015), and is the genotype which has emerged in the past two decades in many developed countries. The geographical distribution of genotypes 1-4 can be seen in Figure 2.1.

Table 2.2 Summary of HEV host species by genotype adapted from Kenney, 2019

Genotype	Hosts identified (common names)	Species infected
1	Humans, Chimpanzees, Monkeys, Horses	*Homo sapiens, Pan troglodytes, Chlorocebus sabaeus*, Chlorocebus pygerythrus*, Erythrocebus patas*, Macaca mulatta, Macaca radiata, Macaca fascicularis, Semnopithecus entellus, Aotus trivirgatus*, Saguinus mystax mystax*, Saimiri sciureus*, Equus caballus ferus*
2	Humans, Monkeys	*Homo sapiens, Chlorocebus pygerythrus*, Erythrocebus patas*, Macaca mulatta, Macaca fascicularis, Aotus trivirgatus*, Saguinus mystax mystax*, Saimiri sciureus**
3	Humans, Monkeys, Hares and Rabbits, Rats, Minks, Mongooses, Pigs, Goats and Sheep, Deer, Dolphins, Horses, Vultures	*Homo sapiens, Erythrocebus patas, Macaca mulatta, Macaca fascicularis, Macaca fuscata, Lepus europaeus, Oryctolagus cuniculus domesticus, Rattus norvegicus, Neovison vison, Herpestes javanicus, Sus scrofa, Sus scrofa domestica, Capra hircus aegagrus, Ovis aries orientalis, Cervus elaphus, Cervus nippon, Capreolus, Tursiops truncatus, Equus africanus, Equus caballus ferus, Gyps himalayensis*
4	Humans, Monkeys, Gerbils, Dogs, Bears, Leopards, Pigs, Cows, Goats, Deer, Cranes, Pheasants	*Homo sapiens, Macaca fascicularis, Macaca mulatta, Meriones unguiculatus*, Canis lupus familiaris, Ursus thibetanus, Neofelis nebulosa, Sus scrofa, Sus scrofa domesticus, Bos taurus primigenuis, Bos grunniens, Capra hircus aegagrus, Ovis aries orientalis, Cervus nippon, Elaphodus cephalophus, Muntiacus reevesi, Balearica regulorum, Lophura nycthemera*
5	Monkeys, Pigs	*Macaca fascicularis, Sus scrofa*
6	Pigs	*Sus scrofa*
7	Humans, Camels	*Homo sapiens, Camelus dromedarius,*
8	Camels	*Camelus bactrianus*

*Infections instigated through experimental conditions

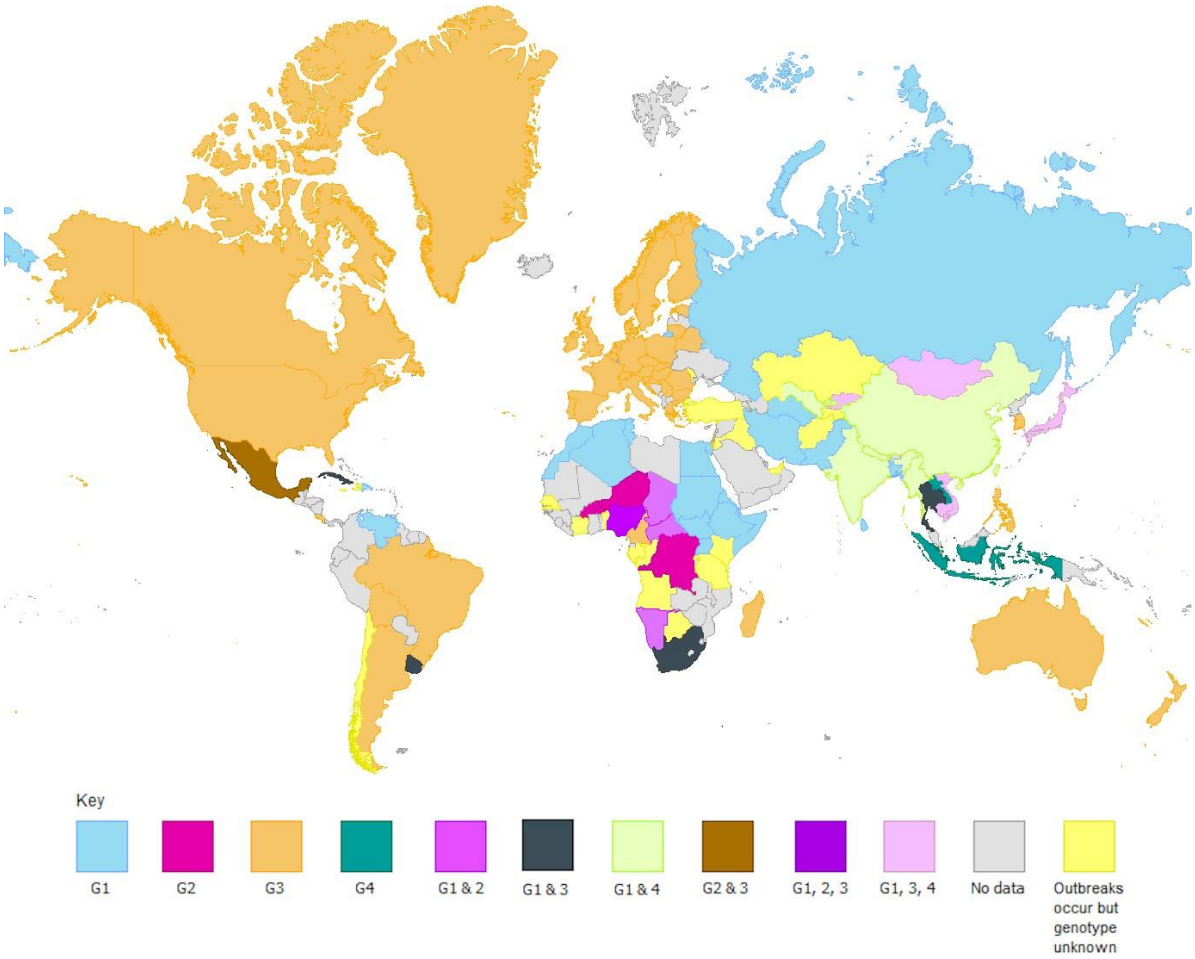


Figure 2.1 The geographical distribution of HEV genotypes 1-4

This figure shows the genotypes of HEV which are endemic to each country, where enough data was available. For graphs which are compatible with the conditions protanopia, deuteranopia and achromatopsia please see supplementary data 2.1 and 2.2. Maps created in ArcMap using the World Countries (generalized) layer package by esri_dm and visualised in GIMP.

G3 HEV is thought to be spread primarily through the consumption of undercooked pork products from infected pigs, however it is unknown if all transmission routes to humans have been identified. The current theories and known routes of transmission can be seen in Figure 2.2. This review will discuss theoretical routes of HEV transmission to humans through foodstuffs and identify areas which require further research for better understanding of the virus.

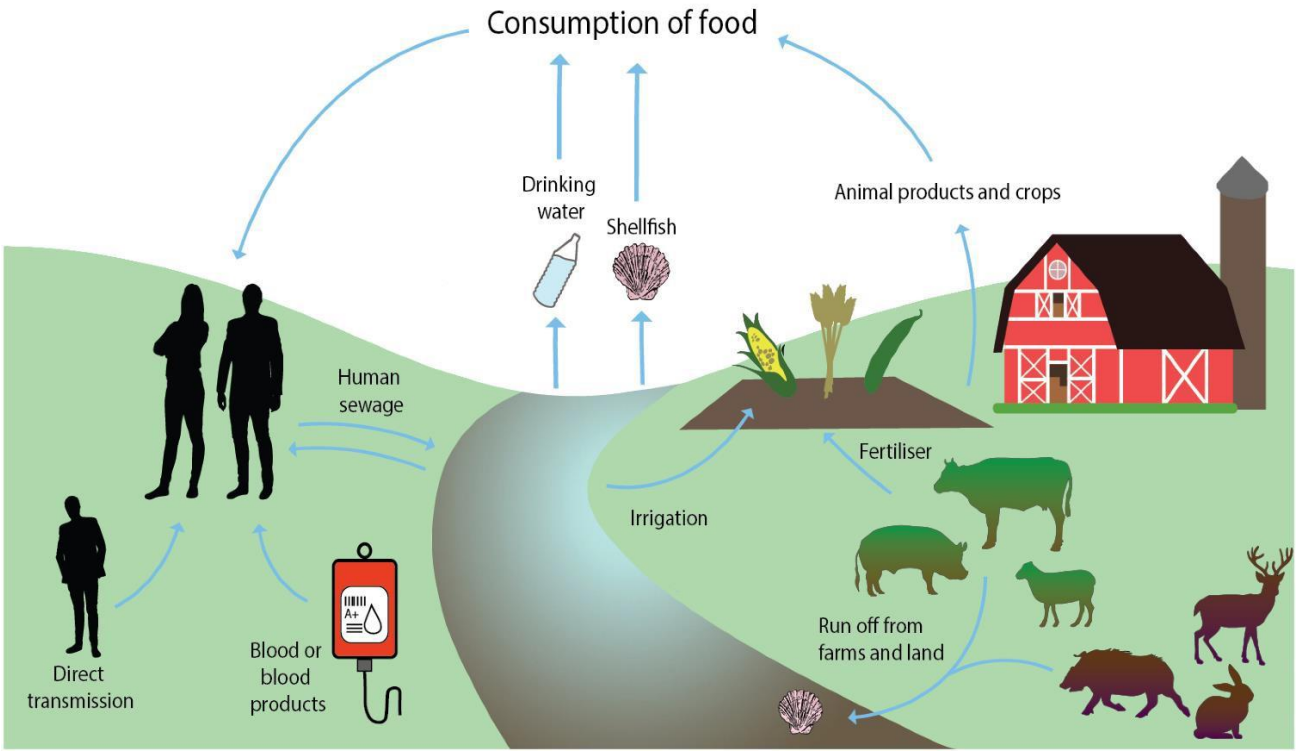


Figure 2.2 Theoretical and confirmed transmission routes of HEV

The figure shows confirmed and theoretical routes of HEV transmission to humans. The theoretical routes of transmission include HEV infections contracted from the consumption of shellfish, sheep, and cows, as well as crops and drinking water, as no confirmed outbreaks from these sources have yet been identified. Illustration created using Adobe Illustrator and edited using GIMP.

HEV in Pigs and Pork Products

Over the past two decades, evidence has accumulated implicating pigs and other animals in the zoonotic transmission of G3 HEV to humans (Tei *et al.*, 2003; Guillois *et al.*, 2015; Lhomme *et al.*, 2013; Rivero-Juarez *et al.*, 2017). In 1998 it was shown that a HEV strain isolated from an acute HEV patient in the USA was capable of

infecting pigs, and that a genetically similar strain isolated from pigs was capable of infecting non-human primates; suggesting a significant possibility that pigs could act as a zoonotic source of HEV (Meng *et al.*, 1998). Since this study, many countries have noted the emergence of HEV cases. At this stage, it was thought that HEV was only endemic in developing countries; (such as those in Asia, Africa, and central America) and that HEV cases in non-endemic areas were obtained through travel. However, studies such as those by Dalton *et al.*, (2007) and Fraga *et al.*, (2017) identified that indigenous cases of HEV were occurring in economically developed countries such as the UK and Switzerland. It is now widely accepted that pigs are a zoonotic source of HEV transmission to humans and may be at least partially responsible for increasing cases worldwide annually, though increased detection and awareness of HEV may also play a small role in the observed increase in cases. Outbreaks of HEV have directly been linked to pork product consumption, including an outbreak in Spain linked to consumption of wild boar (Rivero-Juarez *et al.*, 2017), and another outbreak associated with consumption of a spit-roasted piglet in France (Guillois *et al.*, 2015).Consumption of pork products is now considered a significant risk factor for developing HEV infection, which is concerning considering the seroprevalence levels in European pigs (Said *et al.*, 2014; Slot *et al.*, 2017). Table 2.3 shows a non-exhaustive list of countries that have detected anti-HEV antibodies in pigs, and HEV RNA in pork products.

Table 2.3 A list of the seroprevalence levels of anti-HEV antibodies in pigs and the percentage of pork products found to be HEV-positive by RT-PCR in those locations

Location	Pig sample population anti-HEV antibody seroprevalence	Percentage of tested foodstuffs HEV-positive by RT-PCR
Brazil	63.6% of 357 pigs (Vitral *et al.*, 2005)	1.7% of 118 slaughterhouse livers (Gardinali *et al.*, 2012)
Canada	59.4% of 998 pigs (Yoo *et al.*, 2001)	8.8% of 283 livers, 1.0% of 599 pork chops (Wilhelm *et al.*, 2014)
		47.0% of 76 pork pâtés and 10.5% of 19 retail raw pork livers (Mykytczuk *et al.*, 2017)
France	31.0% of 6565 pigs (Rose *et al.*, 2011); 60% of 1034 pigs (Feurer *et al.*, 2018)	4.0% of 3715 slaughterhouse livers (Rose *et al.*, 2011)
		2.8% of 1034 slaughterhouse livers (Feurer *et al.*, 2018)
		30.0% of 140 figatelli and fitone, 29.0% of 169 liver sausages, 25.0% of 55 quenelles or quenelle paste, 3.0% of 30 dried salted livers (Pavio et al., 2014)
		58.3% of 12 raw liver sausage (Colson *et al.*, 2010)
Germany	49.8% of 1072 pigs (Baechlein *et al.*, 2010)	4.0% of 200 retail livers (Wenzel *et al.*, 2011)

		20.0% of 70 raw sausages and 22.0% of 50 liver sausages (Szabo *et al.*, 2015)
Italy	45.1% of 2700 pigs (Mughini-Gras *et al.*, 2017)	20.8% of 48 slaughterhouse livers (Di Bartolo *et al.*, 2011)
		6.0% of 33 slaughterhouse livers (Di Bartolo *et al.*, 2012)
		13.3% of 15 fresh liver sausages, 7.1% of 14 dried liver sausages (Di Bartolo *et al.*, 2015a)
Japan	57.9% of 2500 pigs (Takahashi *et al.*, 2003)	1.9% of 363 retail livers (Yazaki *et al.*, 2003)
Netherlands	89.0% of 417 organic pigs, 72% of 265 conventionally farmed pigs, 76% of 164 free range pigs (Rutjes *et al.*, 2014)	6.5% of 62 commercial pork livers (Bouwknegt *et al.*, 2007)
		12.7% of 79 livers, 70.7% of 99 liver sausages, 68.9% of 90 liver pâté samples (Boxman *et al.*, 2019)
Spain	20.4% of 1441 pigs (de Oya *et al.*, 2011)	6.0% of 93 sausages, 3.0% of 39 slaughterhouse livers (Di Bartolo *et al.*, 2012)
Switzerland	62.3% of 1001 pigs in 2006, 53.8% of 999 pigs in 2011 (Burri *et al.*, 2014)	1.3% of 160 slaughterhouse livers (Müller *et al.*, 2017)
		11.8% of 102 raw liver sausages (Giannini *et al.*, 2017)

		11.7% of 90 pork liver and raw meat sausages (Moor *et al.*, 2018)
UK	92.8% of 629 pigs, with 20.5% viraemic at slaughter (Grierson *et al.*, 2015)	10.0% of 63 sausages, 3.0% of 40 slaughterhouse livers (Berto *et al.*, 2012)
USA	21.9% of 182 pigs (Owolodun *et al.*, 2013)	11.0% of 127 retail liver (Feagins *et al.*, 2007)

Considering Table 2.3, it is possible that the HEV prevalence in pork sausages may be over- or under-estimated by the fact that the sample sizes for some of these studies are relatively small. It is also important to note that different methods have been used between studies, some of which have been shown to be less sensitive for detecting HEV than others. Interestingly, the seroprevalence of HEV in pigs is significantly higher than the prevalence of HEV RNA in pork products in most cases; this discrepancy is expected as not all pigs would be viraemic at slaughter.

It has been found that HEV generally infects swine asymptomatically at an early point in their life (prior to 6 months of age (de Oya *et al.*, 2011)), and that because of this, many pigs are seropositive at the time of slaughter (Grierson *et al.*, 2015; Rose *et al.*, 2011; de Oya *et al.*, 2011). The slaughter age of pigs is normally slightly before one year of age. The transmission between pigs is suspected to be through a faecal-oral route and this is likely to be due to the high shedding that is seen in pig faeces and urine (Bouwknegt *et al.*, 2009; Halbur *et al.*, 2001). Infection with HEV early in life means that there is a lower chance of the pigs being viraemic at slaughter, and they are therefore less likely to be capable of HEV transmission to humans through the pork food chain. However, whether HEV causes life-long immunity in swine after recovery has been open to debate. In rhesus macaques and humans, the anti-HEV IgG antibodies (characteristic of long term immunity) wane over a variable number of years until they are undetectable, and the period for which individuals may be IgG-positive for varies (Arankalle *et al.*, 1999; Lee *et al.*, 1994). This may not be an issue in pigs as they are normally slaughtered before a year of age, so early life infections would likely allow immunity against subsequent HEV challenge

over their lifetime. However, it has been shown that animals can be re-infected by different HEV strains (De Deus *et al.*, 2008), and whether or not one strain can confer protective immunity to all other HEV strains has also been contested. It has been shown that after infection with one strain of G3 HEV, pigs developed some protective immunity against other strains within the same genotype, and also within G4 (Sanford *et al.*, 2011). However, in rhesus monkeys, it was shown that infection with one strain from a genotype could not confer protective immunity to a strain in a different genotype upon subsequent challenge; and that in some cases, infection with a different strain from the same genotype could also not confer protective immunity (Huang *et al.*, 2008). If there is genetic or environmental variation in host development of immunity to HEV, and some strains of HEV may not provide protection against others, then farms with multiple circulating HEV strains could be more likely to have viraemic pigs at the time of slaughter. This would therefore mean that there could be a higher likelihood of contracting HEV from undercooked pork products or food products containing raw pig components from these sources.

It has been reported that 21% of pigs in the UK tested positive for HEV RNA at the time of slaughter (Grierson *et al.*, 2015), and that in the USA 6.3% of pigs from slaughterhouses were HEV RNA positive (Sooryanarain *et al.*, 2020). As such it is probable that the consumption of raw and undercooked pork products is acting as a transmission route of HEV to humans.

Table 2.4 summarises studies that have investigated the thermal inactivation of HEV in non-food matrix samples. Inactivation condition combinations found sufficient to inactivate virus in these studies are shown in Figure 2.3. Results vary between the different studies, and several factors make comparison difficult. The studies use a variety of different units or expressions of reduction/inactivation. In the study by Huang *et al.*, (1999) a temperature of 56°C for 30 minutes was reported to completely inactivate the virus; however the virus was only left to grow for a relatively short period of time (72 hours). However, it was shown that HEV was still viable following similar heat treatments in cell culture studies with longer growth periods (Emerson et al., 2005; Tanaka *et al.*, 2007). Schielke *et al.*, (2011) used RNase treatment in an attempt to remove viral RNA that had broken from the capsid after heat treatment, assuming this would remove RNA from non-viable virus. However, it

is unknown if any RNA from viable virus could have been lost during this treatment, and the standard deviations seen within the results were relatively large. Without a cell culture component, it is not possible to say with 100% certainty that remaining detected RNA was from viable virus. In addition to these limitations, it is known that HEV is difficult to culture effectively *in vitro*, often requiring large titres of virus to begin the culture, and therefore it is possible that the inactivation requirements for HEV have been underestimated as treatments sufficient to eliminate infectivity *in vitro* may not completely eliminate *in vivo* infectivity. Some researchers have investigated different cell lines and strains of HEV which appear to be more efficiently cultured *in vitro* due to insertions within the HEV genome, however culturing these strains still requires large titres of virus to begin the culturing process (10^6 copies/ml) (Johne *et al.*, 2014; Schemmerer *et al.*, 2016).

Table 2.4 A summary of studies investigating thermal inactivation treatments for HEV in non-food matrix samples

Study	Cell culture or molecular detection?	Genotype	Heat treatment		Growth period	Inactivation/ Reduction
			Temperature (°C)	Time (mins)		
Emerson et al., (2005)	Cell culture, HepG2/C3A cells	1 (strain Akluj[1])	56	60	5-6 days	>80% reduction
		1 (strain SAR55[2])	56	60	5-6 days	~50% reduction
		2 (strain Mex14[3])	60	60	5-6 days	96% reduction
Huang *et al.*, (1999)	Cell culture, A549 cells	3 (strains G93-1*, G93-2*, G93-3*, G93-4*)	56	30	72 hours	<1.0 (TCID50/0.025 ml)
Johne *et al.*, (2016)	Cell culture, A549/D3 cells	3 (strain 47832c[6])	55	1	35 days	~1 log reduction in focus forming units
			70	2		"no infectious virus"
Schielke *et al.*, (2011)	Molecular detection	3 (strain wbGER27[5])	56	15	N/A	74.07% reduction
		3 (strain wbGER27[5])	56	60	N/A	99.90% reduction
Tanaka *et al.*, (2007)	Cell culture, PLC/PRF/5 cells	3 (strain JE03-1760F[4])	70	10	35 days	"no infectious virus"
		3 (strain JE03-1760F[4])	56	30	50 days	"still infectious"

*Accession numbers unknown; [1]AF107909; [2]M80581.1; [3]KX578717.1; [4]AB437319.1; [5]FJ705359.1; [6]KC618403.1

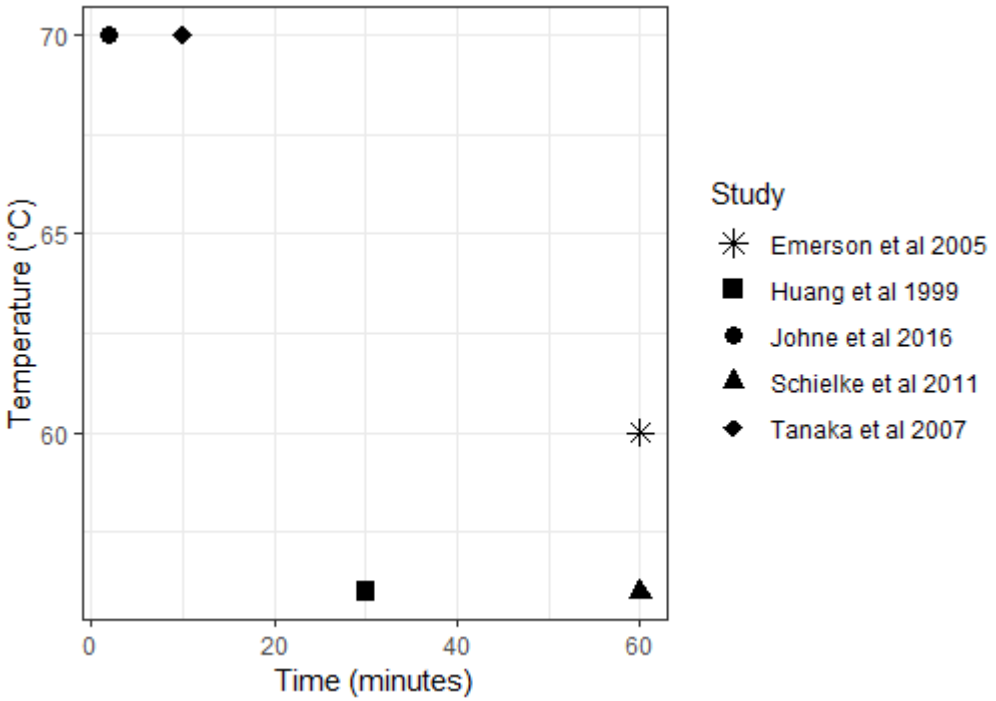


Figure 2.3 A summary of the reported thermal inactivation requirements for HEV from different studies

This graph summarises the observed HEV inactivation requirements for five different studies investigating the effect of heat treatment over time on HEV viability, with the highest reported inactivation requirements being 70°C for 10 minutes, and the lowest being 56°C for 30 minutes. Graph created in R studio.

It is possible that interactions of HEV with organic molecules in food matrices may cause the thermal inactivation temperature to be higher. Some studies have investigated the thermal inactivation of HEV within food stuffs, such as liver and pork sausage (summarised in Table 2.5). These studies have all taken different approaches to heat inactivation of the virus and the food types used, and therefore it is difficult to compare the results and form a definitive answer for the heat treatment required to inactivate the virus within foodstuffs. Feagins *et al.*, (2007) identified that boiling or stir-frying infected pig liver to an internal temperature of 71°C for 5 minutes could prevent infection when the liver was then fed to pigs. However, though the pigs were not infected by this oral dose, it is known that pigs commonly require a high dose of HEV to become infected through the faecal-oral route (Kasorndorkbua *et al.*, 2004), of approximately 10^6 genome copies (Andraud *et al.*, 2013), and therefore it is possible that lower doses of active virus were still present in these food stuffs. The oral infectious dose for humans is unknown. Intravenous inoculation of pigs with the cooked

foodstuffs as carried out by Barnaud *et al.*, (2012) is likely to have provided a more accurate estimate of whether viable virus still existed, especially as intravenous inoculation of pigs has been reported to require much lower HEV doses to cause infection (Dähnert *et al.*, 2018). Imagawa *et al.*, (2018) reported similar inactivation requirements to Feagins *et al.*, (2007). However, the limit of detection for the cell culture system was 10^4 – 10^5 genome copies, and therefore, as with the previous study, viable virus remaining in the minced pork may not have been detected in the cell culture system. In addition, the different food preparations between the studies may have influenced viral stability, as could the different strains of G3 HEV used. The initial viral titre used could also have influenced the results and explain why a longer treatment time was needed in some studies.

Table 2.5 A summary of studies investigating thermal inactivation treatments for HEV in foodstuffs

Study	Food stuff	Cooking method	Temperature (°C)	Time (mins)	Measurement of inactivation	HEV inactivated?
Barnaud *et al.*, (2012)	Pâté preparation (spiked with 10^8 HEV genome copies)	Water bath	62	5	Intravenous administration to pigs	No
				20		No
				120		No
			68	5		No
				10		No
				20		No
			71	5		No
				10		No
				20		Yes
Feagins *et al.*, (2008)	Pig liver (naturally infected)	Incubation	56	60	Oral administration to pigs	No
		Boiling	≥71 (internal)	5		Yes
		Stir fry	≥71 (internal)	5		Yes
Imagawa *et al.*, (2018)	Minced meat (spiked with 10^{10} HEV genome copies)	Boiling or roasting	63	1	Cell culture	No
				5		No
				30		Yes
			65	1		No
				5		Yes
			70	1		No
				5		Yes

Further research is clearly required to investigate HEV inactivation within foods. This may require a more efficient cell culturing method and an assessment of different foods, cooking methods and HEV strains. However, taking the results of studies conducted in non-food and food (pork product) samples together, a conservative measure would be to cook pork products for longer than 20 minutes at temperatures higher than 72°C.

In addition to the potential for HEV to survive some cooking processes, raw pork products are used in some consumables. Raw blood products are commonly used in ready-cooked foods such as processed ham (fibrinogen) and other blood proteins are used as food additives such as emulsifiers. Spray-dried plasma powder (SDPP) is also used in domestic and farm animal foods but this has some heat processing prior to use. SDPP is commonly fed to weaned piglets; although a previous study reported no transmission of HEV in pigs fed spray dried plasma products that were positive for HEV RNA (Pujols *et al.*, 2014), and therefore the heating of spray dried plasma may be sufficient to inactivate the virus present. However, as other porcine products are not subjected to heat processes before they are used, they could constitute a transmission risk to humans through use in food. A study conducted in 2017 found that of 36 liquid porcine products derived from blood, 33 were positive for HEV RNA, and seven of 24 spray dried plasma products were also positive for HEV RNA (Boxman *et al.*, 2017). This is especially significant when blood products from multiple animals are commonly pooled together, meaning that products from one viraemic animal could contaminate a batch and lead to widespread HEV transmission through many different food products.

Pork product consumption has been considered to be a major risk factor in the development of HEV due to the connection to foodborne outbreaks and the fact that HEV in pork products can reach high levels (e.g. $7x10^4$ genome copies/g in liver pâté in the Netherlands, (Boxman *et al.*, 2019)). However, pigs are not the only animals consumed which can act as reservoirs for the virus.

HEV in other Land Animals and Animal Products

In addition to pigs, deer have been reported to be infected with HEV in many different countries (summarised in Table 2.6). It is important to identify the

transmission risk that deer may have to humans, as HEV outbreaks have been directly linked to the consumption of raw deer meat. For example, in Japan, multiple people who had consumed raw Sika deer meat contracted HEV 6-7 weeks later (typical of the HEV incubation period), with the HEV sequence confirmed as 100% identical between the meat and infected patients (Tei *et al.*, 2003). With the presence of HEV in deer being so variable between studies (perhaps due to study limitations such as sample size), further research is required to identify the level of active HEV infection within deer populations through larger prevalence studies. However, with the number of countries that have detected HEV in deer, and the occurrence of foodborne outbreaks from deer meat, deer could be acting as another reservoir for HEV.

Table 2.6 Summary of studies investigating the prevalence of HEV in deer

Study	Location	Deer species	ELISA observed seroprevalence	RT-PCR prevalence
Weger *et al.*, (2017)	Canada	*Odocoileus virginianus* (White-tailed deer)	8.8%	ND†
		Odocoileus hemionus (Mule deer)	4.5%	ND†
		Rangifer tarandus groenlandicus (Barren-ground caribou)	1.7%	ND†
		Rangifer tarandus (Woodland caribou)	5.2%	ND†
Zhang *et al.*, (2015)	China	*Cervus nippon* (Sika deer)	5.4%	ND†
Anheyer-Behmenburg *et al.*, (2017)	Germany	*Capreolus* (Roe deer)	0.0%	6.4%
		Cervus elaphus (Red deer)	0.0%	3.5%
Neumann *et al.*, (2016)		*Cervus elaphus* (Red deer)	2.5%	3.7%
		Capreolus (Roe deer)	6.5%	0.0%
Reuter *et al.*, (2009)	Hungary	*Capreolus* (Roe deer)	ND†	12.2%
Di Bartolo *et al.*, (2017)	Italy	*Cervus elaphus* (Red deer)	13.6%	11.0%

Sonoda *et al.*, (2004)	Japan	*Cervus nippon* (Sika deer)	2.0%	0.0%
Matsuura *et al.*, (2007)		*Cervus nippon* (Sika deer)	2.6%	0.0%
Tomiyama *et al.*, (2009)		*Cervus nippon yesoensis* (Yezo deer)	34.8%	ND†
Spancerniene *et al.*, (2018)	Lithuania	*Capreolus* (Roe deer)	ND†	22.6%
		Cervus elaphus (Red deer)	ND†	6.7%
Medrano *et al.*, (2012)	Mexico	*Odocoileus virginianus* (White-tailed deer)	62.7%	ND†
Rutjes *et al.*, (2010)	The Netherlands	*Cervus elaphus* (Red deer)	8.0%	15.0%
		Capreolus (Roe deer)	12.5%	0.0%
Boadella *et al.*, (2010)	Spain	*Cervus elaphus* (Red deer)	10.4%	N/A*
Kukielka *et al.*, (2016)		*Cervus elaphus* (Red deer)	12.9%	11.1%
Roth *et al.*, (2016)	Sweden	*Capreolus* (Roe deer)	7.0%	0.0%
		Cervus elaphus (Red deer)	7.0%	0.0%

* did not test full sample population which were tested for seropositivity; ND† not done

There have been reports of HEV infections in cattle (*Bos taurus*) from China, where both antibody seroprevalence and HEV RNA of G4 has been identified in multiple studies. Hu and Ma, (2010) showed the presence of G4 HEV RNA in 8.8% of cattle from Xinjiang Autonomous Region. A subsequent study then

identified that 37.1% of tested dairy cows in Yunnan Province were HEV RNA positive, and that 100% of the HEV-positive cows were producing milk that contained HEV RNA (Huang *et al.*, 2016). A further study in Shandong Province also found 3% of yellow cattle to be G4 HEV RNA positive, with 47% seroprevalent for anti-HEV antibodies (Yan *et al.*, 2016). A study in Turkey identified HEV from G1, G3 and G4 in 20.3% of raw milk samples from various domestic animals (cows, sheep, goats, donkeys) (Demirci *et al.*, 2019). Other studies investigating HEV in cattle have produced negative or mixed results; a study in Beijing, China identified 29.4% of cattle were seroprevalent for HEV, but no HEV RNA could be detected (Chang *et al.*, 2009). A study in Burkina Faso also found 26.4% of 72 cattle to be seroprevalent for HEV (Ouoba *et al.*, 2019). Another study in Germany testing 400 milk samples found no evidence of HEV RNA; although G4 HEV is much less commonly reported in Europe than in Asia (Baechlein and Becher, 2017). Likewise, a study in Belgium also found no evidence of HEV RNA in cow milk or faeces (Vercouter *et al.*, 2018). In the USA, Yugo *et al.*, (2019) identified that of 983 cows, 20.4% were seroprevalent for anti-HEV antibodies; however HEV RNA could not be detected in any of the cows. The authors concluded that this may have been because of an antigenically similar relative to HEV rather than due to HEV itself, which could be possible as G4 HEV is not thought to be endemic to the USA; however, this would call into question the specificity of ELISA assays and studies investigating HEV seroprevalence. Notwithstanding the significant number of studies with negative findings, these results are concerning as meat and dairy from cows are consumed worldwide by humans, and the possibility that cows could be a HEV reservoir could have a significant impact on our understanding of HEV transmission to humans. More research worldwide is therefore needed to identify which HEV genotypes are capable of infecting cattle, and to find the prevalence of HEV in cattle and dairy products. This will help to identify the risk of transmission of HEV from cattle to humans.

Goats have also been shown to be potential reservoirs for HEV infection, which is important due to goat meat, milk, and cheese production. In Italy in 2016, 9.2% of goat faecal samples from six farms were found to be positive for HEV RNA, belonging to G3 strains which were highly related to strains found in pigs and humans (Di Martino *et al.*, 2016). Also, in Yunnan province, China, Long *et*

al., (2017) found 70.3% of goat faecal samples to be positive for HEV RNA, with milk samples from these animals also positive for HEV RNA. The strains obtained from these animals were from G4 HEV; and the Huang *et al.*, (2016) and Long *et al.*, (2017) studies highlight that farms with mixed animals may demonstrate a higher risk of HEV transmission. Another study in the Tai'an Region in China identified 4% of goat livers to be HEV positive, with G4 HEV that was similar to cow HEV detected in the same region (Li *et al.*, 2017). In Turkey, 18.5% of goat milk samples were reported positive for HEV RNA (Demirci *et al.*, 2019). Meanwhile in Burkina Faso, 28.4% of 81 goats were found to have anti-HEV antibodies (Ouoba *et al.*, 2019). In the USA however, Sanford *et al.*, (2013) suggested that a HEV-related agent was causing HEV in goats after discovering a seroprevalence of 16% but a lack of any HEV RNA; however this again calls into question the accuracy of HEV ELISAs. In addition, no HEV RNA was detected by conventional RT-PCR in goats that had been experimentally infected with three different HEV strains from G1, G3 and G4 in this study; however the sensitivity of this PCR may be lower than previously reported HEV PCR assays, as assays which target a larger amplicon are generally observed to have lower sensitivity (Debode *et al.*, 2017), and qRT-PCR has generally been observed to be more sensitive for amplicon detection of HEV and other amplicon targets (Zhao *et al.*, 2007; Zemtsova et al., 2015).

Dromedary camels have been implicated in the transmission of G7 HEV to humans. In one case, a patient who regularly consumed camel meat and milk contracted chronic G7 HEV after a liver transplant (Lee *et al.*, 2016). This chronicity is likely to have been opportunistic and influenced by immunosuppressive medication to prevent organ rejection. In a separate paper, HEV was demonstrated to be seroprevalent in 23.1% of dromedary camels which originated in Sudan and Saudi Arabia (El-Kafrawy *et al.*, 2020). Due to the recent discovery of this genotype of HEV, and its implication in human infection, further research is warranted to investigate how widespread camel HEV is within countries which regularly consume camel products to determine the risk such products may have for the foodborne transmission of HEV.

Rabbits and related species e.g., hares are also gaining increasing attention for their potential to transfer HEV to humans through consumption of meat. In

France, five cases of rabbit HEV (defined within Orthohepevirus A, G3ra) were identified in confirmed HEV-positive patients out of 919 from 2015-2016 (Abravanel *et al.*, 2017). Several countries have identified rabbits to be seroprevalent and RNA positive for HEV (table 2.7). The number of observations and the apparent ability of humans to contract rabbit HEV suggests that it is a source of zoonotic HEV transmission. However, with most human cases worldwide belonging to other genotypes and sub-genotypes, rabbits are likely to be the cause of only a minority of cases. Studies have shown mixed results in terms of the ability of rabbits to carry other sub-genotypes of HEV. Zhang *et al.*, (2017) has shown that though rabbits are capable of carrying sub-genotype 3ra, attempts to cause infections with another sub-genotype (3b) were unsuccessful; however, Hammerschmidt *et al.*, (2017) identified a wild rabbit with HEV sub-genotype 3g. Further research is therefore needed to identify which genotypes or sub-genotypes of HEV are capable of infecting rabbits. An interesting observation from the studies summarised in table 2.7 is that concordance in HEV prevalence between different samples from the same animals is often lacking. For example, Burt *et al.*, (2016) found that 60% of liver samples from 32 animals were HEV positive, however only 16% of these 32 animals were faecally shedding the virus. It may therefore be wise to identify standardised testing methods worldwide for identification of infected animals, with a decision made on what samples to test and which assays are best to use, to avoid underestimating HEV prevalence.

Table 2.7 A summary of studies identifying HEV (genotype 3ra) in rabbits and hares

Country	Study	Seroprevalence	RNA prevalence
Burkina Faso	Ouoba *et al.*, (2019)	60.0% of 100 rabbits, 52.6% of 19 hares	ND†
Canada	Xie *et al.*, (2017)	ND†	5.0% of 63 companion rabbit faecal samples, 0.90% of 114 commercial rabbit faecal samples
China	Geng *et al.*, (2011a)	54.6% of 119 farmed rabbits	7.0% of 119 farmed rabbit serum samples
	Geng *et al.*, (2011b)	15.4% of 1094 farmed rabbits	2.0% of 1094 farmed rabbit serum samples
	Xia *et al.*, (2015)	ND†	5.0% of 492 rabbit faecal samples
	Li *et al.*, (2020b)	ND†	15.0% of 120 rabbit faecal samples
	Li *et al.*, (2020c)	7.1% of 70 farmed rabbits	11.4% of 70 farmed rabbit faecal samples
France	Izopet *et al.*, (2012)	ND†	7.0% of 200 farmed rabbit bile samples, 23.0% of 205 wild rabbit liver samples
Germany	Eiden *et al.*, (2016)	30.8% of 13 wild rabbits	30.8% of 13 wild rabbit serum samples

	Hammerschmidt *et al.*, (2017)	37.3% of 164 wild rabbits, 2.2% of 669 wild hares	17.1% of wild rabbit serum samples, 0.0% of wild hare serum samples
	Ryll *et al.*, (2018)	25% of 72 wild rabbits	34.7% of 72 wild rabbit liver samples
	Corman *et al.*, (2019)	0.04% of 2389 wild hares	2.6% of 2389 wild hare serum samples
Italy	Di Bartolo *et al.*, (2016)	3.4% of 206 farmed rabbits, 6.6% of 122 pet rabbits	0.0% of 7 IgG positive farmed rabbit serum samples, 0.0% of 122 pet rabbit serum samples
The Netherlands	Burt *et al.*, (2016)	ND†	23.0% of 35 petting farm rabbit faecal samples, 0% of 10 farmed rabbit liver and faecal samples, 60.0% of 32 wild rabbit liver samples and 16% of wild rabbit faecal samples
Poland	Bigoraj *et al.*, (2020)	6.0% of 482 farmed rabbits	14.9% of 482 farmed rabbit liver samples
South Korea	Ahn *et al.*, (2017)	ND†	6.4% of 264 rabbit faecal samples
USA	Cossaboom *et al.*, (2011)	36.5% of 85 rabbits	16.5% of 85 serum samples, 15.3% of 85 faecal samples

ND† not done

Despite identifying that animal product consumption is a risk factor in the transmission of HEV it is quite possible that there are other food transmission routes. One study in the Netherlands showed that though seroprevalence of HEV antibodies was higher in meat-eaters (22.8%), vegetarians still displayed a relatively high seroprevalence (13.8%) (Slot *et al.*, 2017). This suggests that either they became HEV-positive before becoming vegetarian through animal meat, or they were infected through other transmission routes. Figure 2.2 shows the known and theorised routes of HEV transmission. One transmission route, which is much more tightly controlled now, was the transmission of HEV through blood transfusion (Hewitt *et al.*, 2014), which may be one way to explain the seroprevalence levels in vegetarians. Another explanation could be consumption of dairy products such as milk. It is also possible to contract HEV through organ transplant with an infected organ (Pas *et al.*, 2012), and organ transplant-associated cases commonly result in chronic infections due to immunosuppression medication.

Contamination of the Aquatic Environment

In addition to medical routes of transmission, it is important to consider the impact that HEV within animal farm run off and sewage has on the aquatic environment. It has been suggested that human and farm sewage may have a part to play in other HEV transmission routes, potentially through farm run-off from animal slurry stores or application of animal slurry to crops; and through contamination of surface waters used for irrigation and shellfish farms. Raw human sewage collected at two week intervals in 2014-15 from a sewer which serves the whole of Edinburgh was found to contain HEV in 93% of the samples collected (Smith et al., 2016), and many other countries have also detected HEV in human sewage influent, such as Spain, Switzerland, Portugal and France (Clemente-Casares *et al.*, 2009; Clemente-Casares *et al.*, 2003; Rodriguez-Manzano *et al.*, 2010; Matos *et al.*, 2018; Masclaux *et al.*, 2013). This could therefore mean that when storm overflows discharge into water courses such as rivers and seas, HEV contamination can occur. Because there are many different types of wastewater treatment practises, and many combinations of practices between wastewater treatment plants, it is difficult to know which wastewater treatment plants will be more effective at removing viruses from wastewater. However, other viruses such as adenovirus and

norovirus are commonly found in treated sewage (Bofill-Mas *et al.*, 2006; Campos *et al.*, 2016). It is therefore possible that in addition to storm overflows, inadequate treatment of sewage could result in HEV pollution of the aquatic environment, especially considering that HEV is a single stranded RNA non-enveloped virus like norovirus. Pig farm and slaughterhouse sewage has also been found to be positive for HEV in multiple countries, for example, HEV RNA was detected in sewage from one of twelve slaughterhouses in Spain (Pina *et al.*, 2000), whilst 75% of swine slurry samples collected from Italian pig farms were HEV positive (La Rosa *et al.*, 2017a), and both fresh swine faecal material and pooled stored slurry from pig farms in the USA were shown to contain HEV (Kasorndorkbua *et al.*, 2005). Human sewage, pig farm run off and abattoir outflows could also therefore be contaminating water courses with HEV, which is supported by studies in Italy, the Philippines and Cambodia, showing river water contamination with HEV (Iaconelli *et al.*, 2015; Li *et al.*, 2014; Baez *et al.*, 2017; Rodriguez-Manzano *et al.*, 2010).

Countries within the European Union must conform to EU regulations on how farm manure, animal carcasses and digestive tract content are processed, transported, stored, used as crop fertiliser, and disposed of. The sewage and wastewater that originates on farms must either be discharged to public sewers or treated in a sewage treatment plant on the farm before the effluent can be discharged to surface waters, and a permit is required for the processing and disposal of sewage and wastewater in this way. However, it is possible for farms within EU countries to use sewage and slurry that has been produced on a farm to be spread on crops at the same farm, without prior processing, for the sake of fertiliser or soil quality improvement (European Commission, 2001). Accordingly, the United States also allows manure originating on one farm to be spread on crops from that farm (Environmental Protection Agency, NA). However, manure use and farm practises are likely to be more diverse and potentially problematic in countries within Asia, Africa, and South America.

Previous studies have shown that sewage treatment processes such as long term fermentation and composting are likely to be capable of removing HEV from sewage (García *et al.*, 2014). A study in Switzerland also identified HEV positive influent samples from wastewater treatment plants, but no HEV positive effluent samples, suggesting effective wastewater treatment using a cleaning

and activated sludge process (Masclaux *et al.*, 2013). However, Loisy-Hamon and Leturnier, (2015) detected HEV in treated pig sewage samples from France that had been treated using one of: sawdust composting, slurry dehydration or anaerobic digestion. Other studies have found that river water close to pig farms and pig processing plants had been contaminated with HEV, for example, in Scotland and Italy (Crossan *et al.*, 2012; Idolo *et al.*, 2013; Marcheggiani *et al.*, 2015). Therefore, it is possible that leachate (liquid leaching from solids into the environment) from stored manure and yard run off from farms and abattoirs may be polluting surface waters such as rivers. However, it is unknown whether all of the virus leaching into the environment is viable – for example, viral RNA detected in treated sewage may not indicate viable virus, but remaining RNA.

Crop Contamination

Surface waters from sources such as rivers and groundwater are commonly used as crop irrigation sources throughout the world (Food and Agriculture Organisation, 2011; Food and Agriculture Organisation, 2016). Due to the potential contamination of such water with HEV and other pathogens from faecal matter (whether from human or animal sources), this could cause contamination of irrigated crops. Animal waste (that can potentially be contaminated with HEV as shown above) is also used as crop fertiliser for farms. A small number of studies have found some evidence of crop contamination with HEV. In France, two out of 230 herb and spice samples were positive for HEV RNA (Loisy-Hamon and Leturnier, 2015), a study testing 125 lettuce samples from Greece, Serbia and Poland detected four positive samples (Kokkinos *et al.*, 2012), and in Italy, six of 911 “pre-washed and ready to eat” vegetable samples tested positive for HEV RNA (Terio *et al.*, 2017). Another study in four European countries (Czech Republic, Finland, Poland and Serbia) also detected HEV RNA in one frozen raspberry sample of 38 tested (Maunula *et al.*, 2013). However, it is important to note that no foodborne outbreaks of HEV from contaminated crops have been reported, and the quantities of virus found on the crops is also low enough to call into question whether they would cause illness when consumed. It is also unknown whether the HEV RNA detected originated from viable virus.

HEV in Bivalve Shellfish and other Aquatic Animals

Bivalve molluscs are filter feeding organisms, meaning that they can accumulate and concentrate pathogens from their environment within their tissues. In the EU, bivalve shellfish are tested regularly for faecal contamination, using a faecal indicator, *Escherichia coli*, in accordance with food safety regulations. However, studies have shown that though it functions well as a bacterial faecal indicator, *E. coli* can be a poor indicator of the presence of faecally-derived viruses. Lowther *et al.*, (2012) found that norovirus RNA was present in 76.2% of total UK oyster samples from commercial harvesting areas, with 73.9% of those samples giving *E. coli* results compliant with the end product standard of ≤230 *E. coli*/100g shellfish flesh. Norovirus within oysters is linked to human faecal pollution that has originated from storm overflows and CSOs, or sewage that has received insufficient treatment (Campos *et al.*, 2013; Campos *et al.*, 2016). CSOs release untreated sewage into surface water to prevent overflows within mains drainage, but outfall events can last for several hours or days and are often poorly monitored (Marine Conservation Society, 2011). Considering that farm or abattoir run off, combined sewer overflows, and inadequately treated sewage could be polluting watercourses with HEV, it is also possible for aquatic organisms, such as shellfish, to be affected by HEV contamination. Indeed, studies around the world have found HEV to be present within bivalve shellfish, these are summarised in table 2.8. The study by Rivadulla *et al.*, (2019) also showed shellfish to have as much as 1.1×10^5 RNA copies per gram of shellfish tissue, which is close to the pig ID50, but the human infectious dose is still unknown. It is important to note however, that not all RNA found in the shellfish may have been associated with viable virus. To date, there have been no proven foodborne outbreaks of HEV from shellfish consumption, although an outbreak of HEV on a cruise ship was theorised to have been caused by consumption of shellfish on the basis of a retrospective risk analysis (Said *et al.*, 2009).

Table 2.8 The presence of HEV in shellfish in different countries

Location	Study	Percentage of shellfish HEV-positive
China	Gao *et al.*, (2015)	17.5% of 126 shellfish samples* of various species from production areas
Denmark	Krog et al., (2014)	0% of 29 mussel samples* from 19 production areas
France	Grodzki *et al.*, (2014)	0% of 286 shellfish samples* of various species from two production areas
Italy	La Rosa *et al.*, (2018)	2.6% of 384 shellfish samples* of various species from production areas
Japan	Li et al., (2007)	6.3% of 32 Yamato-Shijimi clam samples*
Scotland	Crossan *et al.*, (2012)	85.4% of 48 individual wild mussels
	O'Hara *et al.*, (2018)	2.9% of 310 retail shellfish samples* (mussels and oysters)
Spain	Mesquita *et al.*, (2016)	14.8% of 81 mussel samples* from a production area
	Rivadulla *et al.*, (2019)	24.4% of 164 mussel, clam, and cockle samples*

*Where the study states that samples of shellfish were tested, it was either stated or assumed in each publication that each "sample" would have been formed by ten or more shellfish individuals and is therefore technically a pooled sample.

HEV has also been found in other aquatic organisms, including dolphins, who present clinical symptoms of HEV infection. A study of 31 dolphins at the National Aquarium, Cuba, found that 32.2% of their dolphins were seroprevalent for HEV during two different studies (Villalba et al., 2017). The cause of the infections within the dolphins was unknown, however, it is possible that contamination of food items such as fish may be the cause, making an investigation of the presence of HEV in such animals important to determine whether there is any risk of HEV to humans from the consumption of fish. It may also be important to investigate the presence of HEV in aquatic mammals as they are used as a food source in some countries.

Conclusion

In summary, the host range of HEV appears to be diverse, having been found within pig, deer, rabbit, cattle, goat, and camels, amongst other animals. HEV has also been detected in shellfish meat because of contamination of their growing waters. Therefore, there is a risk of contracting HEV from undercooked products from these animals (although it is important to note that epidemiological evidence of foodborne transmission for many of these is currently lacking), and there is also potential for other livestock species to be unidentified hosts for the virus. Generally, foodstuffs containing raw meat or shellfish products are more likely to cause a foodborne infection than cooked foods or crops due to no thermal inactivation of the virus through cooking. Cooking in such a way that a minimum internal temperature of 72°C is reached for at least twenty minutes is likely to completely inactivate any HEV present, however this is likely to produce unwanted deterioration of organoleptic qualities in some risky food types e.g., shellfish.

In addition to animal meat, milk from cows, sheep, goats, donkeys, and camels has also been found to contain HEV in some countries, but studies investigating the presence of HEV in milk are much more limited. Because of this, the true risk of HEV transmission from animal milk is yet unknown and requires further research. However, if proven to be a prominent transmission route for the virus, a worrying consideration is that high temperature short time (HTST) pasteurisation of milk products, which is commonly used in the UK and USA,

may be insufficient to reduce infectious HEV within milk, as generally the heat treatment used for HTST pasteurisation is only 72°C for 15 seconds. Other pasteurisation methods, such as ultra-high temperature pasteurisation which utilise treatments of around 135°C for 2-4 seconds, should be more capable of removing viable virus from milk products due to the higher temperature.

Though crops can also become contaminated with HEV, it seems that the risk of contracting HEV from them is much less likely, as confirmed outbreaks from crops have not been identified, and the HEV RNA prevalence and copies of viral RNA present are lower for these foods. However, it may be safe to conduct further research into the contamination of irrigation water, and the presence of HEV in crops from other countries to better assess the risk of contracting HEV from crop contamination.

It has also been shown that marine mammals can be infected with HEV, which is concerning both from an ecosystem and a seafood point of view. If marine mammals are becoming infected naturally, it could be possible that fish and other seafood also become contaminated or infected. Considering that shellfish in many countries have been found to be contaminated with HEV, this is perhaps something that warrants further investigation.

Due to mixed conclusions between and within countries about HEV presence within different hosts or matrices, it appears that there needs to be not only standardised and improved methods for the purpose of HEV detection, but also that further research through larger studies around the world are required to identify the full host range of HEV and the risk of each potential host to transmit the virus to humans (through food or other means). In particular, the suggestion that a HEV-related virus may be causing seroprevalence estimates to be higher than they genuinely are requires investigation.

Further studies identifying both the seroprevalence and the presence of HEV through ELISA and RT-PCR techniques respectively (or similar techniques identifying RNA presence) would be best equipped to identify both the prevalence of the virus within animal populations and the number of active infections within the populations at that point in time. However, sequencing

technologies such as nanopore RNA sequencing within human and animal populations would also be useful to identify similarities between HEV sequences, enabling the identification of infection sources. Some studies investigating the evolution of the virus have already been performed, but are often biased by the large amount of HEV sequences derived from humans (Forni *et al.*, 2018).

Investigations of HEV within food and environmental matrices using whole genome sequencing approaches have been limited so far due to the general observation of low genome copy numbers and fragmented HEV RNA within these matrices. However, techniques utilising methods such as multiplexing RNA extracted samples to obtain a full genome from multiple amplicons, followed by MinION next generation sequencing, which has been successfully applied to sequencing of low levels of zika virus (Quick *et al.*, 2017), could be instrumental in future efforts to identify low levels of HEV in a variety of matrices, including foods.

Globally, HEV is an under-recognised viral threat, which causes an increasing case incidence annually. The best way to tackle a virus is to understand its sources and modes of transmission. Therefore, further research and better understanding of HEV will allow a better assessment of the risk that animal products and other foods may have in the transmission of HEV to humans. In turn, this may allow the introduction of legislative controls to prevent and control the spread of the virus.

References

Abravanel, F., Lhomme, S., El Costa, H., Schvartz, B., Peron, J.-M., Kamar, N. and Izopet, J. (2017) 'Rabbit hepatitis E virus infections in humans, France', *Emerging infectious diseases,* 23(7), pp. 1191.

Ahn, H. S., Park, B. J., Han, S. H., Kim, Y. H., Kim, D. H., Kim, B. S., Lee, J. B., Park, S. Y., Song, C. S. and Lee, S. W. (2017) 'Prevalence and genetic features of rabbit hepatitis E virus in Korea', *Journal of medical virology,* 89(11), pp. 1995-2002.

Andraud, M., Dumarest, M., Cariolet, R., Aylaj, B., Barnaud, E., Eono, F., Pavio, N. and Rose, N. (2013) 'Direct contact and environmental contaminations are responsible for HEV transmission in pigs', *Veterinary research,* 44(1), pp. 102.

Anheyer-Behmenburg, H. E., Szabo, K., Schotte, U., Binder, A., Klein, G. and Johne, R. (2017) 'Hepatitis E virus in wild boars and spillover infection in red and roe deer, Germany, 2013–2015', *Emerging infectious diseases,* 23(1), pp. 130.

Arankalle, V. A., Chadha, M. S. and Chobe, L. P. (1999) 'Long-term serological follow up and cross-challenge studies in rhesus monkeys experimentally infected with hepatitis E virus', *Journal of hepatology,* 30(2), pp. 199-204.

Baechlein, C. and Becher, P. (2017) 'No evidence for zoonotic hepatitis E virus infection through dairy milk in Germany', *Hepatology,* 65(1), pp. 394-395.

Baechlein, C., Schielke, A., Johne, R., Ulrich, R. G., Baumgaertner, W. and Grummer, B. (2010) 'Prevalence of Hepatitis E virus-specific antibodies in sera of German domestic pigs estimated by using different assays', *Veterinary microbiology,* 144(1), pp. 187-191.

Baez, P. A., Lopez, M. C., Duque-Jaramillo, A., Pelaez, D., Molina, F. and Navas, M.-C. (2017) 'First evidence of the Hepatitis E virus in environmental waters in Colombia', *PloS one,* 12(5), pp. e0177525.

Barnaud, E., Rogée, S., Garry, P., Rose, N. and Pavio, N. (2012) 'Thermal inactivation of infectious hepatitis E virus in experimentally contaminated food', *Applied and environmental microbiology,* 78(15), pp. 5153-5159.

Berto, A., Martelli, F., Grierson, S. and Banks, M. (2012) 'Hepatitis E virus in pork food chain, United Kingdom, 2009–2010', *Emerging infectious diseases,* 18(8), pp. 1358.

Bigoraj, E., Kozyra, I., Kwit, E. and Rzeżutka, A. (2020) 'Detection of hepatitis E virus (rabbit genotype) in farmed rabbits entering the food chain', *International Journal of Food Microbiology,* 319, pp. 108507.

Boadella, M., Casas, M., Martín, M., Vicente, J., Segalés, J., De la Fuente, J. and Gortázar, C. (2010) 'Increasing contact with hepatitis E virus in red deer, Spain', *Emerging infectious diseases,* 16(12), pp. 1994.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M. and Girones, R. (2006) 'Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices', *Appl. Environ. Microbiol.,* 72(12), pp. 7894-7896.

Bouwknegt, M., Lodder-Verschoor, F., Van Der Poel, W. H., Rutjes, S. A. and De Roda Husman, A. M. (2007) 'Hepatitis E virus RNA in commercial porcine

livers in The Netherlands', *Journal of food protection,* 70(12), pp. 2889-2895.

Bouwknegt, M., Rutjes, S. A., Reusken, C. B., Stockhofe-Zurwieden, N., Frankena, K., de Jong, M. C., de Roda Husman, A. M. and van der Poel, W. H. (2009) 'The course of hepatitis E virus infection in pigs after contact-infection and intravenous inoculation', *BMC veterinary research,* 5(1), pp. 7.

Boxman, I. L., Jansen, C. C., Hägele, G., Zwartkruis-Nahuis, A., Cremer, J., Vennema, H. and Tijsma, A. S. (2017) 'Porcine blood used as ingredient in meat productions may serve as a vehicle for hepatitis E virus transmission', *International journal of food microbiology,* 257, pp. 225-231.

Boxman, I. L., Jansen, C. C., Hägele, G., Zwartkruis-Nahuis, A., Tijsma, A. S. and Vennema, H. (2019) 'Monitoring of pork liver and meat products on the Dutch market for the presence of HEV RNA', *International journal of food microbiology,* 296, pp. 58-64.

Burri, C., Vial, F., Ryser-Degiorgis, M. P., Schwermer, H., Darling, K., Reist, M., Wu, N., Beerli, O., Schöning, J. and Cavassini, M. (2014) 'Seroprevalence of hepatitis E virus in domestic pigs and wild boars in Switzerland', *Zoonoses and public health,* 61(8), pp. 537-544.

Burt, S. A., Veltman, J., Hakze-van der Honing, R., Schmitt, H. and van der Poel, W. H. (2016) 'Hepatitis E virus in farmed rabbits, wild rabbits and petting farm rabbits in the Netherlands', *Food and environmental virology,* 8(3), pp. 227-229.

Campos, C. J., Avant, J., Lowther, J., Till, D. and Lees, D. N. (2016) 'Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes', *Water research,* 103, pp. 224-232.

Campos, C. J., Kershaw, S. R. and Lee, R. J. (2013) 'Environmental influences on faecal indicator organisms in coastal waters and their accumulation in bivalve shellfish', *Estuaries and coasts,* 36(4), pp. 834-853.

Centers for Disease Control and Prevention. (2020) *Hepatitis E Questions and Answers for Health Professionals*. Available at: https://www.cdc.gov/hepatitis/hev/hevfaq.htm (Accessed: 15 December 2020).

Chang, Y., Wang, L., Geng, J., Zhu, Y., Fu, H., Ren, F., Li, L., Wang, X. and Zhuang, H. (2009) 'Zoonotic risk of hepatitis E virus (HEV): A study of HEV infection in animals and humans in suburbs of Beijing', *Hepatology Research,* 39(12), pp. 1153-1158.

Clemente-Casares, P., Pina, S., Buti, M., Jardi, R., Martín, M., Bofill-Mas, S. and Girones, R. (2003) 'Hepatitis E virus epidemiology in industrialized countries', *Emerging infectious diseases,* 9(4), pp. 449.

Clemente-Casares, P., Rodriguez-Manzano, J. and Girones, R. (2009) 'Hepatitis E virus genotype 3 and sporadically also genotype 1 circulate in the population of Catalonia, Spain', *Journal of water and health,* 7(4), pp. 664-673.

Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal, V., Gallian, P., Heyries, L., Raoult, D. and Gerolami, R. (2010) 'Pig liver sausage as a source of hepatitis E virus transmission to humans', *The Journal of infectious diseases,* 202(6), pp. 825-834.

Corman, V. M., Hilgensloh, L., Voigt, U., Marklewitz, M., Siebert, U., Drosten, C. and Drexler, J. F. (2019) 'Hepatitis E Virus Infection in European Brown Hares,

Germany, 2007–2014', *Emerging infectious diseases,* 25(6), pp. 1233.

Cossaboom, C. M., Córdoba, L., Dryman, B. A. and Meng, X.-J. (2011) 'Hepatitis E virus in rabbits, Virginia, USA', *Emerging infectious diseases,* 17(11), pp. 2047.

Crossan, C., Baker, P. J., Craft, J., Takeuchi, Y., Dalton, H. R. and Scobie, L. (2012) 'Hepatitis E virus genotype 3 in shellfish, United Kingdom', *Emerging infectious diseases,* 18(12), pp. 2085.

Dalton, H., Thurairajah, P., Fellows, H., Hussaini, H., Mitchell, J., Bendall, R., Banks, M., Ijaz, S., Teo, C. G. and Levine, D. (2007) 'Autochthonous hepatitis E in southwest England', *Journal of viral hepatitis,* 14(5), pp. 304-309.

De Deus, N., Casas, M., Peralta, B., Nofrarías, M., Pina, S., Martín, M. and Segalés, J. (2008) 'Hepatitis E virus infection dynamics and organic distribution in naturally infected pigs in a farrow-to-finish farm', *Veterinary Microbiology,* 132(1), pp. 19-28.

de Oya, N. J., de Blas, I., Blázquez, A.-B., Martín-Acebes, M. A., Halaihel, N., Gironés, O., Saiz, J.-C. and Escribano-Romero, E. (2011) 'Widespread distribution of hepatitis E virus in Spanish pig herds', *BMC research notes,* 4(1), pp. 412.

Debode, F., Marien, A., Janssen, É., Bragard, C. and Berben, G. (2017) 'The influence of amplicon length on real-time PCR results', *Base*.

Demirci, M., Yiğin, A., Ünlü, Ö. and Kılıç, S. A. (2019) 'Detection of HEV RNA amounts and genotypes in raw milks obtained from different animals', *Mikrobiyoloji bulteni,* 53(1), pp. 43-52.

Di Bartolo, I., Angeloni, G., Ponterio, E., Ostanello, F. and Ruggeri, F. M. (2015) 'Detection of hepatitis E virus in pork liver sausages', *International journal of food microbiology,* 193, pp. 29-33.

Di Bartolo, I., De Sabato, L., Marata, A., Martinelli, N., Magistrali, C., Monini, M., Ponterio, E., Ostanello, F. and Ruggeri, F. (2016) 'Serological survey of hepatitis E virus infection in farmed and pet rabbits in Italy', *Archives of virology,* 161(5), pp. 1343-1346.

Di Bartolo, I., Diez-Valcarce, M., Vasickova, P., Kralik, P., Hernandez, M., Angeloni, G., Ostanello, F., Bouwknegt, M., Rodríguez-Lázaro, D. and Pavlik, I. (2012) 'Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010', *Emerging infectious diseases,* 18(8), pp. 1282.

Di Bartolo, I., Ponterio, E., Angeloni, G., Morandi, F., Ostanello, F., Nicoloso, S. and Ruggeri, F. (2017) 'Presence of hepatitis E virus in a RED deer (Cervus elaphus) population in Central Italy', *Transboundary and emerging diseases,* 64(1), pp. 137-143.

Di Bartolo, I., Ponterio, E., Castellini, L., Ostanello, F. and Ruggeri, F. M. (2011) 'Viral and antibody HEV prevalence in swine at slaughterhouse in Italy', *Veterinary microbiology,* 149(3-4), pp. 330-338.

Di Martino, B., Di Profio, F., Melegari, I., Sarchese, V., Robetto, S., Marsilio, F. and Martella, V. (2016) 'Detection of hepatitis E virus (HEV) in goats', *Virus research,* 225, pp. 69-72.

Dähnert, L., Eiden, M., Schlosser, J., Fast, C., Schröder, C., Lange, E., Gröner, A., Schäfer, W. and Groschup, M. H. (2018) 'High sensitivity of domestic pigs to

intravenous infection with HEV', *BMC veterinary research,* 14(1), pp. 381.

Eiden, M., Vina-Rodriguez, A., Schlosser, J., Schirrmeier, H. and Groschup, M. H. (2016) 'Detection of hepatitis E virus in archived rabbit serum samples, Germany 1989', *Food and environmental virology,* 8(1), pp. 105-107.

El-Kafrawy, S. A., Hassan, A. M., El-Daly, M. M., Qadri, I., Tolah, A. M., Al-Subhi, T. L., Alzahrani, A. A., Alsaaidi, G. A., Al-Abdullah, N. and Kaki, R. M. (2020) 'Seroprevalence of Dromedary Camel HEV in Domestic and Imported Camels from Saudi Arabia', *Viruses,* 12(5), pp. 553.

Environmental Protection Agency. *Agriculture Nutrient Management and Fertilizer*. USA. Available at: https://www.epa.gov/agriculture/agriculture-nutrient-management-and-fertilizer#Manure (Accessed: 08 September 2020).

Emerson, S. U., Arankalle, V. A. and Purcell, R. H. (2005) 'Thermal stability of hepatitis E virus', *The Journal of infectious diseases,* 192(5), pp. 930-933.

European Commission. (2001) *Survey of wastes spread on land*: European Commission. Available at: https://ec.europa.eu/environment/waste/studies/compost/landspreading_4-6.pdf.

Feagins, A., Opriessnig, T., Guenette, D., Halbur, P. and Meng, X. (2008) 'Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States', *International journal of food microbiology,* 123(1-2), pp. 32-37.

Feagins, A., Opriessnig, T., Guenette, D., Halbur, P. and Meng, X.-J. (2007) 'Detection and characterization of infectious Hepatitis E virus from commercial pig livers sold in local grocery stores in the USA', *Journal of general virology,* 88(3), pp. 912-917.

Feurer, C., Le Roux, A., Rossel, R., Barnaud, E., Dumarest, M., Garry, P. and Pavio, N. (2018) 'High load of hepatitis E viral RNA in pork livers but absence in pork muscle at French slaughterhouses', *International journal of food microbiology,* 264, pp. 25-30.

Food and Agriculture Organisation. (2011) *Irrigation in Southern and Eastern Asia in figures*: Food and Agriculture Organisation. Available at: http://www.fao.org/3/i2809e/i2809e.pdf.

Food and Agriculture Organisation. (2016) *Irrigation in Eastern Europe in figures*: Food and Agriculture Organisation of the United Nations. Available at: http://www.fao.org/3/CA3380EN/ca3380en.pdf (Accessed: 12/08/2020).

Forni, D., Cagliani, R., Clerici, M. and Sironi, M. (2018) 'Origin and dispersal of hepatitis E virus', *Emerging microbes & infections,* 7(1), pp. 1-13.

Fraga, M., Doerig, C., Moulin, H., Bihl, F., Brunner, F., Müllhaupt, B., Ripellino, P., Semela, D., Stickel, F. and Terziroli Beretta-Piccoli, B. (2017) 'Hepatitis E virus as a cause of acute hepatitis acquired in Switzerland', *Liver International*.

Gao, S., Li, D., Zha, E., Zhou, T., Wang, S. and Yue, X. (2015) 'Surveillance of hepatitis E virus contamination in shellfish in China', *International journal of environmental research and public health,* 12(2), pp. 2026-2036.

García, M., Fernández-Barredo, S. and Pérez-Gracia, M. (2014) 'Detection of hepatitis E virus (HEV) through the different stages of pig manure composting

plants', *Microbial biotechnology,* 7(1), pp. 26-31.

Gardinali, N. R., Barry, A. F., Otonel, R. A. A., Alfieri, A. F. and Alfieri, A. A. (2012) 'Hepatitis E virus in liver and bile samples from slaughtered pigs of Brazil', *Memorias do Instituto Oswaldo Cruz,* 107(7), pp. 935-939.

Geng, J., Wang, L., Wang, X., Fu, H., Bu, Q., Zhu, Y. and Zhuang, H. (2011a) 'Study on prevalence and genotype of hepatitis E virus isolated from Rex Rabbits in Beijing, China', *Journal of viral hepatitis,* 18(9), pp. 661-667.

Geng, Y., Zhao, C., Song, A., Wang, J., Zhang, X., Harrison, T. J., Zhou, Y., Wang, W. and Wang, Y. (2011b) 'The serological prevalence and genetic diversity of hepatitis E virus in farmed rabbits in China', *Infection, Genetics and Evolution,* 11(2), pp. 476-482.

Giannini, P., Jermini, M., Leggeri, L., Nüesch-Inderbinen, M. and Stephan, R. (2017) 'Detection of hepatitis E virus RNA in raw cured sausages and raw cured sausages containing pig liver at retail stores in Switzerland', *Journal of food protection,* 81(1), pp. 43-45.

Grierson, S., Heaney, J., Cheney, T., Morgan, D., Wyllie, S., Powell, L., Smith, D., Ijaz, S., Steinbach, F. and Choudhury, B. (2015) 'Prevalence of hepatitis E virus infection in pigs at the time of slaughter, United Kingdom, 2013', *Emerging infectious diseases,* 21(8), pp. 1396.

Grodzki, M., Schaeffer, J., Piquet, J.-C., Le Saux, J.-C., Chevé, J., Ollivier, J., Le Pendu, J. and Le Guyader, F. S. (2014) 'Bioaccumulation efficiency, tissue distribution, and environmental occurrence of hepatitis E virus in bivalve shellfish from France', *Applied and environmental microbiology,* 80(14), pp. 4269-4276.

Guillois, Y., Abravanel, F., Miura, T., Pavio, N., Vaillant, V., Lhomme, S., Le Guyader, F. S., Rose, N., Le Saux, J.-C. and King, L. A. (2015) 'High proportion of asymptomatic infections in an outbreak of hepatitis E associated with a spit-roasted piglet, France, 2013', *Clinical Infectious Diseases,* 62(3), pp. 351-357.

Halbur, P., Kasorndorkbua, C., Gilbert, C., Guenette, D., Potters, M., Purcell, R., Emerson, S., Toth, T. and Meng, X. (2001) 'Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human', *Journal of clinical microbiology,* 39(3), pp. 918-923.

Hammerschmidt, F., Schwaiger, K., Dähnert, L., Vina-Rodriguez, A., Höper, D., Gareis, M., Groschup, M. and Eiden, M. (2017) 'Hepatitis E virus in wild rabbits and European brown hares in Germany', *Zoonoses and public health,* 64(8), pp. 612-622.

Hewitt, P. E., Ijaz, S., Brailsford, S. R., Brett, R., Dicks, S., Haywood, B., Kennedy, I. T., Kitchen, A., Patel, P. and Poh, J. (2014) 'Hepatitis E virus in blood components: a prevalence and transmission study in southeast England', *The Lancet,* 384(9956), pp. 1766-1773.

Horvatits, T., Schulze zur Wiesch, J., Lütgehetmann, M., Lohse, A. W. and Pischke, S. (2019) 'The clinical perspective on hepatitis E', *Viruses,* 11(7), pp. 617.

Hu, G. and Ma, X. (2010) 'Detection and sequences analysis of bovine hepatitis E virus RNA in Xinjiang Autonomous Region', *Bing du xue bao= Chinese journal of virology,* 26(1), pp. 27-32.

Huang, F., Li, Y., Yu, W., Jing, S., Wang, J., Long, F., He, Z., Yang, C., Bi, Y. and Cao, W. (2016) 'Excretion of infectious hepatitis E virus into milk in cows imposes high risks of zoonosis', *Hepatology,* 64(2), pp. 350-359.

Huang, R., Li, D., Wei, S., Li, Q., Yuan, X., Geng, L., Li, X. and Liu, M. (1999) 'Cell culture of sporadic hepatitis E virus in China', *Clinical and diagnostic laboratory immunology,* 6(5), pp. 729-733.

Huang, W., Zhang, H., Harrison, T. J., Lang, S., Huang, G. and Wang, Y. (2008) 'Cross-protection of hepatitis E virus genotypes 1 and 4 in rhesus macaques', *Journal of medical virology,* 80(5), pp. 824-832.

Iaconelli, M., Purpari, G., Della Libera, S., Petricca, S., Guercio, A., Ciccaglione, A., Bruni, R., Taffon, S., Equestre, M. and Fratini, M. (2015) 'Hepatitis A and E viruses in wastewaters, in river waters, and in Bivalve Molluscs in Italy', *Food and environmental virology,* 7(4), pp. 316-324.

Idolo, A., Serio, F., Lugoli, F., Grassi, T., Bagordo, F., Guido, M., Privitera, G., Lobreglio, G. and De Donno, A. (2013) 'Identification of HEV in symptom-free migrants and environmental samples in Italy', *Journal of viral hepatitis,* 20(6), pp. 438-443.

Imagawa, T., Sugiyama, R., Shiota, T., Li, T.-C., Yoshizaki, S., Wakita, T. and Ishii, K. (2018) 'Evaluation of heating conditions for inactivation of hepatitis e virus genotypes 3 and 4', *Journal of food protection,* 81(6), pp. 947-952.

Izopet, J., Dubois, M., Bertagnoli, S., Lhomme, S., Marchandeau, S., Boucher, S., Kamar, N., Abravanel, F. and Guérin, J.-L. (2012) 'Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France', *Emerging infectious diseases,* 18(8), pp. 1274.

Jin, H., Zhao, Y., Zhang, X., Wang, B. and Liu, P. (2016) 'Case-fatality risk of pregnant women with acute viral hepatitis type E: a systematic review and meta-analysis', *Epidemiology & Infection,* 144(10), pp. 2098-2106.

Johne, R., Reetz, J., Ulrich, R., Machnowska, P., Sachsenröder, J., Nickel, P. and Hofmann, J. (2014) 'An ORF 1-rearranged hepatitis E virus derived from a chronically infected patient efficiently replicates in cell culture', *Journal of viral hepatitis,* 21(6), pp. 447-456.

Johne, R., Trojnar, E., Filter, M. and Hofmann, J. (2016) 'Thermal stability of hepatitis E virus as estimated by a cell culture method', *Applied and environmental microbiology,* 82(14), pp. 4225-4231.

Kamar, N., Dalton, H. R., Abravanel, F. and Izopet, J. (2014) 'Hepatitis E virus infection', *Clinical microbiology reviews,* 27(1), pp. 116-138.

Kasorndorkbua, C., Guenette, D., Huang, F., Thomas, P., Meng, X.-J. and Halbur, P. (2004) 'Routes of transmission of swine hepatitis E virus in pigs', *Journal of clinical microbiology,* 42(11), pp. 5047-5052.

Kasorndorkbua, C., Opriessnig, T., Huang, F., Guenette, D., Thomas, P., Meng, X.-J. and Halbur, P. (2005) 'Infectious swine hepatitis E virus is present in pig manure storage facilities on United States farms, but evidence of water contamination is lacking', *Applied and environmental microbiology,* 71(12), pp. 7831-7837.

Kenney, S. P. (2019) 'The current host range of hepatitis E viruses', *Viruses,* 11(5), pp. 452.

Kokkinos, P., Kozyra, I., Lazic, S., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A. M., Kaupke, A. and Legaki, E. (2012) 'Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries', *Food and environmental virology,* 4(4), pp. 179-191.

Krog, J. S., Larsen, L. E. and Schultz, A. C. (2014) 'Enteric porcine viruses in farmed shellfish in Denmark', *International journal of food microbiology,* 186, pp. 105-109.

Kukielka, D., Rodriguez-Prieto, V., Vicente, J. and Sánchez-Vizcaíno, J. (2016) 'Constant hepatitis E virus (HEV) circulation in wild boar and red deer in Spain: an increasing concern source of HEV zoonotic transmission', *Transboundary and emerging diseases,* 63(5), pp. e360-e368.

Kumar, N., Das, V., Agarwal, A., Pandey, A. and Agrawal, S. (2017) 'Fetomaternal outcomes in pregnant women with hepatitis E infection; still an important fetomaternal killer with an unresolved mystery of increased virulence in pregnancy', *Turkish Journal of Obstetrics and Gynecology,* 14(2), pp. 106.

La Rosa, G., Della Libera, S., Brambilla, M., Bisaglia, C., Pisani, G., Ciccaglione, A., Bruni, R., Taffon, S., Equestre, M. and Iaconelli, M. (2017) 'Hepatitis E virus (genotype 3) in slurry samples from swine farming activities in Italy', *Food and environmental virology,* 9(2), pp. 219-229.

La Rosa, G., Proroga, Y., De Medici, D., Capuano, F., Iaconelli, M., Della Libera, S. and Suffredini, E. (2018) 'First detection of hepatitis E virus in shellfish and in seawater from production areas in Southern Italy', *Food and environmental virology,* 10(1), pp. 127-131.

Lee, G.-H., Tan, B.-H., Teo, E. C.-Y., Lim, S.-G., Dan, Y.-Y., Wee, A., Aw, P. P. K., Zhu, Y., Hibberd, M. L. and Tan, C.-K. (2016) 'Chronic infection with camelid hepatitis E virus in a liver transplant recipient who regularly consumes camel meat and milk', *Gastroenterology,* 150(2), pp. 355-357. e3.

Lee, S. D., Wang, Y. J., Lu, R. H., Chan, C. Y., Lo, K. J. and Moeckli, R. (1994) 'Seroprevalence of antibody to hepatitis E virus among Chinese subjects in Taiwan', *Hepatology,* 19(4), pp. 866-870.

Lhomme, S., Dubois, M., Abravanel, F., Top, S., Bertagnoli, S., Guerin, J.-L. and Izopet, J. (2013) 'Risk of zoonotic transmission of HEV from rabbits', *Journal of Clinical Virology,* 58(2), pp. 357-362.

Li, M., Zhang, H., Wang, L., Li, Z., Wang, J., Xu, B., Hao, R., Liu, C., Fu, H. and Rao, H. (2020a) 'The investigation of hepatitis A virus and hepatitis E virus co-infection in humans and animals in China', *Acta Virologica,* 64(1), pp. 20-27.

Li, S., Li, M., He, Q., Liang, Z., Shu, J., Wang, L. and Wang, L. (2020b) 'Characterization of hepatitis E virus natural infection in farmed rabbits', *Journal of Viral Hepatitis*.

Li, S., Liu, M., Cong, J., Zhou, Y. and Miao, Z. (2017) 'Detection and characterization of hepatitis E virus in goats at slaughterhouse in Tai'an region, China', *BioMed research international,* 2017.

Li, T.-C., Miyamura, T. and Takeda, N. (2007) 'Detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (Corbicula japonica) in Japan', *The American journal of tropical medicine and hygiene,* 76(1), pp. 170-172.

Li, T.-C., Yang, T., Shiota, T., Yoshizaki, S., Yoshida, H., Saito, M., Imagawa, T., Malbas, F. F., Lupisan, S. P. and Oshitani, H. (2014) 'Molecular detection of hepatitis E virus in rivers in the Philippines', *The American journal of tropical medicine and hygiene,* 90(4), pp. 764-766.

Loisy-Hamon, F. and Leturnier, G. (2015) 'Autochthonous cases of hepatitis E: where does the virus come from? Impact of pig slurry treatment on reduction of the viral load and prevalence of the virus in food substrates', *EuroReference,* 18, pp. 13-18.

Long, F., Yu, W., Yang, C., Wang, J., Li, Y., Li, Y. and Huang, F. (2017) 'High prevalence of hepatitis E virus infection in goats', *Journal of medical virology,* 89(11), pp. 1981-1987.

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E. and Lees, D. N. (2012) 'Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom', *Applied and environmental microbiology,* 78(16), pp. 5812-5817.

Marcheggiani, S., D'Ugo, E., Puccinelli, C., Giuseppetti, R., D'Angelo, A. M., Gualerzi, C. O., Spurio, R., Medlin, L. K., Guillebault, D. and Weigel, W. (2015) 'Detection of emerging and re-emerging pathogens in surface waters close to an urban area', *International journal of environmental research and public health,* 12(5), pp. 5505-5527.

Marine Conservation Society. (2011) *Combined Sewer Overflows Pollution Policy and Position Statement*: Marine Conservation Society. Available at: https://www.mcsuk.org/downloads/pollution/CSO%20policy.pdf.

Masclaux, F. G., Hotz, P., Friedli, D., Savova-Bianchi, D. and Oppliger, A. (2013) 'High occurrence of hepatitis E virus in samples from wastewater treatment plants in Switzerland and comparison with other enteric viruses', *Water research,* 47(14), pp. 5101-5109.

Matos, A., Mesquita, J. R., Gonçalves, D., Abreu-Silva, J., Luxo, C. and Nascimento, M. S. (2018) 'First detection and molecular characterization of hepatitis E virus in water from wastewater treatment plants in Portugal', *Annals of Agricultural and Environmental Medicine,* 25(2), pp. 364-367.

Matsuura, Y., Suzuki, M., Yoshimatsu, K., Arikawa, J., Takashima, I., Yokoyama, M., Igota, H., Yamauchi, K., Ishida, S. and Fukui, D. (2007) 'Prevalence of antibody to hepatitis E virus among wild sika deer, Cervus nippon, in Japan', *Archives of virology,* 152(7), pp. 1375-1381.

Maunula, L., Kaupke, A., Vasickova, P., Söderberg, K., Kozyra, I., Lazic, S., van der Poel, W. H., Bouwknegt, M., Rutjes, S. and Willems, K. A. (2013) 'Tracing enteric viruses in the European berry fruit supply chain', *International journal of food microbiology,* 167(2), pp. 177-185.

Medrano, C., Boadella, M., Barrios, H., Cantu, A., Garcia, Z., de la Fuente, J. and Gortazar, C. (2012) 'Zoonotic pathogens among white-tailed deer, northern Mexico, 2004–2009', *Emerging Infectious Diseases,* 18(8), pp. 1372.

Meng, X.-J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., Purcell, R. H. and Emerson, S. U. (1998) 'Genetic and experimental evidence for cross-species infection by swine hepatitis E virus', *Journal of virology,* 72(12), pp. 9714-9721.

Mesquita, J. R., Oliveira, D., Rivadulla, E., Abreu-Silva, J., Varela, M. F.,

Romalde, J. L. and Nascimento, M. S. (2016) 'Hepatitis E virus genotype 3 in mussels (Mytilus galloprovinciallis), Spain', *Food microbiology,* 58, pp. 13-15.

Mizuo, H., Yazaki, Y., Sugawara, K., Tsuda, F., Takahashi, M., Nishizawa, T. and Okamoto, H. (2005) 'Possible risk factors for the transmission of hepatitis E virus and for the severe form of hepatitis E acquired locally in Hokkaido, Japan', *Journal of medical virology,* 76(3), pp. 341-349.

Moor, D., Liniger, M., Baumgartner, A. and Felleisen, R. (2018) 'Screening of ready-to-eat meat products for hepatitis E virus in Switzerland', *Food and environmental virology,* 10(3), pp. 263-271.

Mughini-Gras, L., Angeloni, G., Salata, C., Vonesch, N., D'amico, W., Campagna, G., Natale, A., Zuliani, F., Ceglie, L. and Monne, I. (2017) 'Hepatitis E virus infection in North Italy: high seroprevalence in swine herds and increased risk for swine workers', *Epidemiology & Infection,* 145(16), pp. 3375-3384.

Mykytczuk, O., Harlow, J., Bidawid, S., Corneau, N. and Nasheri, N. (2017) 'Prevalence and molecular characterization of the hepatitis E virus in retail pork products marketed in Canada', *Food and environmental virology,* 9(2), pp. 208-218.

Müller, A., Collineau, L., Stephan, R., Müller, A. and Stärk, K. D. (2017) 'Assessment of the risk of foodborne transmission and burden of hepatitis E in Switzerland', *International journal of food microbiology,* 242, pp. 107-115.

Neumann, S., Hackl, S. S., Piepenschneider, M., Vina-Rodriguez, A., Dremsek, P., Ulrich, R. G., Groschup, M. H. and Eiden, M. (2016) 'Serologic and molecular survey of hepatitis E virus in German deer populations', *Journal of wildlife diseases,* 52(1), pp. 106-113.

Ouoba, J. B., Traore, K. A., Rouamba, H., Setondji, K. V.-M., Minoungou, G. L., Ouoba, B. L., Ouedraogo, A., Moctar, S., M'Bengue, A. K. and Kakou, S. N. (2019) 'Prevalence of anti-hepatitis E virus antibodies in domestic animal from three representative provinces of Burkina Faso', *Veterinary and Animal Science,* 7, pp. 100059.

Owolodun, O. A., Giménez-Lirola, L. G., Gerber, P. F., Sanford, B. J., Feagins, A. R., Meng, X.-J., Halbur, P. G. and Opriessnig, T. (2013) 'Development of a fluorescent microbead-based immunoassay for the detection of hepatitis E virus IgG antibodies in pigs and comparison to an enzyme-linked immunoassay', *Journal of virological methods,* 193(2), pp. 278-283.

O'Hara, Z., Crossan, C., Craft, J. and Scobie, L. (2018) 'First Report of the Presence of Hepatitis E Virus in Scottish-Harvested Shellfish Purchased at Retail Level', *Food and environmental virology*, pp. 1-5.

Pas, S. D., Rob, A., Mulders, C., Balk, A. H., van Hal, P. T., Weimar, W., Koopmans, M. P., Osterhaus, A. D. and van der Eijk, A. A. (2012) 'Hepatitis E virus infection among solid organ transplant recipients, the Netherlands', *Emerging infectious diseases,* 18(5), pp. 869.

Pathak, R. and Barde, P. V. (2017) 'Detection of Genotype 1a and 1f of Hepatitis E Virus in Patients Treated at Tertiary Care Hospitals in Central India', *Intervirology,* 60(5), pp. 201-206.

Pavio, N., Merbah, T. and Thebault, A. (2014) 'Frequent hepatitis E virus contamination in food containing raw pork liver, France', *Emerging infectious*

diseases, 20(11), pp. 1925.

Peron, J., Bureau, C., Poirson, H., Mansuy, J., Alric, L., Selves, J., Dupuis, E., Izopet, J. and Vinel, J. (2007) 'Fulminant liver failure from acute autochthonous hepatitis E in France: description of seven patients with acute hepatitis E and encephalopathy', *Journal of viral hepatitis,* 14(5), pp. 298-303.

Pina, S., Buti, M., Cotrina, M., Piella, J. and Girones, R. (2000) 'HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain', *Journal of Hepatology,* 33(5), pp. 826-833.

Pujols, J., Rodríguez, C., Navarro, N., Pina-Pedrero, S., Campbell, J. M., Crenshaw, J. and Polo, J. (2014) 'No transmission of hepatitis E virus in pigs fed diets containing commercial spray-dried porcine plasma: a retrospective study of samples from several swine trials', *Virology journal,* 11(1), pp. 232.

Pérez-Gracia, M. T., García, M., Suay, B. and Mateos-Lindemann, M. L. (2015) 'Current knowledge on hepatitis E', *Journal of clinical and translational hepatology,* 3(2), pp. 117.

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T. F. and Beutler, N. A. (2017) 'Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples', *nature protocols,* 12(6), pp. 1261.

Rein, D. B., Stevens, G. A., Theaker, J., Wittenborn, J. S. and Wiersma, S. T. (2012) 'The global burden of hepatitis E virus genotypes 1 and 2 in 2005', *Hepatology,* 55(4), pp. 988-997.

Reuter, G., Fodor, D., Forgách, P., Kátai, A. and Szűcs, G. (2009) 'Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary', *Journal of Clinical Virology,* 44(4), pp. 277-281.

Rivadulla, E., Varela, M. F., Mesquita, J. R., Nascimento, M. S. and Romalde, J. (2019) 'Detection of hepatitis E virus in shellfish harvesting areas from Galicia (Northwestern Spain)', *Viruses,* 11(7), pp. 618.

Rivero-Juarez, A., Frias, M., Martinez-Peinado, A., Risalde, M., Rodriguez-Cano, D., Camacho, A., García-Bocanegra, I., Cuenca-Lopez, F., Gomez-Villamandos, J. and Rivero, A. (2017) 'Familial hepatitis E outbreak linked to wild boar meat consumption', *Zoonoses and public health*.

Rodriguez-Manzano, J., Miagostovich, M., Hundesa, A., Clemente-Casares, P., Carratala, A., Buti, M., Jardi, R. and Girones, R. (2010) 'Analysis of the evolution in the circulation of HAV and HEV in eastern Spain by testing urban sewage samples', *Journal of water and health,* 8(2), pp. 346-354.

Rose, N., Lunazzi, A., Dorenlor, V., Merbah, T., Eono, F., Eloit, M., Madec, F. and Pavio, N. (2011) 'High prevalence of Hepatitis E virus in French domestic pigs', *Comparative immunology, microbiology and infectious diseases,* 34(5), pp. 419-427.

Roth, A., Lin, J., Magnius, L., Karlsson, M., Belák, S., Widén, F. and Norder, H. (2016) 'Markers for ongoing or previous hepatitis E virus infection are as common in wild ungulates as in humans in Sweden', *Viruses,* 8(9), pp. 259.

Rutjes, S., Bouwknegt, M., Van Der Giessen, J., de Roda Husman, A. and

Reusken, C. (2014) 'Seroprevalence of hepatitis E virus in pigs from different farming systems in The Netherlands', *Journal of food protection,* 77(4), pp. 640-642.

Rutjes, S., Lodder-Verschoor, F., Lodder, W., Van der Giessen, J., Reesink, H., Bouwknegt, M. and de Roda Husman, A. (2010) 'Seroprevalence and molecular detection of hepatitis E virus in wild boar and red deer in The Netherlands', *Journal of virological methods,* 168(1-2), pp. 197-206.

Ryll, R., Eiden, M., Heuser, E., Weinhardt, M., Ziege, M., Höper, D., Groschup, H., Heckel, G., Johne, R. and Ulrich, R. G. (2018) 'Hepatitis E virus in feral rabbits along a rural-urban transect in Central Germany', *Infection, genetics and evolution,* 61, pp. 155-159.

Said, B., Ijaz, S., Chand, M., Kafatos, G., Tedder, R. and Morgan, D. (2014) 'Hepatitis E virus in England and Wales: indigenous infection is associated with the consumption of processed pork products', *Epidemiology & Infection,* 142(7), pp. 1467-1475.

Said, B., Ijaz, S., Kafatos, G., Booth, L., Thomas, H. L., Walsh, A., Ramsay, M. and Morgan, D. (2009) 'Hepatitis E outbreak on cruise ship', *Emerging infectious diseases,* 15(11), pp. 1738.

Sanford, B., Emerson, S., Purcell, R., Engle, R., Dryman, B., Cecere, T., Buechner-Maxwell, V., Sponenberg, D. and Meng, X. (2013) 'Serological Evidence for a Hepatitis E Virus-Related Agent in Goats in the United States', *Transboundary and emerging diseases,* 60(6), pp. 538-545.

Sanford, B. J., Dryman, B. A., Huang, Y.-W., Feagins, A. R., LeRoith, T. and Meng, X.-J. (2011) 'Prior infection of pigs with a genotype 3 swine hepatitis E virus (HEV) protects against subsequent challenges with homologous and heterologous genotypes 3 and 4 human HEV', *Virus research,* 159(1), pp. 17-22.

Schemmerer, M., Apelt, S., Trojnar, E., Ulrich, R. G., Wenzel, J. J. and Johne, R. (2016) 'Enhanced replication of hepatitis E virus strain 47832c in an A549-derived subclonal cell line', *Viruses,* 8(10), pp. 267.

Schielke, A., Filter, M., Appel, B. and Johne, R. (2011) 'Thermal stability of hepatitis E virus assessed by a molecular biological approach', *Virology journal,* 8(1), pp. 487.

Slot, E., Zaaijer, H. L., Molier, M., Van den Hurk, K., Prinsze, F. and Hogema, B. M. (2017) 'Meat consumption is a major risk factor for hepatitis E virus infection', *PloS one,* 12(4), pp. e0176414.

Smith, D. B., Paddy, J. O. and Simmonds, P. (2016) 'The use of human sewage screening for community surveillance of hepatitis E virus in the UK', *Journal of medical virology,* 88(5), pp. 915-918.

Sonoda, H., Abe, M., Sugimoto, T., Sato, Y., Bando, M., Fukui, E., Mizuo, H., Takahashi, M., Nishizawa, T. and Okamoto, H. (2004) 'Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan', *Journal of Clinical Microbiology,* 42(11), pp. 5371-5374.

Sooryanarain, H., Heffron, C. L., Hill, D. E., Fredericks, J., Rosenthal, B. M., Werre, S. R., Opriessnig, T. and Meng, X.-J. (2020) 'Hepatitis E virus in pigs

from slaughterhouses, United States, 2017–2019', *Emerging infectious diseases,* 26(2), pp. 354.

Spancerniene, U., Grigas, J., Buitkuviene, J., Zymantiene, J., Juozaitiene, V., Stankeviciute, M., Razukevicius, D., Zienius, D. and Stankevicius, A. (2018) 'Prevalence and phylogenetic analysis of hepatitis E virus in pigs, wild boars, roe deer, red deer and moose in Lithuania', *Acta veterinaria scandinavica,* 60(1), pp. 13.

Spina, A., Lenglet, A., Beversluis, D., de Jong, M., Vernier, L., Spencer, C., Andayi, F., Kamau, C., Vollmer, S. and Hogema, B. (2017) 'A large outbreak of Hepatitis E virus genotype 1 infection in an urban setting in Chad likely linked to household level transmission factors, 2016-2017', *PLoS One,* 12(11), pp. e0188240.

Szabo, K., Trojnar, E., Anheyer-Behmenburg, H., Binder, A., Schotte, U., Ellerbroek, L., Klein, G. and Johne, R. (2015) 'Detection of hepatitis E virus RNA in raw sausages and liver sausages from retail in Germany using an optimized method', *International journal of food microbiology,* 215, pp. 149-156.

Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F. and Okamoto, H. (2003) 'Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus', *Journal of General Virology,* 84(4), pp. 851-862.

Tanaka, T., Takahashi, M., Kusano, E. and Okamoto, H. (2007) 'Development and evaluation of an efficient cell-culture system for Hepatitis E virus', *Journal of General Virology,* 88(3), pp. 903-911.

Tei, S., Kitajima, N., Takahashi, K. and Mishiro, S. (2003) 'Zoonotic transmission of hepatitis E virus from deer to human beings', *The Lancet,* 362(9381), pp. 371-373.

Terio, V., Bottaro, M., Pavoni, E., Losio, M., Serraino, A., Giacometti, F., Martella, V., Mottola, A., Di Pinto, A. and Tantillo, G. (2017) 'Occurrence of hepatitis A and E and norovirus GI and GII in ready-to-eat vegetables in Italy', *International journal of food microbiology,* 249, pp. 61-65.

Tomiyama, D., Inoue, E., Osawa, Y. and Okazaki, K. (2009) 'Serological evidence of infection with hepatitis E virus among wild Yezo-deer, Cervus nippon yesoensis, in Hokkaido, Japan', *Journal of viral hepatitis,* 16(7), pp. 524-528.

Vercouter, A.-S., Sayed, I. M., Lipkens, Z., De Bleecker, K., De Vliegher, S., Colman, R., Koppelman, M., Supré, K. and Meuleman, P. (2018) 'Absence of zoonotic hepatitis E virus infection in Flemish dairy cows', *International journal of food microbiology,* 281, pp. 54-59.

Villalba, M. C. M., Martínez, D. C., Ahmad, I., Lay, L. A. R., Corredor, M. B., March, C. G., Martínez, L. S., Martínez-Campo, L. S. and Jameel, S. (2017) 'Hepatitis E virus in bottlenose dolphins Tursiops truncatus', *Diseases of aquatic organisms,* 123(1), pp. 13-18.

Vitral, C. L., Pinto, M. A., Lewis-Ximenez, L. L., Khudyakov, Y. E., dos Santos, D. R. and Gaspar, A. M. C. (2005) 'Serological evidence of hepatitis E virus infection in different animal species from the Southeast of Brazil', *Memorias do Instituto Oswaldo Cruz,* 100(2), pp. 117-122.

Wang, Y., Zhang, H., Li, Z., Gu, W., Lan, H., Hao, W., Ling, R., Li, H. and

Harrison, T. J. (2001) 'Detection of sporadic cases of hepatitis E virus (HEV) infection in China using immunoassays based on recombinant open reading frame 2 and 3 polypeptides from HEV genotype 4', *Journal of clinical microbiology,* 39(12), pp. 4370-4379.

Weger, S., Elkin, B., Lindsay, R., Bollinger, T., Crichton, V. and Andonov, A. (2017) 'Hepatitis E Virus Seroprevalence in Free-Ranging Deer in Canada', *Transboundary and emerging diseases,* 64(3), pp. 1008-1011.

Wenzel, J. J., Preiß, J., Schemmerer, M., Huber, B., Plentz, A. and Jilg, W. (2011) 'Detection of hepatitis E virus (HEV) from porcine livers in Southeastern Germany and high sequence homology to human HEV isolates', *Journal of clinical virology,* 52(1), pp. 50-54.

Wilhelm, B., Leblanc, D., Houde, A., Brassard, J., Gagné, M.-J., Plante, D., Bellon-Gagnon, P., Jones, T. H., Muehlhauser, V. and Janecko, N. (2014) 'Survey of Canadian retail pork chops and pork livers for detection of hepatitis E virus, norovirus, and rotavirus using real time RT-PCR', *International journal of food microbiology,* 185, pp. 33-40.

World Health Organisation. (2019) *Hepatitis E*: World Health Organization. Available at: https://www.who.int/news-room/fact-sheets/detail/hepatitis-e (Accessed: 08 September 2021).

Xia, J., Zeng, H., Liu, L., Zhang, Y., Liu, P., Geng, J., Wang, L., Wang, L. and Zhuang, H. (2015) 'Swine and rabbits are the main reservoirs of hepatitis E virus in China: detection of HEV RNA in feces of farmed and wild animals', *Archives of virology,* 160(11), pp. 2791-2798.

Xie, X., Bil, J., Shantz, E., Hammermueller, J., Nagy, E. and Turner, P. V. (2017) 'Prevalence of lapine rotavirus, astrovirus, and hepatitis E virus in Canadian domestic rabbit populations', *Veterinary Microbiology,* 208, pp. 146-149.

Yan, B., Zhang, L., Gong, L., Lv, J., Feng, Y., Liu, J., Song, L., Xu, Q., Jiang, M. and Xu, A. (2016) 'Hepatitis E virus in yellow cattle, Shandong, Eastern China', *Emerging infectious diseases,* 22(12), pp. 2211.

Yazaki, Y., Mizuo, H., Takahashi, M., Nishizawa, T., Sasaki, N., Gotanda, Y. and Okamoto, H. (2003) 'Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food', *Journal of general virology,* 84(9), pp. 2351-2357.

Yin, W., Han, Y., Xin, H., Liu, W., Song, Q., Li, Z., Gao, S., Jiang, F., Cao, J., Bi, S. and Liu, H. (2019) 'Hepatitis E Outbreak in a Mechanical Factory in Qingdao City, China', *Int J Infect Dis*.

Yoo, D., Willson, P., Pei, Y., Hayes, M. A., Deckert, A., Dewey, C. E., Friendship, R. M., Yoon, Y., Gottschalk, M. and Yason, C. (2001) 'Prevalence of hepatitis E virus antibodies in Canadian swine herds and identification of a novel variant of swine hepatitis E virus', *Clin. Diagn. Lab. Immunol.,* 8(6), pp. 1213-1219.

Yugo, D. M., Cossaboom, C. M., Heffron, C. L., Huang, Y. W., Kenney, S. P., Woolums, A. R., Hurley, D. J., Opriessnig, T., Li, L. and Delwart, E. (2019) 'Evidence for an unknown agent antigenically related to the hepatitis E virus in dairy cows in the United States', *Journal of medical virology,* 91(4), pp. 677-686.

Zemtsova, G. E., Montgomery, M. and Levin, M. L. (2015) 'Relative sensitivity of conventional and real-time PCR assays for detection of SFG Rickettsia in blood and tissue samples from laboratory animals', *PloS one,* 10(1).

Zhang, X.-X., Qin, S.-Y., Zhang, Y., Meng, Q.-F., Jiang, J., Yang, G.-L., Zhao, Q. and Zhu, X.-Q. (2015) 'First report of hepatitis E virus infection in sika deer in China', *BioMed research international,* 2015.

Zhang, Y., Gong, W., Song, W. T., Fu, H., Wang, L., Li, M., Wang, L. and Zhuang, H. (2017) 'Different susceptibility and pathogenesis of rabbit genotype 3 hepatitis E virus (HEV-3) and human HEV-3 (JRC-HE3) in SPF rabbits', *Veterinary microbiology,* 207, pp. 1-6.

Zhao, C., Li, Z., Yan, B., Harrison, T. J., Guo, X., Zhang, F., Yin, J., Yan, Y. and Wang, Y. (2007) 'Comparison of real-time fluorescent RT-PCR and conventional RT-PCR for the detection of hepatitis E virus genotypes prevalent in China', *Journal of medical virology,* 79(12), pp. 1966-1973.

Chapter 3

Methods for the Detection of Hepatitis E virus

Abstract

This chapter establishes the development and refinement of materials and methods that were used in the subsequent chapters for detection and quantification of Hepatitis E virus. The detection methods used for other viruses within the thesis were already established elsewhere. The chapter describes a previously published HEV detection method, and the materials developed and used alongside it. It focuses particularly on the development of synthetic controls needed for the quantitative reverse transcription polymerase chain reaction (qRT-PCR). Synthetic DNA and RNA oligonucleotide strings of specific concentrations were created to enable the accurate detection and quantification of HEV within a variety of matrices; as seen in the later chapters utilising this assay. The method refinement also included testing two different probe types, and natural HEV RNA from cell culture to observe the efficacy of the qRT-PCR.

Introduction

RT-PCR is a commonly used tool in the detection of viruses from many different sample matrices, including environmental water samples, sewage, blood, and tissue samples. Though the assays for testing of norovirus and HAV have been developed and standardised for ISO 15216-1:2017, there is currently no “gold standard” HEV detection method which enables 100% of infected or contaminated samples to be detected from clinical and environmental matrices. Many studies have created new quantitative and nested RT-PCR methods in attempts to develop more sensitive and accurate assays, with varying success. One of the most highly used assays was published by Jothikumar *et al.*, (2006); which became popular for its high sensitivity for sequences within HEV genotypes 1-4. With a modification to the qRT-PCR probe by Garson *et al.*, (2012), utilising a minor groove binder quencher, this method became used routinely for HEV detection in many matrices and published studies. It has been used by institutions worldwide, including UK Health Security Agency’s blood screening through the National Health Service (NHS) (Oeser *et al.*, 2019).

HEV blood screening was introduced selectively to the UK in 2016 and became

universal for all blood products in 2017 due to increasing cases. Generally, HEV causes a mild illness, including symptoms such as nausea and jaundice, which people generally recover from without treatment after approximately six weeks. However, some people, such as those who are immunocompromised, can develop chronic HEV infection or complications such as acute liver failure, which can be fatal. Therefore, HEV is classified as a Hazard Group 3* (HG3*) organism; with the '*' meaning that it would be fully classified as a HG3 pathogen if it were to spread through a respiratory route of transmission.

HG3* classification of HEV means that screening for HEV can be performed in Containment Level 2 laboratories (CL2) but intentional work with the virus must only be done in Containment Level 3 (CL3) laboratories. Therefore, samples identified as HEV-positive could not be utilised as positive control material for the qRT-PCR assay, as CL3 laboratories were unavailable during this PhD. In addition to this, sourcing natural HEV RNA was difficult, and obtaining enough to use for controls for each test was not possible. This led to the design and synthesis of DNA and RNA controls for the qRT-PCR assay, in a similar manner to the design of synthetic controls within international standard ISO 15216-1:2017 for the testing of norovirus and HAV in shellfish (International Organization for Standardization, 2017).

HEV, being an RNA virus, requires a reverse transcription step to enable the PCR to amplify the viral cDNA and determine the quantity within samples. This meant that two positive controls were required for this process: DNA and RNA. The DNA control was used to show whether the PCR step was successful and to quantify the amount of HEV in samples through an established PCR standard curve. The RNA control was both to show whether the reverse transcription and PCR steps were successful, and to identify if inhibitors within the samples were impairing the reverse transcription or PCR. As genotype 3 HEV is the most common genotype in England and Wales (Ijaz *et al.*, 2013), but detection of genotypes 1-4 was desired, DNA controls were synthesised for sequences from each of the four genotypes to ensure that the assay could detect all four. To obtain viral RNA and cDNA, samples were first subjected to RNA extraction. These methods are matrix specific and detailed in later chapters.

Method Development for the HEV qRT-PCR

Method details

Testing of total RNA samples within the subsequent chapters required a HEV qRT-PCR assay and the combined qRT-PCR methods published by Jothikumar *et al.*, (2006) and Garson *et al.*, (2012) were suggested by colleagues within UK Health Security Agency (formerly Public Health England) and the Marine Institute (Ireland). Mengo virus was used as an extraction control and was also quantified using qRT-PCR. The qRT-PCR systems used the recipe in Table 3.1 to generate mastermixes of adequate volume for the number of samples being tested. This would, for one well in a PCR plate, yield a mastermix of 20 µl, with 5 µl of RNA extract or DNA control material then added to the mastermix in the plate well.

Table 3.1 Mastermix recipes for a single qRT-PCR reaction

	Volume required (µl)
Reactions	**1**
5' primer	0.25
3' primer	0.45
Probe	1.25
5x Reaction mix	5.00
1:10 ROX reference dye	0.50
Enzymes mix	1.25
H_2O	11.3
Total	20

The primer and probe sequences for the HEV qRT-PCR and the mengo virus qRT-PCR are shown in Table 3.2, as are the cycling parameters for both assays. The final primer concentrations were 0.625 µM for the forward primer and 1.125 µM for the reverse primer. The final probe concentration was 3.125 µM. These concentrations were based on the primer and probe concentrations used for GI norovirus within ISO 15216-1:2017 (International Organization for Standardization, 2017). The annealing temperature for the Jothikumar *et al.*, (2006) assay was modified to be the same as the mengo virus assay (increased from 55°C to 60°C). This was to ensure the annealing temperature was specific to the Invitrogen RNA UltraSense™ One-Step Quantitative RT-PCR System

(calculated to be 61.6°C on the ThermoFisher Tm calculator), and to enable the HEV and mengo virus assays to be run on the same PCR plate, therefore reducing costs for the qRT-PCR assays.

Table 3.2 Primer and probe sequences and details for the qRT-PCR methods

Method	Primer and Probe Sequences	Cycling parameters	Publication
HEV qRT-PCR	FWD: 5'-GGTGGTTTCTGGGGTGAC -3' REV: 5'-AGGGGTTGGTTGGATGAA -3' PROBE: 5'- TGATTCTCAGCCCTTCGC-3' 5'-FAM 3'-MGB	55°C 60 minutes 95°C 5 minutes 45 cycles of: 95°C 15 seconds 60°C 1 minute 65°C 1 minute	Jothikumar *et al.*, (2006); Garson *et al.*, (2012)
mengo virus qRT-PCR	FWD: 5'- GCGGGTCCTGCCGAAAGT -3' REV: 5'-GAAGTAACATATAGACAGACGCACAC -3' PROBE: 5'- ATCACATTACTGGCCGAAGC -3' 5'-FAM 3'-MGB	55°C 60 minutes 95°C 5 minutes 45 cycles of: 95°C 15 seconds 60°C 1 minute 65°C 1 minute	Pintó et al., (2009)

Mengo virus was used as an RNA extraction control, using a relative quantification method. The mengo virus was cultured within mammalian cells (FRhK-4) by members of Cefas staff, and used as a whole virus extraction efficiency control, rather than an RNA only control (due to RNA stability). This meant that the quantity of mengo virus RNA could not be determined using instruments such as the Invitrogen Qubit or Thermo Scientific Nanodrop due to the presence of mammalian RNA. Therefore, mengo virus supernatant from the cell culture was seeded into each sample and a molecular grade water only control (reference extraction) prior to RNA extraction. The reference extraction was then used to create a standard curve to assess the relative quantity of the mengo virus within the samples. This standard curve is a dilution series (1:10) from the neat reference extraction to a concentration of 10^{-3}. Samples had to reach an extraction efficiency percentage of ≥0.5% to be deemed to give reliable quantitative data. The extraction efficiency threshold was less than the usual 1% threshold set in ISO 15216- 1:2017 (International Organization for

Standardization, 2017). This was due to an issue with the mengo virus batch which had been cultured for use. When compared to previous culture batches, the batch in use consistently under-estimated the extraction efficiency. Therefore, as a temporary measure, the extraction efficiency when using this batch was reduced to 0.5% instead of 1%. Under the guidelines in ISO 15216-1:2017, samples under the 0.5% extraction efficiency threshold that gave positive results for one or more target virus were considered valid positive results but were non-quantifiable.

A standard curve was also included for the HEV assay, using synthetic control DNA at concentration 21.8 pg/µl, corresponding to 10^5 copies/µl. The control was diluted serially (1:10) to 10 copies/µl. It also acted as a positive PCR control for quantification of HEV RNA in samples. The creation of the synthetic control DNA is described below. Standard curves for both mengo virus and HEV had to conform to $r^2 \leq 0.99$ and a slope between -3.1 and -3.6 for sample test results to be deemed acceptable; values outside of these ranges were deemed to be caused by human error in preparation of the qRT-PCR mastermix and the assays were repeated. C_T values were calculated based on the specific samples being tested and can be seen in the subsequent chapters.

Additionally, a control for measuring inhibition of the sample matrix to the RT-PCR was also performed, utilising wells distinct to the sample HEV detection wells, which contained 5 µl of sample, 20 µl of HEV mastermix, and 1 µl of the synthetic HEV RNA control (4.97×10^4 copies/µl). C_T values resulting from these reactions were then compared to the C_T value obtained from a well containing 5 µl of molecular grade water, 20 µl of HEV mastermix, and 1 µl of the synthetic RNA control.

The difference in C_T values was converted into an inhibition percentage by referring to the standard curve. Samples had to have an RT-PCR inhibition of ≤75% for the quantification to be considered reliable. Under the guidelines in ISO 15216-1:2017, samples over the 75% inhibition threshold that gave positive results for one or more target virus would be regarded as a valid positive detection, but non-quantifiable. ISO 15216-1:2017 was followed in this regard because HEV is a single stranded non-enveloped virus like norovirus and HAV; though it must be noted that the method is not accredited for HEV. In addition to the positive controls, the extracted negative control was tested for both mengo

virus and HEV (acting as a control for cross contamination during RNA extraction), and a negative water control was also tested for mengo virus and HEV (acting as a control for cross contamination during plate preparation). An example plate showing the different controls can be seen in Figure 3.1. Sample preparation, qRT-PCR plate preparation and the qRT-PCR itself were all performed in separate rooms.

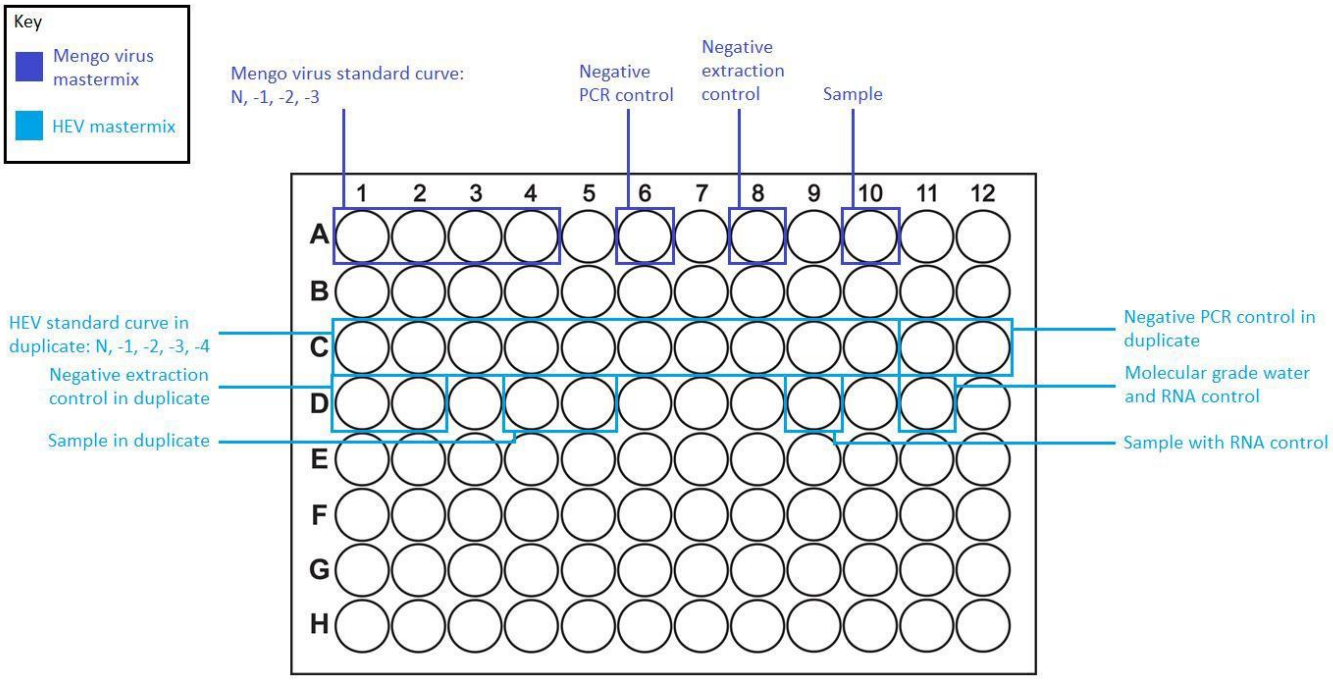


Figure 3.1 Example qRT-PCR plate for HEV and mengo virus showing the different controls

The controls used for an example sample on a 96 well PCR plate. The wells selected in dark blue are the mengo virus qRT-PCR mastermix and the light blue wells are the HEV qRT-PCR mastermix. Where standard curves are shown: N = Neat concentration; -1 = 10^{-1} dilution; -2 = 10^{-2} dilution; -3 = 10^{-3} dilution, -4 = 10^{-4} dilution.

The HEV assay was originally reported to have a detection limit of 4 genome copies (Jothikumar *et al.*, 2006). We were unable to confirm the sensitivity of the assay within the Cefas or University laboratories as this would have required multiple Hepatitis E virus strains, which could not be handled within the CL2 facilities available. The assay was determined to be specific to HEV in the study by Jothikumar *et al.*, (2006), which tested RNA from five viruses (22 different strains) with the assay primers and probe. They found that the assay generated no false positive results. In addition, an *in silico* analysis using the alignment tool

Clustal Omega (EMBL-EBI CLUSTAL multiple sequence alignment (Madeira *et al.*, 2019) showed no evidence of correct alignment of primers or probe to genomes of other viruses (Table 3.3). The alignment files can be found as FASTA files in the in the supplementary data 3.1 – 3.8.

Table 3.3 Specificity of the HEV assay primers and probe against other viruses that could be present in sample matrices

Test	*In vitro* or *in silico* analysis	Positive result?	Study
Adenovirus	*In vitro*	No	Jothikumar *et al.*, (2006)
Enterovirus	*In vitro*	No	
Hepatitis A virus	*In vitro*	No	
Norovirus	*In vitro*	No	
Rotavirus	*In vitro*	No	
Astrovirus	*In silico alignment*	Does not align	This PhD
Coronavirus	*In silico alignment*	Does not align	
Epstein-Barr virus	*In silico alignment*	Does not align	
Human betaherpes 5	*In silico alignment*	Does not align	
Mengo virus	*In silico alignment*	Does not align	
MS2 Escherichia phage	*In silico alignment*	Does not align	
Sapovirus	*In silico alignment*	Does not align	
Piscine novirhabdovirus	*In silico alignment*	Does not align	

Synthetic Control DNA Strings

Fifty-seven sequences were chosen for each of the first four genotypes of HEV from the NCBI nucleotide database. The sequences were chosen based on locations, with an aim to capture the diversity seen between different countries around the world. These were aligned alongside the primer and probe sequences of the qRT-PCR assay by Jothikumar *et al.*, (2006) to identify mismatches. Most sequences within the alignment were genotype 3 as this is the primary target and G3 HEV is also highly diverse. There were only two genotype 2 sequences due to a lack of publicly available sequences. The alignment was created again using Clustal Omega (Madeira *et al.*, 2019), which was viewed in MEGA7. The mismatches can be viewed in Figure 3.2. Overall, 12/57 of the sequences may not have been detected by the primer or probe due to a single mismatch in these sequences and one sequence may not have been detected due to one mismatch in each of the forward primer region and the probe region. The mismatches were relatively rare, and it was theorised that even with these single mismatches, primers and probes should still be able to bind.

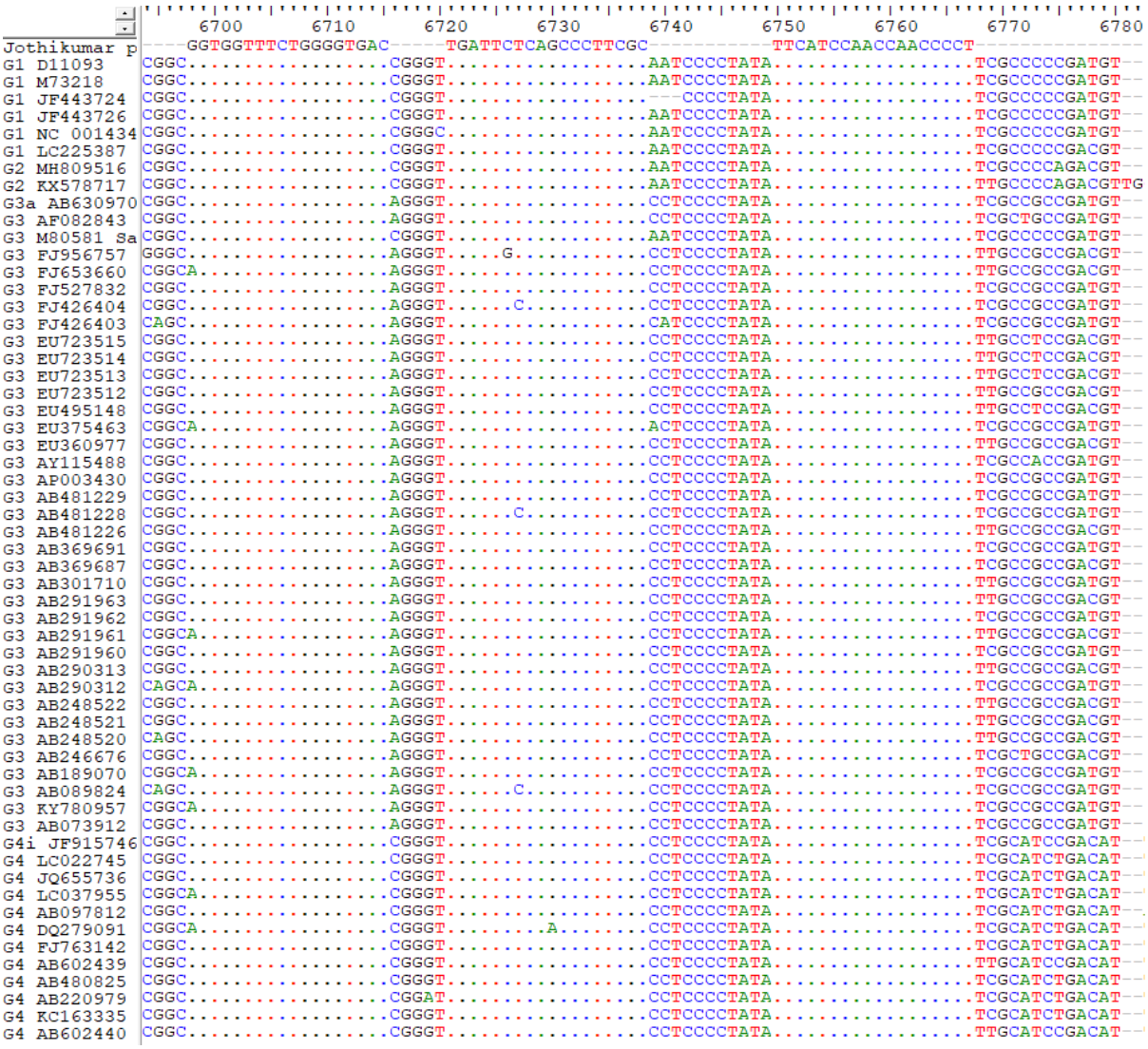


Figure 3.2 Sequence alignment of 57 strains from HEV genotypes 1-4 (G1-4) and the Jothikumar *et al.*, (2006) qRT-PCR primers and probe

Alignment for 57 HEV strains from four genotypes alongside the Jothikumar *et al.*, (2006) forward primer probe, and reverse primer. The reverse primer is reverse complimented. Accession numbers for genotype 1 strains in order of appearance: D11093; M80581; JF443724; JF443726; NC_001434; LC225387. Genotype 2 strains in order of appearance: MH809516; KX578717. Genotype 3 strains in order of appearance: AB630970; M73218; AF082843; FJ956757; FJ653660; FJ527832; FJ426404; FJ426403; EU723515; EU723514; EU723513; EU723512; EU495148; EU375463; EU360977; AY115488; AP003430; AB481229; AB481228; AB481226; AB369691; AB369687; AB301710; AB291963; AB291962; AB291961; AB291960; AB290313; AB290312; AB248522; AB248521; AB248520; AB246676; AB189070; AB089824; KY780957; AB073912. Genotype 4 strains in order of appearance: JF915746; LC022745; JQ655736; LC037955; AB097812; DQ279091; FJ763142; AB602439; AB480825; AB220979; KC163335; AB602440.

To create the controls for each genotype for the qRT-PCR assay, a single sequence from each of the four genotypes was aligned using a new multiple

sequence alignment alongside the Jothikumar assay primers and probes (Figure 3.3). These sequences were then used to design the synthetic DNA and RNA. DNA strings and plasmids were ordered from GeneArt by ThermoFisher Scientific (United Kingdom).

Figure 3.3 Sequence alignment of the area that the qRT-PCR primers and probe will bind to for genotypes 1-4

Alignment of four strains from each of the four target genotypes with the Jothikumar *et al.*, (2006) primers and probe. The Jothikumar row shows the forward primer, probe and then reverse primer. The reverse primer is reverse complimented. Accession numbers for strains: Genotype 1 D11093. Genotype 2 KX578717. Genotype 3 EU360977. Genotype 4 AB097812.

The genotype 1 (G1) and 2 (G2) control DNA sequences were ordered as synthetic DNA strings; however, the genotype 3 (G3) and 4 (G4) sequences were ordered as inserts within plasmids due to high GC content. All sequences consisted of the HEV amplicon region in addition to a primer region for conventional PCR amplification, and the plasmids contained the SfiI restriction enzyme site for the G3 and G4 plasmids to enable them to be linearised for more efficient PCR amplification of the insert. The primers which were used for the conventional PCR amplification are summarised in Table 3.4, and the full annotated sequences for the DNA controls can be seen in supplementary data 3.9. The plasmid maps for G3 and G4 can be seen in supplementary data 3.10 and 3.11.

Table 3.4 Primers for conventional PCR amplification of region of interest from strings and plasmids

Method	Primer Sequences	Cycling parameters	Publication
DNA amplification	FWD: 5'-GCTATGACCATGATTACGCCAA-3' REV: 5'-TGTAAAACGACGGCCAGTGAA-3'	94°C for 3 minutes 40 cycles of: 94°C 1 minute 55°C 1 minute 72°C 1 minute Then: 72°C for 7 minutes 4°C hold	Maguire *et al.*, (1999)

DNA strings (genotypes 1 and 2) were resuspended in 100 µl of 1% Tris-EDTA buffer (100% Tris-EDTA buffer solution, Sigma-Aldrich) and were serially diluted (1:10) from neat to 10^{-9}. These were then subjected to PCR (by the method stated in Table 3.4) and gel electrophoresis to visualise the PCR products. The gel result of the best amplified concentrations can be seen in Figure 3.4. The correct sized band (216bp) was excised from the gel and purified using the Qiagen QIAquick Gel Extraction kit according to the manufacturer's instructions.

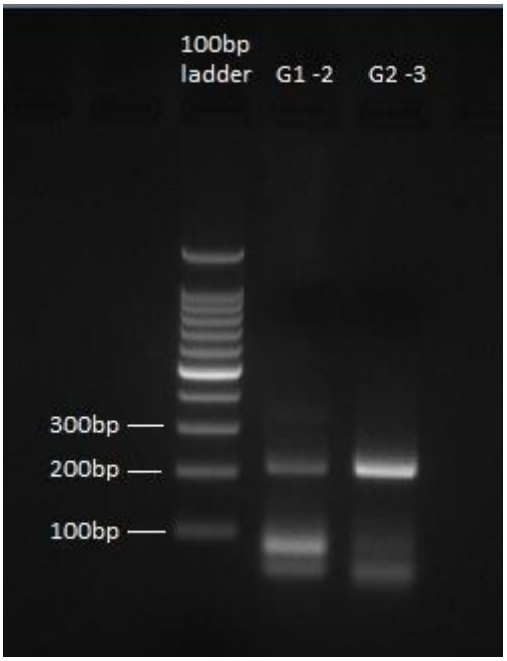


Figure 3.4 A gel to show the PCR products produced from the G1 and G2 strings

Gel electrophoresis showing the PCR products yielded from the 10^{-2} concentration of the G1 DNA and the 10^{-3} concentration of the G2 DNA alongside a Promega 100 bp DNA ladder.

After purification, the DNA was quantified using an Invitrogen Qubit 3.0 with the Qubit dsDNA high-sensitivity (HS) kit. The genome copies per microlitre (copies/µl) were calculated and the DNA was diluted to 10^5 copies/µl for use as standards for the qRT-PCR standard curve. The DNA was tested, in the form of a standard curve, using the HEV detection qRT-PCR and was determined to be adequate for use as standard curve DNA control material.

Synthetic Control DNA Plasmids

The genotype 3 and 4 control DNA were ordered as plasmids due to high GC content in parts of the sequence. The plasmids were dissolved in 50 µl of Molecular Grade water. They were then linearized using the ThermoFisher Scientific SfiI restriction enzyme and purified using the Qiagen QIAquick PCR Purification kit.

The linearised plasmids were then subjected to the PCR stated in Table 3.4, and gel electrophoresis to identify amplified bands. The gel picture showing the G3 and G4 conventional PCR products, in addition to a repeated conventional PCR of gel extracted G1 and G2 products, can be seen in Figure 3.5.

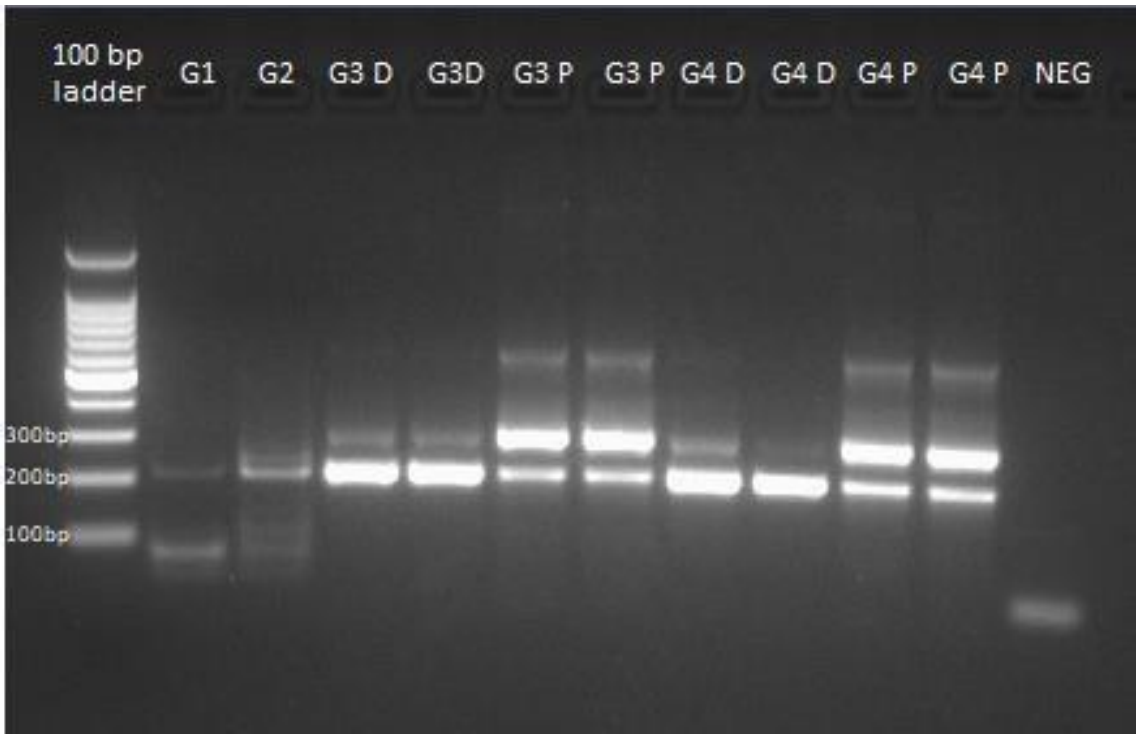


Figure 3.5 A gel showing the PCR products from G1 and G2 gel strings, and G3 and G4 digested and undigested plasmids

Gel electrophoresis showing PCR products from genotypes 1-4. From left to right, the first lane is a 100 base pair ladder, beginning at 100 bp. Lane 2, labelled G1, is for genotype 1 and lane 3, labelled G2, is for genotype 2. Genotypes 1 and 2 were a repeated PCR on gel extracted PCR products from Figure 3.4 to identify if non-specific binding PCR products could be removed using a gel extraction. This was not the case, so the previous gel extracted products were used as the controls (Figure 3.4). Lanes four and five, labelled G3 D, are for a genotype 3 digested plasmid (linearized). Lanes 6 and 7, labelled G3 P are for undigested (non-linearised) genotype 3 plasmid. Lanes 8 and 9, labelled G4 D, were for digested genotype 4 plasmid. Lanes nine and ten, labelled G4 P, were for undigested genotype 4 plasmid. Lane 11 was a PCR negative control containing water instead of plasmid material. The digested plasmids gave better desired bands at around 216 bp and less non-specific binding products. These were therefore used as controls.

The desired bands from the digested plasmids were excised from the gel and purified using the Qiagen QIAquick Gel Extraction kit. The products were analysed on an Invitrogen Qubit 3.0 using the Invitrogen Qubit dsDNA high sensitivity kit to identify the DNA concentration. The DNA copies/µl were then calculated (using http://cels.uri.edu/gsc/cndna.html) and diluted to 10^5 copies/µl before a standard curve was tested using the HEV qRT-PCR. The results showed the DNA was adequate for use as standard curve material; the 10^5 copies/µl concentration of the DNA gave a C_T value of ~20. Due to HEV in the UK belonging mostly to genotype 3 HEV; the G3 control DNA was henceforth used for qRT-PCR testing of samples.

Synthetic control RNA

A synthetic plasmid was used to create an RNA control. It contained a T7 polymerase promoter region to allow transcription, in addition to the same region of interest as the G3 synthetic DNA, and an XBaI restriction site. The insert sequence can be seen in supplementary data 3.12, and the plasmid map can be seen in supplementary data 3.13. The following steps were performed according to the manufacturer protocols. The plasmid was dissolved in 50 µl of molecular grade water and digested using the Thermo Scientific XBaI restriction enzyme. It was purified using a Qiagen QIAquick PCR purification kit. The linearised plasmid was then transcribed using an Invitrogen MAXIscript™ T7/T3 Transcription Kit for T7 bacteriophage. DNA contamination was removed using a Qiagen RNeasy mini kit with a DNase step (using Qiagen RNase-free DNase Set). RNA was quantified using the Invitrogen Qubit 3.0 with the Invitrogen Qubit RNA High Sensitivity kit. RNA was then diluted to a concentration of $4.97x10^4$ copies/µl for use as the inhibition control RNA.

A DNA contamination check was performed prior to the inhibition control use, where a qRT-PCR mastermix was prepared and split into two aliquots. One aliquot was subjected to 95°C for 5 minutes, and one was not heat treated. The heat step for aliquot one denatures the reverse transcriptase to assess whether there is DNA amplification using the heated mastermix (suggesting DNA contamination). The results showed no DNA contamination when tested. It was subsequently concluded the transcribed RNA could be used for the RNA control. The full control sequences for the DNA and RNA controls can be seen in Figure 3.6.

```
>HEV_G1_DNA_Control
gctatgacca tgattacgcc aatccggcgg tggtttctgg ggtgaccggg ttgattctca gcccttcgca atcccctata ttcatccaac caaccccttc
gcccccgatgt caccgctgcg gccggggctg gacctcgtgt tcgccaaccc gcccgaccac tcggctccgc ttggcgtgac caggcccagc gcccttcact
ggccgtcgtt ttaca

>HEV_G2_DNA_Control
gctatgacca tgattacgcc aaaccggcgg tggtttctgg ggtgaccggg ttgattctca gcccttcgca atcccctata ttcatccaac caaccccttt
gccccagacgt tgccgctgcg tccgggtctg gacctcgcct tcgccaacca gcccggccac ttggctccac ttggcgagat caggcccagc gcccttcact
ggccgtcgtt ttaca

>HEV_G3_DNA_Control
gctatgacca tgattacgcc aatccggcgg tggtttctgg ggtgacaggg ttgattctca gcccttcgcc ctcccctata ttcatccaac caaccccttt
gccgccgacgt cgtatcacaa tccggggctg gagctcgccc tcgacaaccg gcccgccccc tcggctcctc ttggcgcgac cagtcccagc gcccttcact
ggccgtcgtt ttaca

>HEV_G4_DNA_Control
gctatgacca tgattacgcc aagccggcgg tggtttctgg ggtgaccggg ttgattctca gcccttcgcc ctcccctata ttcatccaac caaccccttc
gcatctgacat accaaccgca gccgggtctg gagctcgccc tcggcagccg gcccgtccac tcggctccgc ttggcgtgac cagtcccagc gcccttcact
ggccgtcgtt ttaca

>HEV_G3_RNA_control
tcatattaag ttgggtaacg ccagggtttt cccagtcacg acgttgtaaa acgacggcca gtgaattgta atacgactca ctatagggcg atccggcggt
ggtttctggg gtgacagggt tgattctcag cccttcgccc tcccctatat tcatccaacc aacccctttg ccgccgacgt cgtatcacaa tccggggctg
gagctcgccc tcgacaaccg gcccgccccc tcggctcctc ttggcgcgac cagtcccagc gcctatgtat agatcttcat
```

Figure 3.6 The full control sequences for the synthetic DNA controls and synthetic RNA control

The full sequences for the DNA and RNA controls synthesized for use in the HEV qRT-PCR assay, beginning with the DNA strings and then the DNA plasmid insert.

Experiments with FAM-TAMRA and FAM-MGB probes

Many laboratories use the Jothikumar *et al.*, (2006) assay with Garson *et al.*, (2012) probe modification as using an FAM-MGB-NFQ probe instead of a FAM-TAMRA probe reduced the risk of false negative results whilst also giving improved amplification curves. FAM is the fluorophore 6-Carboxyfluorescein, whilst TAMRA is the quencher Tetramethylrhodamine and MGB-NFQ is a 'Minor Groove Binder Non-Fluorescent Quencher'. A comparison of the FAM-TAMRA and FAM-MGB-NFQ probes revealed that C_T values for the FAM-MGB-NFQ assay were often slightly lower (despite the same serially diluted standards being used for each mastermix) and that for some genotypes (G1 and G3), the 10^{-4} concentration appeared to have amplified better under the FAM-MGB-NFQ probe than the FAM-TAMRA probe (see Table 3.5). Therefore, the FAM-MGB-NFQ probe was chosen for sample testing as a refinement to the HEV qRT-PCR.

Table 3.5 Results of the probe comparisons for synthetic DNA controls

Genotype	FAM-TAMRA probe CT average	MGB-NFQ probe CT average	CT Difference
1	39.52	36.885	2.635
2	36.7	36.4	0.3
3	39.565	37.67	1.895
4	36.945	36.555	0.39

Use of the qRT-PCR on natural HEV RNA

Cultured HEV RNA was used to test the qRT-PCR method (kindly provided by Reimar Johne and Eva Trojnar from Bundesinstitut für Risikobewertung). This HEV strain was originally obtained from an infected patient. The concentration of the RNA was unknown, but had a neat C_T value of ~20, which is lower than the neat standard curve DNA control (C_T ~21). The RNA was diluted serially (1:10) from neat to 10^{-4} and then tested to determine how well the natural HEV was amplified and how sensitive the assay was when compared to the synthetic DNA used in the standard curve. The assay could detect the 10^{-4} dilution of the RNA, giving C_T values of ~34 for this RNA concentration. The results can be seen in table 3.5.

Table 3.6 C_T values for the natural HEV RNA in comparison to the synthetic dsDNA

Dilution	dsDNA average CT	Natural HEV RNA average CT
Neat	21.73	20.29
10^{-1}	25.61	24.07
10^{-2}	29.54	27.42
10^{-3}	32.97	31.12
10^{-4}	36.05	34.75

Discussion

Originally the methods published by Jothikumar *et al.*, (2006) and Garson *et al.*, (2012) were suggested by colleagues within UK Health Security Agency (UKHSA)and the Marine Institute (Ireland). The combined methods have been used in many other institutions and for many different purposes, such as universal blood screening for HEV in the UK (Oeser *et al.*, 2019), the detection of HEV in pigs in Italy (De Sabato *et al.*, 2019), and the detection of HEV in shellfish in Spain (Mesquita *et al.*, 2016); plus many other recorded uses. Because of the popularity of the assay, the professional recommendations, and its reported sensitivity, it was decided that synthetic control material (DNA and RNA) would be created to test the method.

The synthesised DNA and RNA were to act as controls for the HEV qRT-PCR and were created in a similar format to the controls used for norovirus and hepatitis A virus testing within ISO 15216-1:2017. The synthetic control materials were created not only because the laboratories available during this PhD were not of a high enough containment level to allow HEV-positive samples to be used, but also because a significant source of natural HEV RNA extracted from cell culture would have been needed otherwise; and this could not be obtained. We were able to successfully create synthetic DNA and RNA control materials for the HEV qRT-PCR for the purposes of sample testing. Additionally, we found that using a MGB-NFQ probe could slightly improve the curves and CT values given for the 10^{-4} dilution of some genotypes of HEV. This modification was the same as that used by Garson *et al.*, 2012 to improve the binding of the probe to HEV DNA. Garson *et al.*, 2012 showed that the increased temperature difference between the primers and probe (introduced by using the minor groove binder) made the probe less susceptible to providing false negative results when tested against different HEV sequences. They also observed improved amplification curves, with higher plateau values. We therefore determined that the FAM-MGB-NFQ probe was a good refinement to the method.

A possible limitation of this method is that there are some sequences which show some different bases in the probe binding region. This may lead to reduced probe binding and therefore lower sensitivity of the method. However, when Garson *et al.*, 2012 used a MGB-NFQ probe instead of a FAM-TAMRA

probe, they found that the probe was less sensitive to mismatches at singular loci within the probe binding region, and less false negative results. To avoid changing the detection sensitivity, reported by Jothikumar *et al.*, 2006 to be four genome copies, I decided not to change the probe sequence. This was under the consideration thatt the MGB-NFQ probe may account for such singular mismatches.

In summary, I have outlined the method which will be used for the detection of HEV, using qRT-PCR, in subsequent chapters. I have selected this HEV qRT-PCR detection method for several reasons. There is very high homology between HEV sequences in the primer and probe regions; the assay has been previously reported to have a very high sensitivity; and it has been used by many different studies and programs previously. This method was chosen to be the HEV detection method for the variety of different matrices within the subsequent chapters of this PhD. I was able to successfully create DNA and RNA control materials and run tests of the type of probe which would maximise the chances of obtaining true positive results. I also used the synthetic control materials and limited amounts of natural HEV RNA to test the qRT-PCR method and show that this is a sensitive and specific qRT-PCR method which would be useful in subsequent studies to detect and quantify these viruses potentially in various matrices.

References

De Sabato, L., Amoroso, M. G., Ianiro, G., Esposito, C., De Grossi, L., Fusco, G., Barone, A., Martini, E., Ostanello, F. and Di Bartolo, I. (2019) 'Detection of Hepatitis E Virus in Livers and Muscle Tissues of Wild Boars in Italy', *Food and environmental virology*, pp. 1-8.

Garson, J. A., Ferns, R. B., Grant, P. R., Ijaz, S., Nastouli, E., Szypulska, R. and Tedder, R. S. (2012) 'Minor groove binder modification of widely used TaqMan probe for hepatitis E virus reduces risk of false negative real-time PCR results', *Journal of virological methods,* 186(1), pp. 157-160.

Ijaz, S., Said, B., Boxall, E., Smit, E., Morgan, D. and Tedder, R. S. (2013) 'Indigenous hepatitis E in England and Wales from 2003 to 2012: evidence of an emerging novel phylotype of viruses', *The Journal of infectious diseases,* 209(8), pp. 1212-1218.

International Organization for Standardization. (2017)*: ISO 15216-1: 2017: Microbiology of food and animal feed—Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR.* Geneva.

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. and Hill, V. R. (2006) 'A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus', *Journal of virological methods,* 131(1), pp. 65-71.

Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A. R., Potter, S. C. and Finn, R. D. (2019) 'The EMBL-EBI search and sequence analysis tools APIs in 2019', *Nucleic acids research,* 47(W1), pp. W636-W641.

Maguire, A. J., Green, J., Brown, D. W., Desselberger, U. and Gray, J. J. (1999) 'Molecular epidemiology of outbreaks of gastroenteritis associated with small round-structured viruses in East Anglia, United Kingdom, during the 1996–1997 season', *Journal of Clinical Microbiology,* 37(1), pp. 81-89.

Mesquita, J. R., Oliveira, D., Rivadulla, E., Abreu-Silva, J., Varela, M. F., Romalde, J. L. and Nascimento, M. S. (2016) 'Hepatitis E virus genotype 3 in mussels (Mytilus galloprovinciallis), Spain', *Food microbiology,* 58, pp. 13-15.

Oeser, C., Vaughan, A., Said, B., Ijaz, S., Tedder, R., Haywood, B., Warburton, F., Charlett, A., Elson, R. and Morgan, D. (2019) 'Epidemiology of Hepatitis E in England and Wales: A 10-Year Retrospective Surveillance Study, 2008–2017', *The Journal of Infectious Diseases,* 220(5), pp. 802-810.

Pintó, R. M., Costafreda, M. I. and Bosch, A. (2009) 'Risk assessment in shellfish-borne outbreaks of hepatitis A', *Appl. Environ. Microbiol.,* 75(23), pp. 7350-7355.

Chapter 4

Surveillance of faecally-derived viruses within sewage

Abstract

Norovirus causes an estimated 3 million cases in the UK each year, and whilst the reported number of HEV cases in the UK have been increasing yearly since 2003, the true burden of HEV cases is unknown due to asymptomatic illness. Norovirus and HEV are shed in the faeces and urine of infected individuals, and sewage can provide an estimate of the pathogenic burden of viruses (as demonstrated during the SARS-CoV-2 pandemic). Sewage also provides an onwards transmission risk as, unlike SARS-CoV-2, these viruses can be infective in excreta. In this study, we investigated the presence of HEV and norovirus in sewage from wastewater treatment plants in Southern England. All influent samples (70) and 62/70 (88.6%) effluent samples tested positive for the presence of norovirus RNA, whilst 32/70 (45.7%) influent samples and 11/70 (16.4%) of effluent samples were HEV positive. A positive correlation was observed between GI and GII norovirus, and total norovirus and HEV. We also showed that three of seven wastewater treatment plants had ineffective treatment practices for removing norovirus RNA from sewage. This is the first study to our knowledge to identify the presence of HEV in sewage in England and considering the recent publication of sewage spill data from combined sewer overflows, suggests that HEV in sewage could be entering the aquatic environment.

Introduction

Enteric pathogens impose a significant burden on global healthcare, with a study by the World Health Organization identifying that nine major pathogens caused 1.8 billion cases of gastroenteritis in 2010, and 599,000 deaths (Pires *et al.*, 2015). Identifying the routes of transmission of these pathogens and understanding how to prevent transmission is an essential part of reducing gastroenteritis cases and deaths. Sewage has long been known to harbour many pathogens, as they are often spread through a faecal-oral route of transmission. Faecal shedding of these pathogens leads to their presence in sewage at high concentrations, which can then lead to the contamination of aquatic environments during treated or raw sewage release into surface waters.

In 2010, norovirus was estimated to have caused 677 million cases and 213,515 deaths globally (Pires *et al.*, 2015). In comparison, the World Health Organization estimates that HEV causes 20 million infections globally per year, and 44,000 deaths in 2015 (World Health Organization, 2019). In 2005, hepatitis E virus (HEV) genotypes 1 and 2 alone were responsible for 2 million hepatitis cases (World Health Organization, 2019). Norovirus is a well-known enteric pathogen, which has been identified in sewage and surface waters in many countries. In the Netherlands, norovirus was detected in river water as well as treated and untreated sewage in high quantities (maximum concentrations of $4.9x10^6$, $7.5x10^6$ and $8.5x10^8$ PCR detectable units per millilitre respectively) (Lodder and de Roda Husman, 2005). In Japan Iwai *et al.*, (2009) found that GI norovirus reached a maximum of $2.3x10^9$ copies/ml of raw sewage, and GII reached $7.1x10^{10}$ copies/ml. In the UK, GI norovirus was found to have a maximum of $5.6x10^4$ copies/ml of sewage influent, and GII was found to have a maximum of $4.5x10^5$ copies/ml (Campos *et al.*, 2016). The concentrations of norovirus in sewage and surface waters varies considerably between country and depends on season. Studies have also identified that norovirus RNA is difficult to remove from sewage, and that some wastewater treatment processes are more effective than others (Campos *et al.*, 2016; Kingsley *et al.*, 2017; Sima *et al.*, 2011). The release of treated sewage and raw sewage from storm overflows is a common occurrence, with a report from the Environment Agency in the UK identifying that combined sewer overflows operated for over 3 million hours collectively in 2020 (Environment Agency, 2020a). This can lead to contamination of surface waters with norovirus (Di Bartolo *et al.*, 2015b; La Rosa *et al.*, 2017b; Sedji *et al.*, 2018; Haramoto *et al.*, 2005; Wyn-Jones *et al.*, 2011). This release of norovirus into aquatic environments can then lead to the contamination of recreational waters, and shellfish harvesting areas (Smith *et al.*, 2012). In the latter case, bivalve shellfish can bioaccumulate norovirus from their growing waters, and this can lead to foodborne outbreaks and sporadic cases of norovirus. It is estimated that there are 11,800 cases of shellfish-borne norovirus in the UK per year (Hassard *et al.*, 2017). In addition, consumption of surface waters (for drinking) can also lead to norovirus outbreaks (Di Bartolo *et al.*, 2015b). Furthermore, norovirus outbreaks from recreational use of surface waters such as lakes and canals have also been reported (Kauppinen *et al.*, 2017; Schets *et al.*, 2018; Wade *et al.*, 2018; Zlot *et al.*, 2015).

Hepatitis E virus (HEV) has also been detected in sewage in many different countries. HEV in sewage can originate from humans and other hosts of the virus, including pigs, deer and rabbits (Kenney, 2019), where wastewater treatment plants (WWTPs) also serve farms or abattoirs. In India, 55.6% of 144 sewage influent samples were positive for genotype 1 (Vivek *et al.*, 2013). In Italy, 16% of 118 influent samples were positive for genotype 1 and 3 (La Rosa *et al.*, 2010). And in the UK, genotype 3 was found in 93% (14/15) of WWTP influent samples in Edinburgh (Smith *et al.*, 2016). Surface waters have also been identified to be contaminated with HEV in some countries; this can be due to sewage release into surface waters, contamination with farm run-off, or possibly even from wild animals. A study in Germany identified 27/90 (30%) samples of water from two rivers to be HEV positive (Beyer *et al.*, 2020); whilst a study in Iowa, USA identified 9/20 (45%) river water samples to be HEV positive before and after pig manure application to nearby fields (Givens *et al.*, 2016). Similar to norovirus, HEV has been identified in both influent and effluent as well as shellfish in previous studies (Beyer *et al.*, 2020; Rivadulla *et al.*, 2019; O'Hara *et al.*, 2018). HEV has also been detected in pig slurry samples from UK farms, having been identified in two of nine pig slurry lagoons in the UK (McCreary et al., 2008). Contamination of water courses and ground water with HEV from animal slurry and human sewage is a common concern. However a UK-wide investigation into the presence of HEV in sewage and environmental waters has not yet been completed.

Surveillance of wastewater is increasingly seen as a useful tool to monitor the emergence of outbreaks of disease. Sewage is likely to be a good representation of the true transmission of viruses within the population as many viruses (including SARS-CoV-2, HEV and norovirus) can cause asymptomatic infections, which are likely to be missed by surveillance. The sewerage system is used by by approximately 96% of the population, with the remainder served by private treatment works, cesspits and septic tanks (DEFRA, 2002). Therefore they may be used to identify new outbreaks before symptomatic cases arise (Medema *et al.*, 2020a). During the SARS-CoV-2 pandemic, sewage surveillance has been used to not only track outbreaks as they are happening, or before symptomatic cases are reported (Medema *et al.*, 2020b; Agrawal et al., 2021), but also for monitoring of variant abundance within the population and comparison to other areas (Martin *et al.*, 2020; Crits-Christoph *et al.*, 2021). Identifying viruses such as SARS-CoV-2, HEV and

norovirus within sewage could also be used to identify if contamination of surface waters with treated or untreated sewage could lead to infections in persons who use surface waters for recreational activities, and who consume shellfish. An investigation into HEV and norovirus in sewage is an important step towards our understanding of the transmission of these viruses in the UK. Due to previously identified presence of HEV in sewage in Scotland (Smith *et al.*, 2016), the aim of this study was to test WWTP influent and effluent samples from Southern England for HEV, and to provide a comparison of its presence with that of norovirus. We also aimed to identify whether the treatment processes used by the WWTPs were effective for norovirus and HEV removal.

Materials and Methods

Sewage study samples

We collected 140 paired samples, comprising of 70 influent and 70 effluent samples, from seven WWTPs in Southern England between October 2019 and February 2020. Each influent sample consisted of a litre of coarsely screened influent, and each effluent sample was one litre of final effluent. Ten pairs of influent and effluent samples were collected from each WWTP over the collection period. Samples were collected weekly during this period, or fortnightly due to holiday periods. Details of the WWTPs are shown in Table 4.1 (sites and locations have been anonymised). Samples were stored in cooled boxes during transport to the Cefas Weymouth laboratory on the same day. Upon arrival at the laboratory, samples were either stored overnight at 4°C, or frozen at -20°C for a maximum of two weeks before processing. The counties which the samples were collected from can be seen in Figure 4.1.

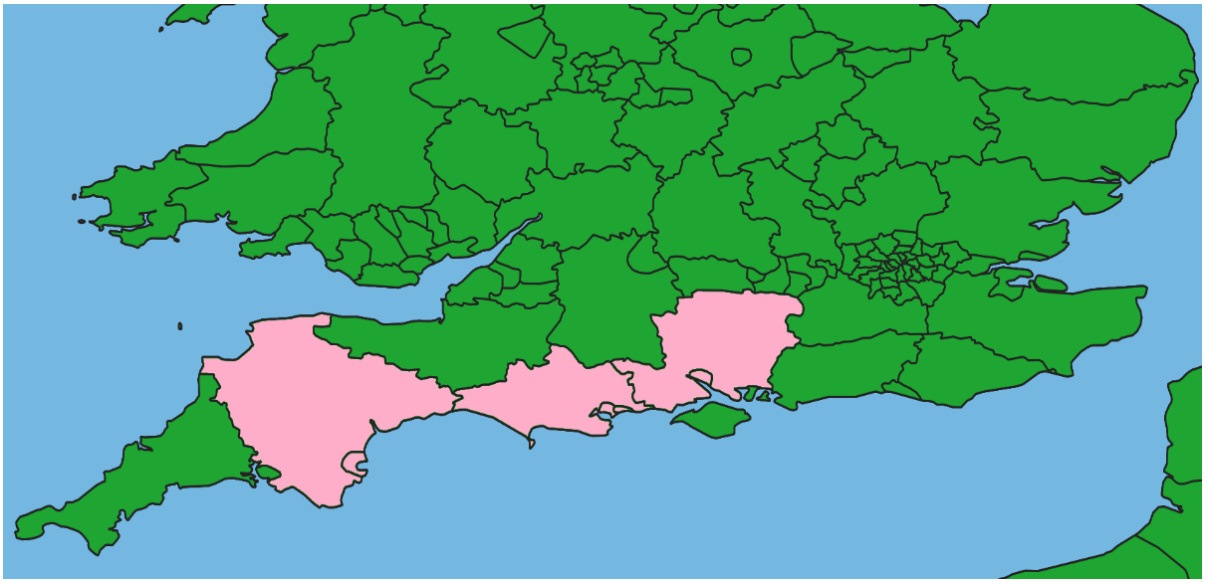


Figure 4.1 Counties from which the influent and effluent samples were collected for HEV and norovirus testing

Figure showing the South of England with counties outlined. The counties highlighted in pink are those containing WWTPs involved in the study, and those in green were not included but may have formed part of the catchments of some of the WWTPs.

Table 4.1 Characteristics of the WWTPs involved in the studies

WWTP number	Population equivalent	Dry weather flow (m^3/day)	Treatment level (type)
1	33,822	9,450	Secondary (trickling filter)
2	166,837	40,486	Tertiary (UV)
3	178,531	55,000	Tertiary (UV)
4	166,931	47,700	Tertiary (sand filter and UV)
5	141,213	40,007	Secondary (activated sludge)
6	22,352	4,910	Tertiary (membrane filtration)
7	93,303	32,141	Secondary (aeration)

Sewage treatment occurs within two or three stages, depending on the size of the

population equivalent being served by the WWTP. All WWTPs use a primary treatment of coarse filters and grit screens to remove solids, which go to landfill. The next stage is a secondary treatment, which usually involves a biological digestion stage using bacteria which are used to break down the sewage and possibly remove nitrogen and phosphorus, followed by a settlement stage where the clear water is separated from a sludge layer. This sludge layer is then treated before being used to generate biogas, or to generate fertilizer. However, some WWTPs use a secondary filtration option to break down or separate sewage. Some WWTPs then use a tertiary treatment if they are considered to be large enough, this can be in the form of a UV treatment, or it can be additional filtration or nutrient removal (usually nitrogen and phosphorus) (Severn Trent Water, none). Table 4.1 provides the available information on the types of treatments used in the WWTPs in this study, where data is lacking it is unavailable.

Sewage concentration

Samples were concentrated using a modified ultracentrifugation method (Puig *et al.*, 1994; Cross, 2004). Briefly, 40 ml of sewage sample was mixed by vigorous inversion then split equally into two ultracentrifuge bottles. Mengo virus (10 µl) was then added to both tubes to act as a control to measure the RNA extraction efficiency later. The sample bottles were then spun at 152,000 x g at 4°C for one hour and the supernatant was discarded. The pellet from one of the sample bottles was resuspended in 2ml of 0.25M glycine buffer (pH 9.5; glycine powder from Sigma-Aldrich). This suspension was then added to the pellet from the second sample bottle and the second pellet resuspended with the first. The sample was then put on ice for twenty minutes before adding 2ml of cold (4°C) phosphate buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer; PBS tablets from VWR). Samples were then spun in the ultracentrifuge at 6090 x g for 20 minutes and at 4°C. The supernatant from the bottles was then transferred to clean bottles, and 18ml of cold PBS added before a final spin at 152,000 x g for one hour at 4°C. The pellet from this final spin was resuspended in 1ml of cold PBS before it was used in subsequent RNA extraction.

Virus and RNA extraction

The total RNA extraction began by adding 500 µl of the concentrated sewage pellet to 2 ml of lysis buffer (Biomerieux), and this was left to incubate at room temperature

for 10 minutes. The method for RNA extraction is then the same for that used for extraction of viral RNA from shellfish, described in Lowther *et al.*, (2012). Following the lysis buffer incubation, 50 µl of magnetic silica beads from the NucliSENS Magnetic Extraction Reagents kit (Biomerieux) were added, and the mixture was left to incubate for ten minutes at room temperature. The sample was then centrifuged at 1500 x g for 2 minutes and the supernatant discarded. The silica beads remaining were washed in buffers whilst held in a NucliSENS Minimag (Biomerieux). Wash buffer 1 was applied and removed using an aspirator after 30 seconds of wash spinning within the Minimag; and this step was repeated. Wash buffer 2 was then applied in the same way and repeated. Wash buffer 3 was applied for 15 seconds of wash spinning and removed, and finally 100 µl of elution buffer was added to resuspend the magnetic beads. The elution mix was incubated on a thermoshaker at 60°C and 1400 rpm for five minutes, before applying to a magnet to separate the buffer (containing extracted RNA) from the silica beads. The 100 µl of RNA extract was then transferred to a clean tube for use in subsequent qRT-PCR. A reference extraction was performed alongside the samples, consisting of 10 µl of mengo virus (same batch as used in the ultracentrifugation) in 500 µl of molecular grade water; and a negative extraction control of 500 µl of molecular grade water was also included during each extraction.

qRT-PCR methods

Sewage RNA extracts were tested for the mengo virus extraction control and presence of HEV and norovirus using qRT-PCR. The mengo virus and norovirus assays are described within the international standard for quantification of viruses in foods ISO 15216-1:2017. The details for these assays and the HEV assay are shown in Table 4.2. Reagents from the Invitrogen RNA UltraSense™ One-Step Quantitative RT-PCR System were used to prepare qRT-PCR mastermixes, and amplification was carried out using a QuantStudio 3 Real-Time PCR System. Two (technical) replicatesof RNA extract were tested for each target virus detection assay, and one technical replicate was tested for the extraction control assay. Each technical replicate in each assay consisted of 20 µl of mastermix with 5 µl of RNA extract as template. For all qRT-PCR assays the final primer concentrations were 0.625 µM for the forward primer and 1.125 µM for the reverse primer.

Table 4.2 Primer and probe sequences and details for the qRT-PCR methods

Target	Primer and Probe Sequences	Cycling parameters	Paper
HEV	FWD: 5'-GGTGGTTTCTGGGGTGAC-3' REV: 5'-AGGGGTTGGTTGGATGAA-3' PROBE: 5'- TGATTCTCAGCCCTTCGC-3' 5'-FAM 3'-MGB-NFQ	55°C 60 minutes 95°C 5 minutes 45 cycles of: 95°C 15 seconds 60°C 1 minute 65°C 1 minute	Jothikumar *et al.*, (2006); Garson *et al.*, (2012)
Norovirus GI	FWD: 5'-CGCTGGATGCGNTTCCAT-3' REV: 5'-CCTTAGACGCCATCATCATTTAC-3' PROBE: 5'-TGGACAGGAGATCGC-3' 5'-FAM 3'-MGB-NFQ	55°C 60 min 95°C 5 minutes 45 cycles of: 95°C 15 seconds 60°C 1 minute 65°C 1 minute	da Silva *et al.*, (2007); Svraka *et al.*, (2007); Hoehne and Schreier, (2006)
Norovirus GII	FWD: 5'-ATGTTCAGRTGGATGAGRTTCTCWGA-3' REV: 5'-TCGACGCCATCTTCATTCACA-3' PROBE: 5'-AGCACGTGGGAGGGCGATCG-3' 5'-FAM 3'-TAMRA	55°C 60 min 95°C 5 minutes 45 cycles of: 95°C 15 seconds 60°C 1 minute 65°C 1 minute	Loisy *et al.*, (2005); Kageyama *et al.*, (2003)
Mengo virus	FWD: 5'-GCGGGTCCTGCCGAAAGT-3' REV: 5'-GAAGTAACATATAGACAGACGCACAC-3' PROBE: 5'-ATCACATTACTGGCCGAAGC-3' 5'-FAM 3'-MGB-NFQ	55°C 60 min 95°C 5 minutes 45 cycles of: 95°C 15 seconds 60°C 1 minute 65°C 1 minute	Pintó, Costafreda and Bosch, (2009)

Several controls were included within the qRT-PCR to minimise experimental error. Mengo virus was used as an extraction control for all samples through a relative quantification method. The mengo virus was cultured within mammalian FRhK-4 cells and was used as a whole virus extraction efficiency control after harvesting by spiking into sewage prior to concentration and RNA extraction. A reference extraction (molecular grade water with 10 µl mengo virus) was performed alongside, and the RNA from this was used to prepare a standard curve (serially diluted by a factor of 10,

to 10^{-3}) to assess the extraction efficiency of samples. One technical replicate for each of the four dilutions were tested. The extraction efficiency threshold was set to 0.5% as discussed in chapter 3. Samples under the extraction efficiency threshold that gave an otherwise valid positive virus target result were regarded as positive, but non-quantifiable; under the guidelines in ISO 15216-1:2017.

A standard curve was also included for the norovirus and HEV assays, using synthetic control linear dsDNA preparations, which acted as positive PCR controls and calibration standards for absolute quantification of virus RNA. The synthetic DNA controls for norovirus (GI and GII) were created following the ISO 15216-1:2017 guidelines, and the same principles were applied for creating the HEV synthetic DNA in chapter 3. The sequence composition for the norovirus (GI and GII), and HEV control material are detailed in the annex, within supplementary data 4.1 and 3.9 respectively.

For detection assay standard curves, control DNA of concentration 10^5 copies/µl was diluted serially (by a factor of 10) to 10 copies/µl. Two technical replicates for each of the five dilutions were tested, using 20 µl of mastermix with 5 µl of standard DNA. Standard curves had to conform to an r^2 value of 0.99 and a slope of between -3.1 and -3.6 to be deemed valid. Non-conformance to these values was deemed to be caused by human error in preparation of qRT-PCR mastermixes and assays were repeated. Virus concentrations (in copies/ml) were calculated from sample C_T values, utilising the slope and intercept of the standard curves, in addition to the volume of concentrated sewage tested (500 µl), and the concentration factors employed during the concentration and extraction methods. The calculation of copies/ul of template from C_T values can be seen in supplementary data 4.2.

In addition, controls for measuring inhibition to the norovirus and HEV qRT-PCRs, caused by the sample matrix, were also included, utilising synthetic RNA. Additional reaction wells (separate to those for virus detection) were prepared, containing 5 µl of sample RNA extract, 20 µl of mastermix, and 1 µl of synthetic RNA control (creation of the HEV RNA control is described in chapter 3, following the same method used in ISO 15216-1:2017 for creation of the norovirus RNA controls). C_T values for these reactions were then compared to the C_T value obtained from a well containing 5 µl of molecular grade water, 20 µl of mastermix, and 1 µl of the synthetic RNA control. The difference in C_T values was converted into an RT-PCR inhibition percentage by reference to the standard curve. Sample qRT-PCR inhibition had to be ≤75%

(approximately 2 C_T values difference). Samples over the 75% inhibition threshold were not considered to provide reliable quantitative data, however an otherwise valid positive result within such samples would be regarded as a positive detection, but non-quantifiable, as outlined in ISO 15216-1:2017.

For negative controls, an extracted negative control was tested for mengo virus, HEV and norovirus (a control for cross contamination during RNA extraction), and a negative qRT-PCR control was also tested (a control for cross contamination during plate preparation). Sample preparation, qRT-PCR plate preparation and the thermocycling all occurred in separate laboratory rooms to minimise contamination risk.

Statistical Analysis

All statistical analysis and graphs were produced in R version 3.4.3 (R Core Team, 2018). All data was initially tested for normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965). The data was found not to be normally distributed when $\log_{10}$ transformed, or when untransformed, and zeroes were meaningful data, therefore data was analysed using non-parametric tests throughout.

Mean virus copies/ml sewage was calculated using the absolute quantification values obtained for all samples, including negative samples (0 copies/ml sewage). Total norovirus refers to the addition of GI and GII copies/ml sewage.

Spearman's rank correlation was used to check for correlation between levels of GI and GII norovirus, and total norovirus and HEV in samples, and to determine if there were correlations between WWTP population equivalent and average total norovirus, average HEV, and number of HEV positive samples (Jerrold, 2005). Fisher's exact test was used to identify if norovirus or HEV positive samples were more likely to originate from certain WWTPs (Fisher, 1934). A Kruskal-Wallis test was used to identify if there were significant differences in the distribution of total norovirus and HEV levels between the WWTPs (Kruskal and Wallis, 1952). A paired one-tailed Wilcoxon test was also used to identify if the median norovirus in influent was greater than the median norovirus in treated effluent to determine whether the sewage treatment practices for each WWTP were adequate for removal of norovirus RNA. Additionally, we used a one-tailed (greater) Wilcoxon test to determine whether the log reduction of norovirus levels was larger for WWTPs using tertiary sewage treatment methods than those using secondary methods. Log reductions were calculated for pairs of influent and effluent samples collected on the same day from the same

WWTP. To enable the log reduction to be calculated for pairs where effluent samples were negative for norovirus, these samples were given a censored value of 0.5 copies/ml, which is slightly lower than the lowest positive result.

Results

Extraction efficiency and Inhibition

Of the 140 samples collected from the seven WWTPs, four gave invalid extraction results (<0.5%) and three different samples gave invalid inhibition percentage results (>75%). These samples were re-extracted from the concentrated sewage pellet but still failed the quality control parameters on retesting. Two of these invalid samples were influent samples which tested positive for HEV and GI and GII norovirus. Three of the invalid samples (one effluent and two influent samples) tested positive for GI and/or GII norovirus. The two remaining invalid samples were effluent samples which were negative for all viruses. This left 133 samples for quantitative data analysis. The range of extraction efficiency values obtained for these 133 samples was 0.53 – 65.1% and the range of percentage inhibition for these samples was 0.0 – 74.6%. The extraction efficiencies for the samples can be seen in supplementary data 4.3. Of these 133 samples, 66 were influent samples and 67 were effluent samples.

Norovirus and HEV in sewage

For norovirus, all influent samples (70) and 63/70 (90%) of effluent samples tested positive for the presence of RNA from at least one norovirus genogroup. GI norovirus RNA was detected in 67/70 (95.7%) and 58/70 (82.9%) of influent and effluent samples respectively; whilst GII norovirus RNA was detected in 69/70 (98.6%) and 60/70 (85.7%) of influent and effluent samples. For HEV, 32/70 (45.7%) of influent samples were HEV positive, as were 11/70 (16.4%) of effluent samples. Table 4.3 shows the percentage of influents and effluents from each site which tested positive for at least one genogroup of norovirus, or HEV, utilising all data (including samples which were positive but non-quantifiable).

Table 4.3 Virus detection results by WWTP

WWTP	Population Equivalent	Effluents GI norovirus positive	Influents GI norovirus positive	Effluents GII norovirus positive	Influents GII norovirus positive	Influents HEV positive	Effluents HEV positive
1	33822	100% (10/10)	88.89% (8/9*)	100% (10/10)	100% (9/9*)	33% (3/9*)	10% (1/10)
2	166837	100% (10/10)	100% (10/10*)	100% (10/10)	100% (10/10*)	70% (7/10)	20% (2/10)
3	178531	90% (9/10)	100% (10/10)	80% (8/10)	100% (10/10)	50% (5/10)	10% (1/10)
4	166931	100% (10/10)	100% (10/10*)	100% (10/10)	90% (9/10*)	67% (6/9*)	10% (1/10)
5	141213	77.78% (7/9*)	100% (10/10*)	100% (9/9*)	100% (10/10*)	50% (5/10*)	33% (3/9*)
6	22352	22.22% (2/9*)	100% (10/10*)	33.33% (3/9*)	100% (10/10*)	50% (5/10)	22% (2/9*)
7	93303	100% (10/10*)	90% (9/10)	100% (10/10*)	100% (10/10)	10% (1/10)	11% (1/9*)
Totals	N/A	82.8% (58/70)	95.7% (67/70)	85.7% (60/70)	97.1% (68/70)	45.7% (32/70)	16.4% (11/70)

*Samples which failed quality controls and were negative for target viruses not included. Samples with invalid quality controls but positive target virus detections are included as they are positive detections but are non-quantifiable according to ISO 15216-1:2017 (International Organization for Standardization, 2017).

The presence of both GI and GII norovirus was common within the influents and effluents, with 66/70 (94.3%) and 56/70 (80%) of influents and effluents respectively containing both genogroups. All three target viruses (HEV, GI and GII norovirus) were detected in 30/70 (42.9%) of influents and 9/70 (12.9%) of effluents. All samples containing HEV also contained GII norovirus. Only one influent sample and two effluent samples containing HEV and GII norovirus did not also contain GI norovirus. Figure 4.2 shows the comparison of GI and GII norovirus and HEV quantities found in the influent and effluent samples. A significant positive correlation was found between GI and GII norovirus levels within both influent and effluent samples (Spearman's rank correlation, effluent Rho= 0.74, $P < 0.0001$; influent Rho= 0.62, $P < 0.0001$). Significant positive correlations were also observed between levels of total norovirus and HEV (effluent Rho= 0.46, $P < 0.0001$; influent Rho= 0.57, $P < 0.0001$).

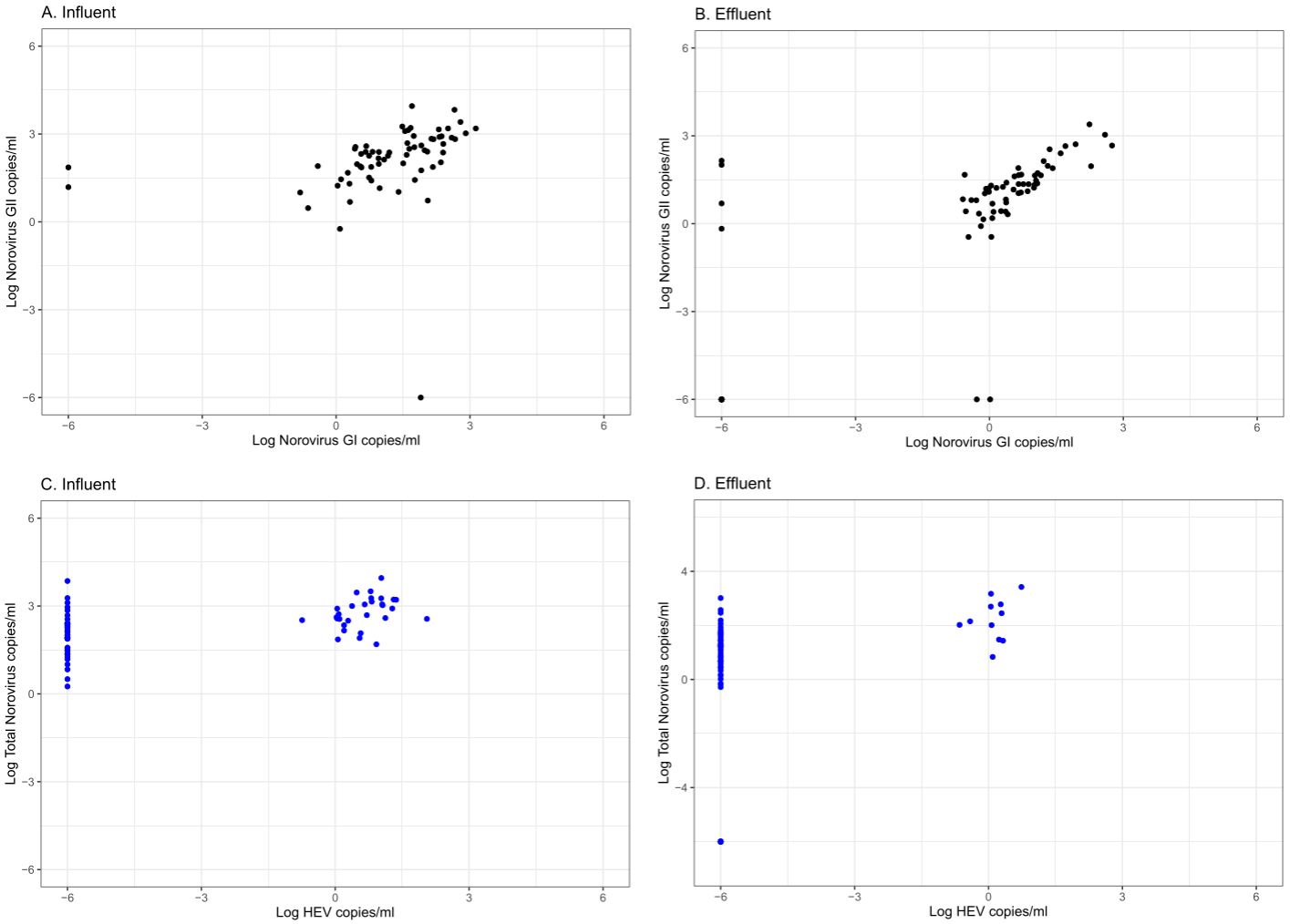


Figure 4.2 Comparison of levels of GI and GII norovirus and total norovirus and HEV within the influent and effluent samples collected

A and B show the comparison of GI and GII norovirus levels in influent and effluent samples from Southern England. C and D show a comparison of total norovirus and HEV levels in influent and effluent samples. The axes have all been logged to better present the data. Where samples had no detected virus, a value of 0.000001 was assigned for log calculations, giving a result of -6.

Considering only the samples with valid results, the average GI norovirus was 112 copies/ml and 26 copies/ml in influent and effluent respectively. For GII, the average was 638 copies/ml and 105 copies/ml in influent and effluent respectively. The mean total norovirus was 751 copies/ml for influents, and 131 copies/ml for effluents. For HEV, the averages were 4.4 copies/ml and 0.27 copies/ml in influent and effluent respectively. The minimum GI and GII norovirus and HEV in influent and effluent samples was 1 copy/ml of sewage. The maximum GI was 1,345 and 559 copies/ml of sewage for influent and effluent respectively (GI C_T range 29-41). The maximum for GII was 8,984 and 2,460 copies/ml of sewage in influent and effluent samples

respectively (GII C_T range 27-41). For HEV, the maximum was 113 copies/ml and copies/ml in influent and effluent respectively (C_T range 34 – 44). The minimum total norovirus in positive influent and effluent samples was 2 copies/ml and 1 copy/ml respectively, whilst the maximum was 9,034 copies/ml and 2,634 copies/ml in influent and effluent samples respectively. The raw data for norovirus and HEV in the influent and effluent samples can be seen in supplementary data 4.4 – 4.7. Figure 4.3 shows the mean total norovirus by the WWTPs they were sourced from. The average norovirus levels from all WWTPs, in influent or effluent samples, were correlated with the population equivalents from all WWTPs. Additionally, the average HEV levels from all WWTPs were also correlated with the population equivalents. No significant correlation was observed between the average total norovirus or HEV and the population equivalent for either influent or effluent samples (see Table 4.4).

Table 4.4 Results of correlation between average total norovirus or HEV and population equivalent

Virus	**Sample type**	**Rho value**	***p* value**
Norovirus	Influent	-0.39	0.396
	Effluent	-0.11	0.840
HEV	Influent	-0.14	0.783
	Effluent	-.043	0.354

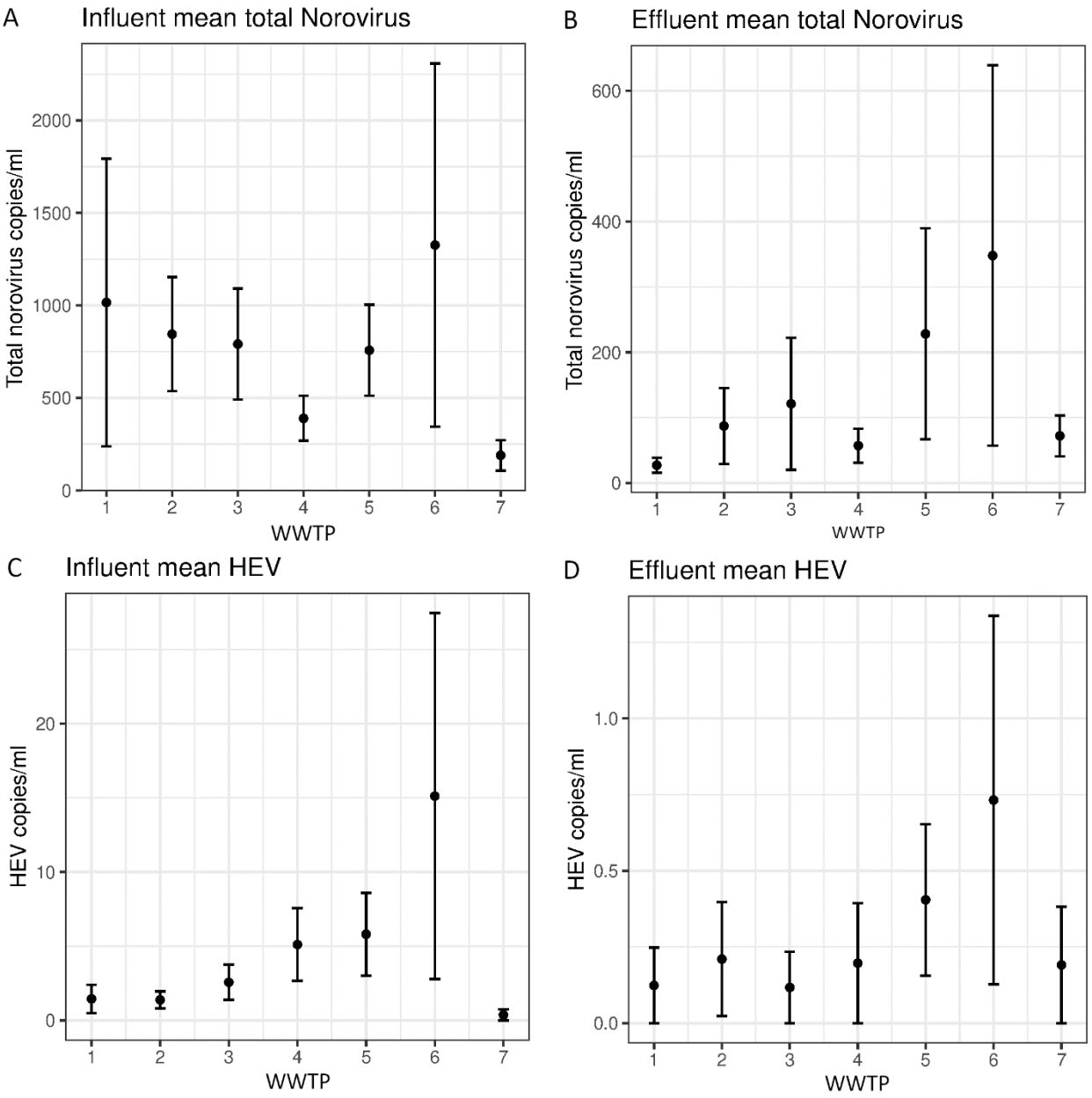


Figure 4.3 The mean total norovirus and HEV in the influent and effluent samples from each WWTP

The graphs A and B show the mean total norovirus observed from each WWTP, with SEM error bars. Graphs C and D show the mean HEV observed from each WWTP, also with SEM error bars. The axes for HEV are lower than those for norovirus due to the lower levels seen for this virus.

For norovirus, a Fisher's exact test to identify if certain WWTPs were more likely to provide norovirus positive samples was not possible on the influent data as all samples were norovirus positive, but there was no significant association for the effluent samples (Fisher's exact test, $p = 0.143$). No WWTPs were more likely to provide HEV positive samples either (Fisher's exact test, p values 0.371 and 0.114 for

influents and effluents respectively). We observed no significant difference in total norovirus or HEV levels between WWTPs. For norovirus, the *p* values obtained were 0.088 and 0.229 (Kruskal-Wallis tests) for the influent and effluent tests respectively, for HEV, the *p* values obtained were 0.358 and 0.810 for influent and effluent respectively (Kruskal-Wallis tests).

We identified that four of the WWTPs involved in the study showed a significant difference in the median levels of norovirus between paired influent and effluent samples (one-tailed Wilcoxon tests). Table 4.5 shows the *p* values obtained from these Wilcoxon tests. The same test was not performed for HEV data due to the low quantities observed in the sewage samples. Significant differences were seen only for WWTPs 1, 2, 3, and 4. In an assessment of whether secondary or tertiary sewage treatment was more effective for reducing norovirus from influent to effluent, the log difference between paired influent and effluent samples was calculated and showed that tertiary treatment had a greater effect on the removal of norovirus than secondary treatment (one-sided Wilcoxon test p = 0.042). The average log reduction of norovirus for each WWTP can also be seen in Table 4.5.

Table 4.5 Statistical significance of the difference in median norovirus levels between influent and effluent samples for each WWTP

WWTP	Treatment level (type)	*p* value	Average log reduction of norovirus
1	Secondary (trickling filter)	0.0059	1.18
2	Tertiary (UV)	0.0020	1.50
3	Tertiary (UV)	0.0186	1.44
4	Tertiary (sand filter and UV)	0.0273	0.86
5	Secondary (activated sludge)	0.0820	0.94
6	Tertiary (membrane filtration)	0.2305	1.58
7	Secondary (aeration)	0.0820	0.43

Discussion

Norovirus in sewage

Within sewage we observed that 100% of influent and 90% of effluent samples were positive for the presence of norovirus from either or both GI and GII. GI was identified in 95.7% and 82.9% of influent and effluent samples respectively, whilst GII was identified in 98.6% and 85.7%. These results are very similar to those in a previous study on WWTPs in England by Campos *et al.*, (2016), which identified GI norovirus in 94.7% of influent samples, and GII norovirus in 98.7%. These results show the ubiquitous nature of norovirus in sewage, and are likely to be a result of the estimated 3 million cases which occur in the UK each year (Gherman *et al.*, 2020). However, this is likely to be a result of the time of year in which the study was done, as norovirus is known to have a pattern of seasonality, with much higher cases in the winter months than summer months. This has been observed not only within human cases, but also sewage and shellfish (Public Health England, 2021a; Campos *et al.*, 2016; Lowther et al., 2012). Therefore, these results may not be representative of summer months. We identified that GII was present at higher average and maximum concentrations than GI in both influent and effluent, but that the prevalence of the two genogroups in influent and effluent was almost identical. It is possible that the higher levels of GII are the result of more GII noroviruses circulating within the population than GI, and indeed GII noroviruses have been the dominant genogroup detected within reported cases in 2019 (Public Health England, 2020). GI cases are comparatively few. However, the similar prevalence of GI and GII noroviruses within these sewage samples suggests that there is still a significant presence of GI noroviruses within the community, which could perhaps be explained by asymptomatic GI cases. The fact that both GI and GII noroviruses were detected in 94.3% and 80% of influents and effluents respectively also indicates a large amount of community transmission of both genogroups of norovirus. The significant correlations observed between levels of GI and GII norovirus in influent and effluent samples suggest that risk factors for both genogroups within the population are the same.

We observed no significant correlation between the population equivalent served by the WWTPs and the average total norovirus observed for each WWTP; this was surprising as it was expected that WWTPs serving lower population equivalents would observe less average total norovirus. This may be a result of similar proportions of

infected and non-infected people within the communities served by the smaller and larger WWTPs, leading to similar concentrations of norovirus seen in samples between WWTPs. Several factors may influence the dynamics of norovirus infections within the local community, such as population density and the origins or nature of the outbreak – for example an outbreak caused by a contaminated water supply may affect more people than an outbreak from consuming oysters in a restaurant (Riera-Montes *et al.*, 2011; Coutts *et al.*, 2017). We did not observe any WWTPs to be more likely to provide norovirus positives than others, or any significant variation of total norovirus levels between WWTPs, although mean norovirus levels varied from 189 – 1,326 copies/ml in influent samples and 27 – 348 copies/ml in effluent samples. These results could be explained by the high variability of norovirus levels on a weekly basis within individual WWTPs, similar proportions of infected and non-infected people within populations served by both smaller and larger WWTPs, or perhaps could be influenced by factors such as rainfall dilution of sewage, and population density within the areas served by each WWTP; though there is no evidence to suggest any particular cause. However, the data is limited by the fact that there is limited information on what was being fed into the WWTPs. Some of the catchments had hospitals which may have fed into the WWTPs (five of the seven WWTPs) however it is also possible that these hospitals may have had their own sewage treatment facilities. Additionally, some WWTPs were in more industrial and urban areas (the larger WWTPs) and some were in more community-based rural areas. Additionally, the study was undertaken only over the winter months and was not conducted over a full year to observe the effects of seasonality.

We also identified that 100% of influents from each WWTP and all effluent samples from all WWTPs, bar one (WWTP 6), were norovirus positive. Of the seven WWTPs, four had significantly lower median total norovirus in their treated effluent than untreated influent. This means that these sites had more efficient treatment practises for removal of norovirus RNA than the other three. Of these four WWTPs, three had a tertiary level of treatment, including UV, and one had a secondary level of treatment, including a trickling filter. This suggests that the methods of wastewater treatment employed by these WWTPs were more effective for removing norovirus RNA from sewage. An analysis of the percentage reduction of norovirus in paired samples from WWTPs using secondary and tertiary treatment showed that tertiary treatment of the sewage resulted in greater percentage reductions of norovirus within the sewage.

These results are in agreement with the study by Campos *et al.*, (2016), which found that use of tertiary UV treatment following biological treatment was the most effective for removal of norovirus RNA from sewage. Other studies have also identified limited success when using secondary treatment practices to remove viruses, including Lodder and de Roda Husman, (2005) in the Netherlands. This same pattern was also observed for *E. coli*, where geometric mean *E. coli* levels were 3.2 $\log_{10}$ higher in secondary treated effluent that tertiary (UV treated) effluent (Campos *et al.*, 2016). Sima et al., (2011) in France also observed a generally more limited ability for secondary treatment practices to remove norovirus than *E. coli* in colder months. Sewage treatment practices vary widely not only between WWTPs in the UK, but also between countries. Some countries have limited wastewater treatment practices (such as Libya) or may not fully comply with wastewater treatment laws (such as Portugal) and often discharge raw sewage into surface waters (Alsadey and Mansour *et al.*, 2020; European Commission, 2007). The USA, uses similar systems to the UK, implementing a primary, secondary and possible additional advanced level of treatment (United States Government, 2018). However, Japan commonly uses small scale aeration-filtration systems (known as Gappei-shori Johkaso (Mizuochi *et al.*, 2008). Removal of viruses and bacteria from sewage is likely to vary significantly between countries, not only because of differences in seasonality of norovirus between different countries, but also because of differing sewage treatment methods, degrees of sewage treatment, and environmental conditions.

HEV in sewage

This study is the first to identify HEV in sewage in England, though at low levels. We observed that 45.7% of influent and 16.4% of effluent samples in this study were positive for HEV. The levels of HEV identified within this study were low, ranging from 1 – 113 copies/ml of sewage. The median levels of the virus in influent and effluent were 0 copies/ml of sewage, and the standard deviation was 14.5 and 0.82 for influent and effluent respectively. This is potentially lower than the figures reported by Smith et al., (2016), who identified HEV in untreated influent in Edinburgh at quantities <2,000 copies/ml of sewage, but a range of values was not provided for more complete comparison, so it is difficult to say with confidence whether the levels reported here are genuinely lower. We did not observe a significant correlation between HEV presence and the size of the population equivalent. We also found that no WWTP was more likely to provide a HEV positive sample, and there was no significant

difference in the HEV levels between WWTPs. These results are likely to have been affected by the low HEV levels detected, as the maximum level identified was 113 copies/ml of sewage. But it could suggest that other factors may influence the presence and levels of HEV in sewage (such as weather conditions, population density, and the prevalence and transmission of the virus within the populations served), though there is no evidence for this at present.

One factor which is beginning to be explored is the possibility of sewer-dwelling animals such as rats contributing to the burden of HEV within sewage. It has been theorized that sewer-dwelling rats can become infected with human HEV, which can then infect livestock animals such as pigs. Some studies have identified that human HEV is capable of infecting rats. A study in the USA identified genotype 3 HEV in 34/446 rat liver samples (Lack *et al.*, 2012), and one study in Norway identified that 10/56 rats from a farm had genotype 3 HEV within their tissues which was identical to the pig HEV strains prevalent on the farm (Kanai *et al.*, 2012). However other studies have shown no infection of rats with human HEV strains under experimental conditions (Purcell *et al.*, 2011; Schlosser *et al.*, 2019). A study of rats from farms in the UK in 2018 showed no evidence that they were carrying human HEV (Grierson *et al.*, 2018). However, the potential for sewer-dwelling rats to be carrying and excreting HEV within the sewers cannot be ruled out and should be considered as an additional factor which may have influenced the results of this study.

We identified a significant positive correlation between the levels of norovirus and HEV. This was emphasised by the presence of GII norovirus in all HEV positive samples, and the presence of norovirus (GI and GII) and HEV in 42.9% of influent and 12.9% of effluent samples. A correlation between levels of norovirus and HEV is unsurprising, and suggests a common source for both viruses, and that levels of norovirus and HEV vary in similar ways. This may suggest common risk factors for both viruses, though it is unknown what this may be. Considering that these WWTPs are fed primarily by human populations, this may suggest a human origin for both viruses, but animal sources of HEV cannot be ruled out as there are animal farms in the study areas. To determine whether the HEV originated from humans or animals, sequencing of the HEV positive samples was required (see chapter 6). Importantly, for all WWTPs, we reported at least one influent and one effluent sample containing HEV RNA, which shows that HEV is prevalent in humans and/or animals across the area covered by the study area (seven WWTPs within an area of approximately 4,500 km^2).

Due to low levels of HEV within the sewage samples, an assessment of the efficacy of wastewater treatment on HEV levels between influent and effluent samples was not conducted. However, the detection of HEV within influent and effluent suggests that HEV could be entering surface waters, especially considering the recent report of over 3 million hours of raw sewage release into surface waters in 2020 (Environment Agency, 2020a).

To compare the levels of norovirus and HEV within sewage, total norovirus reached a maximum of 9,034 copies/ml and 2,634 copies/ml in influent and effluent respectively, which is 80 times higher than maximum levels of HEV in influent, and 527 times higher in effluent. This is representative of the relative incidence of norovirus cases yearly, compared to HEV cases. From June 2017 to June 2018, norovirus cases reached 6,719 in England and Wales (Public Health England, 2018a), whereas in 2018 HEV cases reached 1,002 in England and Wales (Public Health England, 2019). However, many cases of norovirus go unreported, with an estimate of the true number of norovirus cases at 3 million in the UK annually (Gherman et al., 2020), and many people are also infected asymptomatically (Phillips et al., 2010), which may partially explain this discrepancy. In comparison, HEV cases are likely to be under-reported due to the possibility of asymptomatic infection (Guillois et al., 2015). However, as symptomatic HEV illness is comparatively more severe than norovirus, and HEV illness is a notifiable disease to public health agencies, this would suggest that under-reporting of HEV illness is less of an issue than it is for norovirus.A correlation between HEV and norovirus levels was identified as significant within sewage. This means that levels of HEV and norovirus may vary similarly. With the discrepancy between norovirus and HEV cases in the UK yearly, HEV is much less likely to be present in human sewage than norovirus, and unlikely to be at high levels. Due to the low levels of HEV identified in the samples in this study, the aquatic environment in England is not likely to have high levels of HEV within it, and this should limit the possibility of onward transmission through consumption of shellfish, or through recreational water activities which result in the consumption of surface waters. However, this must be investigated further.

Limitations

We experienced sample limitations related to the quality controls. Several samples failed to reach the extraction efficiency threshold (0.5%) or exceeded the inhibition

threshold (75%), and the range of extraction efficiencies and inhibition percentages was also large. Environmental samples commonly have difficulty in obtaining high extraction efficiencies (Li *et al.*, 2018). This may be due to the presence of inhibitory compounds and degrading enzymes within sewage (Sanchez-Galan *et al.*, 2021), and it is a common difficulty encountered when analysing sewage samples. An RNA integrity control was not possible to assess for sewage due to the unlikelihood of intact mRNA being present in the samples due to RNases. This could mean that viral RNA within the samples may have degraded prior to analysis. The fact that some samples yielded positive results suggests that the virus capsids were fully in tact when in the sewage samples were collected. Samples were processed as quickly as possible to minimise the effects of this, and presence of norovirus in high quantities within some samples suggests that RNases did not overly deplete viral RNA from the samples. Norovirus was also detected in the majority of samples, suggesting there was no significant problem with RNA quality.

Another limitation of the study is that we were unable to test sewage samples from areas outside of southern England, and therefore the prevalence of norovirus and HEV identified in these samples cannot be representatively applied to the whole of the UK. In addition, influent and effluent samples were collected within ten minutes of each other at each WWTP, and therefore influent entering the WWTP would not have been fully treated by the time effluent samples were collected. Treatment times between WWTPs vary depending on the methods of treatment, however a previous study on a WWTP using a membrane bioreactor as the secondary treatment process had a residence time of 20 hours (Sima *et al.*, 2011). This means that the influent samples may not have been representative of the norovirus levels which were present prior to the treatment of the paired effluent samples. However, we considered that this was still a useful measurement of real time reductions in norovirus RNA as times when virus levels in influent had been both increasing and decreasing are likely to have been captured across the dataset, meaning that inflation and reduction of the difference between influent and effluent norovirus RNA levels should have been cancelled out.

Additionally, the data is limited by the fact that the exact treatment processes used by each WWTP were not provided by the water companies involved in the study. Though it was observed that tertiary treatment of influent was more efficient than WWTPs with secondary treatments only, it is difficult to know if this was observed because of the

use of tertiary UV treatments, or if it is because of the secondary treatments being used at these sites, or a combination. However, it has been observed elsewhere that tertiary treatment (with UV) is more effective for virus removal than secondary treatment alone (Campos *et al.*, 2016). The data may also be limited by the effect of storage on the samples, as storage at 4°C overnight or immediate freezing at -20°C may have caused some viral particles to degrade, and therefore the amount of virus detected in the samples may be lower. The samples were also only collected over the winter between 2019 and 2020, representing the season where norovirus levels are highest, and therefore do not represent the warmer months where norovirus levels are lower.

The state of the viruses within the sewage is also unknown. Whilst qRT-PCR is an incredibly useful technique for detection and quantification of viruses such as HEV and norovirus in sewage, it is unknown whether the viruses detected are in a viable or non-viable state, and this study did not have the resources or facilities required for culturing the viruses from the samples. It is also unknown whether the viruses are in an aggregated state or disaggregated state. It is theorized that norovirus would be disaggregated in water as the pH of water is close to the isoelectric point of norovirus (Van Abel *et al.*, 2017); however, sewage is a complex biological matrix which may lead the virus particles to “stick” to the organic matter and possibly aggregate together, as they have in previous high-protein matrices (Teunis *et al.*, 2008a). Additionally, it is unknown whether the viruses would be in a quasi-enveloped or vesicle-cloaked state within the sewage samples. HEV is considered to exist in a quasi-enveloped state within blood and tissue culture supernatant (Takahashi *et al.*, 2010; Yin *et al.*, 2016; Capelli *et al.*, 2019), whilst norovirus has been observed to exist in both a “naked” virion state, and also a vesicle-cloaked state containing multiple particles (Santiana *et al.*, 2018). Both the quasi-envelope and the vesicles are made up of host cell lipids (Takahashi *et al.*, 2008; Santiana *et al.*, 2018). However, the quasi-envelope for HEV is not maintained in the faeces, due to exposure to bile (Primadharsini *et al.*, 2021; Takahashi *et al.*, 2008), and quasi-envelopment is thought to maintain the infectivity of virus particles (Capelli *et al.*, 2019). On the contrary, vesicle-cloaked norovirus particles have been detected in the faeces (Santiana et al., 2018), and the cloaking of norovirus particles within sewage is thought to provide improved transmission for the virus to other hosts as viable norovirus was cultured from the vesicles (Santiana *et al.*, 2018). This would suggest

that HEV is not quasi-enveloped within sewage and may also be less infectious than norovirus within sewage due to the lack of a protective-quasi-envelope. These observations make determining the viability of viruses within sewage ever-more important, however this was not possible within this study.

Conclusion

This study has shown the ubiquitous nature of norovirus and identified the presence of HEV in sewage from Southern England for the first time. With farm run off and thousands of litres of treated and untreated sewage being released into UK waters yearly, and the confirmed presence of HEV in sewage in Scotland and England, the next important question to ask is whether HEV presence in sewage could be leading to its transmission through the UK aquatic environment to humans, perhaps through recreational water, such as swimming, or the consumption of shellfish from the aquatic environment.

References

Alsadey, S. and Mansour, O. (2020) 'Wastewater Treatment Plants in Libya: Challenges and Future Prospects', 6, pp. 76-80.

Agrawal, S., Orschler, L. and Lackner, S. (2021) 'Long-term monitoring of SARS-CoV- 2 RNA in wastewater of the Frankfurt metropolitan area in Southern Germany', *Scientific reports,* 11(1), pp. 5372-5372.

Beyer, S., Szewzyk, R., Gnirss, R., Johne, R. and Selinka, H.-C. (2020) 'Detection and characterization of hepatitis E Virus genotype 3 in wastewater and urban surface waters in Germany', *Food and environmental virology*, pp. 1-11.

Campos, C. J., Avant, J., Lowther, J., Till, D. and Lees, D. N. (2016) 'Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes', *Water research,* 103, pp. 224-232.

Capelli, N., Marion, O., Dubois, M., Allart, S., Bertrand-Michel, J., Lhomme, S., Abravanel, F., Izopet, J. and Chapuy-Regaud, S. (2019) 'Vectorial Release of Hepatitis E Virus in Polarized Human Hepatocytes', *J Virol,* 93(4).

Coutts, S. P., Sturge, K., Lalor, K., Marshall, J. A., Bruggink, L. D., Subasinghe, N. and Easton, M. (2017) 'An outbreak of foodborne norovirus gastroenteritis linked to a restaurant in Melbourne, Australia, 2014', *Western Pac Surveill Response J,* 8(2), pp. 12-16.

Crits-Christoph, A., Kantor, R. S., Olm, M. R., Whitney, O. N., Al-Shayeb, B., Lou, Y. C., Flamholz, A., Kennedy, L. C., Greenwald, H., Hinkle, A., Hetzel, J., Spitzer, S., Koble, J., Tan, A., Hyde, F., Schroth, G., Kuersten, S., Banfield, J. F. and Nelson, K. L. (2021) 'Genome Sequencing of Sewage Detects Regionally Prevalent SARS-CoV-2 Variants', *mBio,* 12(1), pp. e02703-20.

Cross, L. J. (2004) *The development and application of quantitative methods for norovirus detection in sewage effluents and seawater.* University of Surrey (United Kingdom).

da Silva, A. K., Le Saux, J.-C., Parnaudeau, S., Pommepuy, M., Elimelech, M. and Le Guyader, F. S. (2007) 'Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II', *Appl. Environ. Microbiol.,* 73(24), pp. 7891-7897.

DEFRA (2002). 'Sewage Treatment in the UK'. Department for Environment, Food and Rural Affairs. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/69582/pb6655-uk-sewage-treatment-020424.pdf (Accessed: 03 July 2022)

Di Bartolo, I., Pavoni, E., Tofani, S., Consoli, M., Galuppini, E., Losio, M. N., Ruggeri, F. M. and Varisco, G. (2015) 'Waterborne norovirus outbreak during a summer excursion in Northern Italy', *New Microbiol,* 38(1), pp. 109-12.

Environment Agency. (2020) 'Event Duration Monitoring - Storm Overflows - 2020' [Database of CSO use throughout UK in 2020]. Available at: https://environment.data.gov.uk/dataset/21e15f12-0df8-4bfc-b763-45226c16a8ac (Accessed 23 Jun 2021).

European Commission., European Union. (2007) *Portugal: Commission*

continues legal action over environmental and human health infringements. Brussels: European Commission.

Fisher, R. A. (1934) 'Statistical methods for research workers', *Statistical methods for research workers.,* (5th Ed).

Garson, J. A., Ferns, R. B., Grant, P. R., Ijaz, S., Nastouli, E., Szypulska, R. and Tedder, R. S. (2012) 'Minor groove binder modification of widely used TaqMan probe for hepatitis E virus reduces risk of false negative real-time PCR results', *Journal of virological methods,* 186(1), pp. 157-160.

Gherman, I., Holland, D., Clifford, R., Mahmoudzadeh, N., Uy, F., Adkin, A., Kainth, B., Cook, P., Day, A. and Wilson, A. (2020) 'Technical Report: Review of Quantitative Risk Assessment of foodborne norovirus transmission', *Science,* 1, pp. 1.

Givens, C. E., Kolpin, D. W., Borchardt, M. A., Duris, J. W., Moorman, T. B. and Spencer, S. K. (2016) 'Detection of hepatitis E virus and other livestock-related pathogens in Iowa streams', *Science of the Total Environment,* 566, pp. 1042-1051.

Grierson, S., Rabie, A., Lambert, M., Choudhury, B. and Smith, R. P. (2018) 'HEV infection not evident in rodents on English pig farms', *Veterinary Record,* 182(3), pp. 81-81.

Guillois, Y., Abravanel, F., Miura, T., Pavio, N., Vaillant, V., Lhomme, S., Le Guyader, S., Rose, N., Le Saux, J.-C. and King, L. A. (2015) 'High proportion of asymptomatic infections in an outbreak of hepatitis E associated with a spit-roasted piglet, France, 2013', *Clinical Infectious Diseases,* 62(3), pp. 351-357.

Haramoto, E., Katayama, H., Oguma, K. and Ohgaki, S. (2005) 'Application of cation- coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan', *Applied and Environmental Microbiology,* 71(5), pp. 2403-2411.

Hassard, F., Sharp, J. H., Taft, H., LeVay, L., Harris, J. P., McDonald, J. E., Tuson, K., Wilson, J., Jones, D. L. and Malham, S. K. (2017) 'Critical review on the public health impact of norovirus contamination in shellfish and the environment: a UK perspective', *Food and environmental virology,* 9(2), pp. 123-141.

Hoehne, M. and Schreier, E. (2006) 'Detection of Norovirus genogroup I and II by multiplex real-time RT-PCR using a 3'-minor groove binder-DNA probe', *BMC infectious diseases,* 6(1), pp. 69.

Iwai, M., Hasegawa, S., Obara, M., Nakamura, K., Horimoto, E., Takizawa, T., Kurata, T., Sogen, S.-i. and Shiraki, K. (2009) 'Continuous presence of noroviruses and sapoviruses in raw sewage reflects infections among inhabitants of Toyama, Japan (2006 to 2008)', *Applied and environmental microbiology,* 75(5), pp. 1264-1270.

Jerrold, Z. (2005) 'Spearman rank correlation', *Encyclopedia of Biostatistics,* 7.

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. and Hill, V. R. (2006) 'A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus', *Journal of virological methods,* 131(1), pp. 65-71.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., Takeda, N. and Katayama, K. (2003) 'Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse

transcription-PCR', *Journal of clinical microbiology,* 41(4), pp. 1548-1557.

Kanai, Y., Miyasaka, S., Uyama, S., Kawami, S., Kato-Mori, Y., Tsujikawa, M., Yunoki, M., Nishiyama, S., Ikuta, K. and Hagiwara, K. (2012) 'Hepatitis E virus in Norway rats (Rattus norvegicus) captured around a pig farm', BMC Res Notes, 5, pp. 4.

Kauppinen, A., Al-Hello, H., Zacheus, O., Kilponen, J., Maunula, L., Huusko, S., Lappalainen, M., Miettinen, I., Blomqvist, S. and Rimhanen-Finne, R. (2017) 'Increase in outbreaks of gastroenteritis linked to bathing water in Finland in summer 2014', *Eurosurveillance,* 22(8), pp. 30470.

Kenney, S. P. (2019) 'The current host range of hepatitis E viruses', *Viruses,* 11(5), pp. 452.

Kingsley, D. H., Fay, J. P., Calci, K., Pouillot, R., Woods, J., Chen, H., Niemira, B. A. and Van Doren, J. M. (2017) 'Evaluation of Chlorine Treatment Levels for Inactivation of Human Norovirus and MS2 Bacteriophage during Sewage Treatment', *Applied and environmental microbiology,* 83(23), pp. e01270-17.

Kruskal, W. H. and Wallis, W. A. (1952) 'Use of ranks in one-criterion variance analysis', *Journal of the American statistical Association,* 47(260), pp. 583-621.

La Rosa, G., Della Libera, S., Iaconelli, M., Proroga, Y., De Medici, D., Martella, V. and Suffredini, E. (2017) 'Detection of norovirus GII. 17 Kawasaki 2014 in shellfish, marine water and underwater sewage discharges in Italy', *Food and environmental virology,* 9(3), pp. 326-333.

La Rosa, G., Pourshaban, M., Iaconelli, M., Vennarucci, V. S. and Muscillo, M. (2010) 'Molecular detection of hepatitis E virus in sewage samples', *Applied and environmental microbiology,* 76(17), pp. 5870-5873.

Lack, J. B., Volk, K. and Van Den Bussche, R. A. (2012) 'Hepatitis E virus genotype 3 in wild rats, United States', *Emerg Infect Dis,* 18(8), pp. 1268-73.

Li, D., Butot, S., Zuber, S. and Uyttendaele, M. (2018) 'Monitoring of foodborne viruses in berries and considerations on the use of RT-PCR methods in surveillance', *Food Control,* 89, pp. 235-240.

Lodder, W. J. and de Roda Husman, A. M. (2005) 'Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands', *Applied and environmental microbiology,* 71(3), pp. 1453-1461.

Loisy, F., Atmar, R., Guillon, P., Le Cann, P., Pommepuy, M. and Le Guyader, F. (2005) 'Real-time RT-PCR for norovirus screening in shellfish', *Journal of virological methods,* 123(1), pp. 1-7.

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E. and Lees, D. N. (2012) 'Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom', *Applied and environmental microbiology,* 78(16), pp. 5812-5817.

Martin, J., Klapsa, D., Wilton, T., Zambon, M., Bentley, E., Bujaki, E., Fritzsche, M., Mate, R. and Majumdar, M. (2020) 'Tracking SARS-CoV-2 in Sewage: Evidence of Changes in Virus Variant Predominance during COVID-19 Pandemic', *Viruses,* 12(10), pp. 1144.

Medema, G., Been, F., Heijnen, L. and Petterson, S. (2020a) 'Implementation of environmental surveillance for SARS-CoV-2 virus to support public health

decisions: Opportunities and challenges', *Current Opinion in Environmental Science & Health*.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R. and Brouwer, A. (2020b) 'Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands', *Environmental Science & Technology Letters,* 7(7), pp. 511-516.

Mizuochi, M. (2008). 'Small-Scale Domestic Wastewater Treatment Technology in Japan , and the Possibility of Technological Transfer'.

O'Hara, Z., Crossan, C., Craft, J. and Scobie, L. (2018) 'First Report of the Presence of Hepatitis E Virus in Scottish-Harvested Shellfish Purchased at Retail Level', *Food and environmental virology*, pp. 1-5.

Phillips, G., Tam, C. C., Rodrigues, L. and Lopman, B. (2010) 'Prevalence and characteristics of asymptomatic norovirus infection in the community in England', *Epidemiology & Infection,* 138(10), pp. 1454-1458.

Pintó, R. M., Costafreda, M. I. and Bosch, A. (2009) 'Risk assessment in shellfish-borne outbreaks of hepatitis A', *Appl. Environ. Microbiol.,* 75(23), pp. 7350-7355.

Pires, S. M., Fischer-Walker, C. L., Lanata, C. F., Devleesschauwer, B., Hall, A. J., Kirk, M. D., Duarte, A. S., Black, R. E. and Angulo, F. J. (2015) 'Aetiology-specific estimates of the global and regional incidence and mortality of diarrhoeal diseases commonly transmitted through food', *PloS one,* 10(12), pp. e0142927.

Primadharsini, P. P., Nagashima, S. and Okamoto, H. (2021) 'Mechanism of Cross-Species Transmission, Adaptive Evolution and Pathogenesis of Hepatitis E Virus', Viruses, 13(5).

Public Health England. (2018) *PHE National norovirus and rotavirus Report*: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/724329/Norovirus_update_2018_weeks_22_to_26.pdf.

Public Health England. (2019) *Hepatitis E: symptoms, transmission, treatment and prevention*. gov.uk: Public Health England. Available at: https://www.gov.uk/government/publications/hepatitis-e-symptoms-transmission-prevention-treatment/hepatitis-e-symptoms-transmission-treatment-and-prevention (Accessed: 27 August 2019).

Public Health England. (2020) *PHE National norovirus and rotavirus Report*, GOV.UK: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/871753/Norovirus_update_2020_weeks_08_to_09.pdf.

Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G. and Girones, R. (1994) 'Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification', *Appl. Environ. Microbiol.,* 60(8), pp. 2963-2970.

Purcell, R. H., Engle, R. E., Rood, M. P., Kabrane-Lazizi, Y., Nguyen, H. T., Govindarajan, S., St Claire, M. and Emerson, S. U. (2011) 'Hepatitis E virus in rats, Los Angeles, California, USA', Emerg Infect Dis, 17(12), pp. 2216-22.

R Core Team (2018) *R: A Language and Environment for Statistical Computing* (Version 1.1.383). Available at: https://www.R-project.org/.

Riera-Montes, M., Brus Sjölander, K., Allestam, G., Hallin, E., Hedlund, K. O. and Löfdahl, M. (2011) 'Waterborne norovirus outbreak in a municipal drinking-water supply in Sweden', *Epidemiol Infect,* 139(12), pp. 1928-35.

Rivadulla, E., Varela, M. F., Mesquita, J. R., Nascimento, M. S. and Romalde, J. L. (2019) 'Detection of hepatitis E virus in shellfish harvesting areas from Galicia (Northwestern Spain)', *Viruses,* 11(7), pp. 618.

Sanchez-Galan, J. E., Ureña, G., Escovar, L. F., Fabrega-Duque, J. R., Coles, A. and Kurt, Z. (2021) 'Challenges to Detect SARS-CoV-2 on Environmental Media, the Need and Strategies to Implement the Detection Methodologies in Wastewaters', *Journal of Environmental Chemical Engineering*, pp. 105881.

Santiana, M., Ghosh, S., Ho, B. A., Rajasekaran, V., Du, W. L., Mutsafi, Y., De Jesus-Diaz, D. A., Sosnovtsev, S. V., Levenson, E. A., Parra, G. I., Takvorian, P. M., Cali, A., Bleck, C., Vlasova, A. N., Saif, L. J., Patton, J. T., Lopalco, P., Corcelli, A., Green, K. Y. and Altan-Bonnet, N. (2018) 'Vesicle-Cloaked Virus Clusters Are Optimal Units for Inter-organismal Viral Transmission', *Cell Host Microbe,* 24(2), pp. 208-220.e8.

Schets FM, van den Berg HHJL, Vennema H, Pelgrim MTM, Collé C, Rutjes SA, Lodder WJ. 'Norovirus Outbreak Associated with Swimming in a Recreational Lake Not Influenced by External Human Fecal Sources in The Netherlands, August 2012'. Int J Environ Res Public Health. 2018 Nov 14;15(11):2550. doi: 10.3390/ijerph15112550. PMID: 30441782; PMCID: PMC6267268.

Schlosser, J., Dahnert, L., Dremsek, P., Tauscher, K., Fast, C., Ziegler, U., Groner, A., Ulrich, R. G., Groschup, M. H. and Eiden, M. (2018) 'Different Outcomes of Experimental Hepatitis E Virus Infection in Diverse Mouse Strains, Wistar Rats, and Rabbits', Viruses, 11(1).

Sedji, M. I., Varbanov, M., Meo, M., Colin, M., Mathieu, L. and Bertrand, I. (2018) 'Quantification of human adenovirus and norovirus in river water in the north-east of France', *Environmental science and pollution research,* 25(30), pp. 30497-30507.

Severn Trent Water. (None) *Making it clear: Sewage treatment*. Available at: https://www.stwater.co.uk/content/dam/stw/my-severn-trent/documents/Sewage-treatment-poster.pdf (Accessed: 26 June 2022)

Shapiro, S. S. and Wilk, M. B. (1965) 'An analysis of variance test for normality (complete samples)', *Biometrika,* 52(3/4), pp. 591-611.

Sima, L. C., Schaeffer, J., Le Saux, J.-C., Parnaudeau, S., Elimelech, M. and Le Guyader, F. S. (2011) 'Calicivirus removal in a membrane bioreactor wastewater treatment plant', *Applied and environmental microbiology,* 77(15), pp. 5170-5177.

Smith, A. J., McCarthy, N., Saldana, L., Ihekweazu, C., McPhedran, K., Adak, G. K., Iturriza-Gómara, M., Bickler, G. and O'Moore, É. (2012) 'A large foodborne outbreak of norovirus in diners at a restaurant in England between January and February 2009', *Epidemiol Infect,* 140(9), pp. 1695-701.

Smith, D. B., Paddy, J. O. and Simmonds, P. (2016) 'The use of human sewage screening for community surveillance of hepatitis E virus in the UK', *Journal of medical virology,* 88(5), pp. 915-918.

Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B. and Koopmans, M. (2007) 'Etiological role of viruses in outbreaks of acute

gastroenteritis in The Netherlands from 1994 through 2005', *Journal of clinical microbiology,* 45(5), pp. 1389-1394.

Takahashi, M., Tanaka, T., Takahashi, H., Hoshino, Y., Nagashima, S., Mizuo, H., Yazaki, Y., Takagi, T., Azuma, M. and Kusano, E. (2010) 'Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: characterization of HEV virions in blood circulation', Journal of clinical microbiology, 48(4), pp. 1112-1125.

Takahashi, M., Yamada, K., Hoshino, Y., Takahashi, H., Ichiyama, K., Tanaka, T. and Okamoto, H. (2008) 'Monoclonal antibodies raised against the ORF3 protein of hepatitis E virus (HEV) can capture HEV particles in culture supernatant and serum but not those in feces', Archives of virology, 153(9), pp. 1703.

Teunis, P. F., Moe, C. L., Liu, P., Miller, S. E., Lindesmith, L., Baric, R. S., Le Pendu, J. and Calderon, R. L. (2008) 'Norwalk virus: how infectious is it?', *J Med Virol,* 80(8), pp. 1468-76.

United States Government, U.S House of Representatives. (2018) *The United States Code.* USA: Office of the Law Revision Counsel of the U.S. House of Representatives.

Van Abel, N., Schoen, M. E., Kissel, J. C. and Meschke, J. S. (2017) 'Comparison of Risk Predicted by Multiple Norovirus Dose–Response Models and Implications for Quantitative Microbial Risk Assessment', *Risk Analysis,* 37(2), pp. 245-264.

Vivek, R., Zachariah, U. G., Ramachandran, J., Eapen, C. E., Rajan, D. P. and Kang, (2013) 'Characterization of hepatitis E virus from sporadic hepatitis cases and sewage samples from Vellore, south India', *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 107(6), pp. 363-367.

Wade TJ, Augustine SAJ, Griffin SM, Sams EA, Oshima KH, Egorov AI, Simmons KJ, Eason TN, Dufour AP. 'Asymptomatic norovirus infection associated with swimming at a tropical beach: A prospective cohort study'. PLoS One. 2018 Mar 28;13(3):e0195056. doi: 10.1371/journal.pone.0195056. PMID: 29590196; PMCID: PMC5874074.

World Health Organisation. (2019) *Hepatitis E*: World Health Organization. Available at: https://www.who.int/news-room/fact-sheets/detail/hepatitis-e (Accessed: 08 September 2021).

Wyn-Jones, A. P., Carducci, A., Cook, N., D'agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R. and Höller, C. (2011) 'Surveillance of adenoviruses and noroviruses in European recreational waters', *Water research,* 45(3), pp. 1025-1038.

Yin, X., Ambardekar, C., Lu, Y. and Feng, Z. (2016) 'Distinct entry mechanisms for nonenveloped and quasi-enveloped hepatitis E viruses', *Journal of virology,* 90(8), pp. 4232-4242.

Zlot A, Simckes M, Vines J, Reynolds L, Sullivan A, Scott AK, McLuckie JM, Kromer D, Hill VR, Yoder JS, Hlavsa MC; Centers for Disease Control and Prevention (CDC). Norovirus outbreak associated with a natural lake used for recreation - Oregon, 2014. MMWR Morb Mortal Wkly Rep. 2015 May 15;64(18):485-90. PMID: 25974632; PMCID: PMC4584822.

Chapter 5

Surveillance of Faecally-Derived Viruses within Shellfish, Cetaceans, and Seals from the UK

Abstract

Faecal pollution of the aquatic environment is known to have a wide variety of potentially negative health outcomes, including bacterial and viral pathogen contamination of shellfish on a global scale. One of the most common pathogenic viruses detected in sewage and shellfish is norovirus. In many countries, Hepatitis E virus (HEV) is an emerging viral threat and has been detected in shellfish in several countries. Zoonotic transmission of human or animal HEV to marine mammals is also a possibility. Here I have assessed the presence of HEV and norovirus in British shellfish and examined whether marine mammals in English waters were infected with HEV. We identified that 5/91 (5%) shellfish samples, within an archived sample set harvested between 2009 and 2018, were HEV positive, however 0/85 freshly harvested ready-to-eat shellfish were HEV positive. Of the ready-to-eat shellfish, 67/85 (79%) of samples were positive for the presence of norovirus RNA, with quantities ranging from 1 – 1,525 copies/g of shellfish digestive glands in positive samples. We also identified the presence of HEV RNA in 34/105 (32%) of cetaceans studied. The results show that contamination of shellfish with norovirus is an ongoing problem, and that HEV contamination of shellfish occurs at a much lower frequency. We also identified HEV presence within wild cetaceans, and this suggests that HEV may be infecting marine mammals, with detrimental effects.

Introduction

Bivalve shellfish are filter feeding organisms that can concentrate microorganisms from surrounding waters within their tissues (Lees, 2000; Lowther *et al.*, 2012). Pathogens such as norovirus (from faecal pollution in surface waters) are capable of accumulating within shellfish to a level that has the potential to cause illness in humans (Hardstaff *et al.*, 2019). Surface waters can be contaminated through farm run-off from animals, and from human sewage (treated or untreated). With the number of pollution incidents due to release of untreated wastewater in the UK reaching 2,204 in 2019, the equivalent of one every 45 minutes (Environment Agency, 2020b), regular monitoring of faecal pollution within shellfish is necessary as pathogen loads within the water can vary significantly from day to day. For this reason, routine testing of shellfish

for the presence of *E. coli* (a faecal indicator) is performed in all European Union member states, the UK, and in other countries worldwide. Shellfish for human consumption are often relayed (grown in cleaner waters for a period of time) or depurated (placed in tanks temporarily with circulating water which is UV treated) to reduce their pathogen loads. However, this has often proved to be ineffective for the removal of viruses such as norovirus. For example, Ueki *et al.*, (2007) showed that norovirus did not decrease significantly during a 10-day depuration period; whereas Choi and Kingsley, (2016) showed that norovirus was not significantly reduced over six weeks in 7°C water (similar to relaying conditions).

Oysters are often indicated as the source of foodborne norovirus outbreaks since they are usually consumed raw, and therefore the lack of food processing such as cooking means there is no removal or reduction of the viable virus within the shellfish before consumption. Norovirus has been detected in surface waters and shellfish of various species' worldwide (Wyn-Jones *et al.*, 2011), and identified in marine, riverine and estuarine environments in several countries; Hassard *et al.*, (2017) provides a summary of locations which have identified norovirus in these same environments. Contamination of surface waters near to shellfish harvesting areas commonly leads to contamination of shellfish. In the UK, norovirus was detected in samples of river water and shellfish from the same locations (Campos *et al.*, 2015). Also in the UK, 433/630 (69%) ready-to-eat shellfish samples were identified as positive for the presence of GI and GII norovirus RNA (Lowther *et al.*, 2018). In Italy, 36/253 (14%) shellfish samples (of various species) were found to be contaminated with norovirus RNA (La Bella *et al.*, 2017). In China, 112/840 (13.3%) samples of shellfish were positive for norovirus RNA (Ma *et al.*, 2013), in addition to six marine water sites in Hong Kong being positive for eleven different genotypes of GI and GII norovirus (Yang *et al.*, 2012). Many other countries have also reported norovirus in their shellfish, with a global range of prevalence from 0-95.6% (depending on locality, time of testing and meteorological factors) (Razafimahefa et al., 2020), and outbreaks of norovirus caused by shellfish consumption have also been confirmed in many countries (Potasman et al., 2002). Norovirus has also been identified within surface waters worldwide (Gibson *et al.*, 2011; Lodder and de Roda Husman, 2005; Marcheggiani *et al.*, 2015; Wyn-Jones *et al.*, 2011).

Norovirus is an issue for shellfish suppliers worldwide, but it is not the only enteric virus found in shellfish which causes human disease. Hepatitis A virus (HAV), hepatitis

E virus (HEV) and sapovirus have also been found to contaminate shellfish, and foodborne outbreaks from the consumption of shellfish have been linked to HAV and sapovirus. Several countries have identified HAV within shellfish. In Spain, Manso *et al.*, (2010) identified that 29/68 (42%) of farmed and 40/92 (44%) of wild shellfish samples (160 total) were positive for HAV RNA, whilst in 2002 an outbreak of HAV was determined to have originated at least partly from shellfish (Sánchez *et al.*, 2002). In France, a HAV outbreak was also determined to have been caused by consumption of oysters from a faecally contaminated shellfish harvesting area (Guillois-Bécel *et al.*, 2009). Additionally, the largest ever foodborne outbreak linked to shellfish occurred in China, where HAV infections in approximately 290,000 people were associated with the consumption of clams harvested from a faecally polluted area (Tang *et al.*, 1991). In the UK, regular testing of viruses within shellfish does not take place, though there has been significant attention on testing for norovirus in shellfish with several large national surveys carried out. Systematic studies on HAV presence in shellfish are less common, though there have been some historical reports of HAV presence in UK shellfish, with three samples from sites prohibited from harvesting shellfish for food testing positive (Formiga-Cruz *et al.*, 2002), and a plausible link was found between UK mussels and an outbreak of HAV within the Netherlands (Boxman *et al.*, 2016). However, no large, or recent systematic study of HAV in UK shellfish has been performed.

Sapovirus is also a human pathogen, spread through the faecal-oral route, which is starting to become more widely researched due to its presence in shellfish in several countries. A foodborne outbreak of sapovirus and norovirus was identified in Japan, where a sequence similarity of 99.3-100% was observed between sapoviruses isolated from clam packaging liquid and from stool samples of people who had consumed the clams (Iizuka *et al.*, 2010). Sapovirus has also been identified in surveillance studies of shellfish. For example, 30/168 (18%) of shellfish samples from north-western Spain (Varela *et al.*, 2016), and 54/289 (19%) of shellfish samples from northern Italy (Fusco *et al.*, 2019), were identified as positive for the presence of sapovirus RNA. Again, however, no studies to date have investigated sapovirus presence in UK shellfish.

HEV has also been detected within both sewage and shellfish. One study found an outbreak of HEV on a cruise ship to have been caused most likely by the consumption of shellfish through a retrospective analysis (Said *et al.*, 2009), however consumption

of shellfish has not been conclusively linked to any HEV outbreaks. Rivadulla *et al.*, (2019) identified G3 HEV in 41/164 (24%) of shellfish samples in Spain, and Gao *et al.*, (2015) observed G4 HEV in 22/126 (17%) shellfish samples in China. G3 HEV was also found in 14/15 (93%) untreated influent samples in Edinburgh (Smith *et al.*, 2016); and in chapter 4, we also identified 31/70 (45%) influent samples and 11/70 (16%) effluent samples in a South England study to be positive for HEV RNA. Shellfish in Scotland have also been identified to contain G3 HEV; a study of individual wild Scottish shellfish identified 41/48 (85%) to be G3 HEV positive, though one sampling site was downstream from a pig slaughterhouse (Crossan *et al.*, 2012). Another Scottish study identified 9/310 (3%) of retail shellfish from a Scottish supermarket to be G3 HEV-positive (O'Hara *et al.*, 2018)G3 HEV seems to be the most common genotype detected in shellfish within the UK and Europe, whereas G3 and G4 HEV are detected frequently in shellfish in Asia (Li *et al.*, 2007; Gao *et al.*, 2015; La Rosa *et al.*, 2018; Mesquita *et al.*, 2016; Rivadulla *et al.*, 2019; Crossan *et al.*, 2012; O'Hara *et al.*, 2008). This also reflects human cases, where G4 cases in Asia are much more common than in Europe (Kitaura *et al.*, 2020; Oeser *et al.*, 2019; Hakze-van der Honing *et al.*, 2011). The vast majority of cases in the UK are from G3 and G1 HEV, with G4 having occurred in only three cases between 2014 and 2017 (Oeser *et al.*, 2019). Due to the zoonotic nature of HEV, shellfish may become contaminated with HEV from human faecal pollution, or animal sources (e.g., animal waste within farm run off). However, no UK-wide study on HEV in shellfish has been carried out to date.

Although many studies worldwide have investigated the presence of G3 and G4 HEV in shellfish, studies which investigate the presence of this known zoonotic pathogen have rarely been conducted to identify if other marine and aquatic organisms could be affected by it. In Cuba, a study identified that 10/31 (32%) of captive dolphins were seroprevalent for HEV antibodies, with 5/31 (16%) of these also positive for G3 HEV RNA by qRT-PCR (Villalba *et al.*, 2017). The study indicated that perhaps the food sources for the dolphins could have been contaminated with HEV, leading to infections and subsequent seropositivity. However, no studies have been conducted to identify if wild cetaceans could be impacted by the presence of HEV in surface waters, and to our knowledge, no studies investigating HEV presence in other aquatic organisms (other than shellfish) have been conducted.

Due to the presence of HAV, sapovirus and HEV in surface waters and shellfish from

many countries worldwide, it is important to investigate these viruses from a UK-wide context. This will identify if they are present in the UK aquatic environment, and therefore determine if they could be a risk to human health in the UK through shellfish consumption. We decided to test whether shellfish samples from certain suppliers were more likely to provide shellfish containing norovirus to identify if certain supply lines may be more susceptible to causing norovirus outbreaks. We investigated whether norovirus was more likely and in higher levels in certain regional locations than others to identify whether there may be safer regions in which to harvest shellfish. We also identified the number of samples with over 500 copies/g of norovirus. This is because the European Union is considering introducing legislature which will limit the amount of total norovirus in shellfish samples to ≤500 copies/g, meaning that shellfish with levels higher than this will be prohibited from sale (Younger, A. Personal Communication, 2021). Therefore we looked to see how many samples would be affected by imposing such a limit, and how many suppliers would also be affected, as shellfish farmers wishing to trade in the EU from the UK would also be subject to these limits.Faecal pollution of surface waters is the root cause of illness from consumption of virally contaminated shellfish and can also lead to illness through water consumption during recreational water activities, so identifying the prevalence and levels of the mentioned viruses is important in the context of human health risks. In addition, with some viruses, e.g., HEV, it may be leading to infections within aquatic organisms, which could impact wildlife populations and possibly cause or contribute to stranding events. Therefore, the main aims of this study were to identify whether UK ready-to-eat shellfish were contaminated with norovirus, sapovirus, HAV and HEV; and whether wild beached cetaceans and seals were infected with HEV by testing liver samples from these mammals.

Materials and Methods

Shellfish harvesting area pilot study samples

A selection of archived shellfish samples, stored at -20°C since collection, were chosen in a biased dataset for HEV testing. A dataset of 91 shellfish samples (87 oyster and four mussel) from the frozen archive were selected. The samples were selected from ten harvesting areas which historically had high norovirus concentrations in shellfish samples (range of geometric means for sites 102 – 2,243 copies/g) to identify if HEV was present in shellfish from more faecally polluted harvesting areas. This norovirus data was obtained by other CEFAS colleagues when

samples were originally received. Classification of harvesting areas is dependent on the detection of faecal indicator bacteria within sampled shellfish from the harvesting area. There are four classifications which a harvesting area can obtain depending on the results of *E. coli* testing, classes A, B and C, or prohibited. A Class A harvesting area will not have more than 230 *E. coli* per gram of flesh and intravalvular liquid in 80% of samples collected during a review period, and the remaining 20% will not have more than 700 *E. coli* per gram of flesh and intravalvular liquid. A Class B harvesting area will not have more than 4,600 *E. coli* per 100 g of flesh and intravalvular liquid in 90% of samples during a review period, and the remaining 10% will not have more than 46,000 *E. coli* per 100 g of flesh and intravalvular liquid. Shellfish from Class B must be relayed or depurated to meet Class A requirements. A Class C harvesting area will not have more than 46,000 E. coli per 100g of flesh and intravalvular liquid, and must be relayed or depurated to meet Class A requirements. If E. coli levels are higher than 46,000 E. coli per 100g of flash and intravalvular liquid, the site is prohibited from harvesting shellfish for consumption. All of the harvesting areas in this study were Class B areas, bar one site which was class C (UK Government, 2021). Table 5.5 shows metadata such as harvesting area classification for each anonymized harvesting area. These comprised of 72 samples which were positive for norovirus RNA (of one or both genogroups) and 19 samples in which norovirus RNA was not detected. Within norovirus positive samples, the range of norovirus quantities was 1 – 14,160 copies/g of digestive glands. Each sample consisted of a pool of digestive glands from 10-15 shellfish harvested from 17 different locations, including commercial harvesting areas. Digestive glands were pooled instead of whole oysters as the digestive gland is where most virus particles accumulate, and other shellfish tissues can be inhibitory to PCR techniques. These samples were obtained in 2009, 2010, and 2018, shucked and dissected immediately after arrival at the lab to remove the digestive glands, which were then pooled, and RNA extracted before testing for norovirus. The remainder of the glands were frozen at -20°C until they were RNA extracted and qRT-PCR tested as part of this study. Harvesting locations have been anonymised to prevent loss of reputation for businesses.

Shellfish retail study samples

Ready to eat Pacific oysters (*Crassostrea gigas*) were purchased from eleven different retail suppliers, in March 2019 over a period of two weeks using normal purchasing

channels accessible to the public. It was assumed that most shellfish purchased would have been subject to post-harvest processing such as depuration and relaying (in accordance with UK hygiene regulations for class B and C sites), and most suppliers stated this on their websites. It was not possible to identify the source harvesting sites of all the shellfish samples; all identified harvesting locations were in England and Scotland. Unfortunately, no shellfish could be sourced from suppliers in Northern Ireland or Wales. Of the 11 shellfish suppliers, nine gave harvesting locations for their shellfish (12 locations in total as one supplier harvested from seven different locations, and some suppliers harvested from the same locations). The remaining two suppliers did not disclose harvesting area locations. A sample consisted of 12 oysters purchased from a single supplier on a single date, except in the case of one sample which consisted of 10 oysters, due to lack of availability from the supplier. Pools of 10 or 12 were selected as these represent a typical serving in restaurants or supermarkets, in addition the use of at least 10 animals in a pool is required by the ISO standard method for viruses in foods as it ensures the result is representative of the whole batch (International Organization for Standardization, 2017). A total of 85 pooled samples were purchased during the study period to study HEV and norovirus presence within ready to eat shellfish from different suppliers and determine if certain suppliers or regions were more likely to provide HEV or norovirus-positive shellfish samples. This was intended to be 88 samples (8 from each supplier, one per day from Monday to Thursday over two weeks); however, three samples did not arrive. Only eleven suppliers could be identified for the study. Harvesting locations have been anonymised to prevent loss of reputation for businesses. Approximate locations have been given in Table 5.1. Post-harvest processing treatments were not known or provided by all retailers as this is not a legal requirement, however it is likely that most shellfish samples would have been relayed or depurated as most shellfish harvesting areas in the UK are Class B.

Table 5.1 Generalised harvesting locations of the shellfish suppliers involved in the study

Supplier Number	Regional Location
1	Southeast England[a]
2	Southwest England[b]
3	Southwest England[b]
4	Southwest England[b]
5	Southeast England[a]
6	Unknown
7	Northeast England[c]
8	Scotland
9	Southeast England[a]
10*	Southeast England[a] (four sites), Southwest England[b] (two sites), Channel Islands (one site)
11	Unknown

*All suppliers harvested from one location except for Supplier 10.

[a] Southeast England is defined as all coastal counties from Hampshire to Norfolk.

[b] Southwest England is defined as all coastal counties from Somerset to Dorset.

[c] Northeast England is defined as all coastal counties from Lincolnshire to Northumberland.

Shellfish methods

Shellfish sample preparation

On receipt of the sample, oysters were shucked (opened) and dissected to remove the digestive glands. Shellfish were processed following the international standard (ISO 15216-1:2017) for the quantification of norovirus and Hepatitis A virus (HAV) in foods (International Organization for Standardization, 2017); the method is also suitable for analysis of other single-stranded RNA viruses, such as HEV and sapovirus, by substituting virus-specific primers and probes; however it is not validated for use for these viruses. After dissection and pooling of the oyster digestive glands they were then refrigerated at 4°C for up to 12 hours before being subjected to RNA extraction.

Virus RNA extraction

Mengo virus was cultured within mammalian FRhK-4 cells, and then used as a whole virus extraction efficiency control by spiking into samples prior to RNA extraction.

Samples were extracted alongside a reference extraction (molecular grade water with 10 µl mengo virus). The RNA extraction process began with a proteinase K step to catabolize lipids and denature proteins within the tissues of the gland and allow viral particles to be released. Ten microlitres of an extraction efficiency control, mengo virus, was added to 2 g of chopped pooled digestive glands. The mengo virus batch was recorded and the remaining mengo virus in the batch was frozen at -80°C. Two millilitres of a 100 µg/ml proteinase K (Merck, item number P6656) solution were then added to the digestive glands, and samples were incubated at 37°C for 1 hour on a platform shaker at 320 rpm. The sample was then incubated at 60°C for fifteen minutes, before centrifuging at 3,000 x g for five minutes. The supernatant was collected, and the volume recorded for future calculation of the extraction efficiency and virus concentrations. Sample supernatant was then stored at -20°C for a maximum of 25 days until RNA extraction, adhering to ISO 15216-1:2017.

The method for RNA extraction from the sample supernatant using NucliSENS reagents is described within ISO 15216-1:2017, and was detailed in Lowther *et al.*, (2012). The method utilises a mengo virus control for determining the extraction efficiency. For this purpose, a reference extraction was performed alongside samples, consisting of 10 µl of mengo virus (same batch as used in the initial proteinase K step) in 500 µl of molecular grade water; and a negative extraction control of 500 µl of molecular grade water was also included during each extraction. The extraction process yielded 100 µl of RNA extract per sample, which was frozen at -80°C prior to testing.

Cetacean and seal methods

Cetaceans and seals that beach on UK shores are routinely collected for post-mortem examination by members of the Cetacean Strandings Investigation Programme (CSIP) to identify causes of death. The Cornwall branch of CSIP freezes liver portions of approximately 10 grams in size from all animals collected at -80°C. Between 2017 and 2019 CSIP provided liver samples from a total of 105 cetacean and 66 seal samples collected between 2013 to 2019 for HEV analysis as part of this study. The samples were from 73 common dolphins (*Delphinus delphis)*, 27 harbour porpoises (*Phocoena phocoena)*, 3 striped dolphins (*Stenella coeruleoalba)*, one Risso's dolphin (*Grampus griseus),* one short-finned pilot whale (*Globicephala macrorhynchus)* and 66 grey seals (*Halichoerus grypus)*. The liver samples were transferred to the Cefas laboratory

within cool boxes. 30 mg portions of liver were removed and stored in Invitrogen RNAlater overnight. The liver portions were then subjected to RNA extraction using the Qiagen RNeasy MiniKit, following the manufacturers protocol for purification of total RNA from animal tissues. Briefly, the 30 mg liver portions were removed from the RNAlater and placed in 600 µl of RLT buffer, with 10 µl of mengo virus as an extraction control. The tissue sections were then homogenised using an Ultra-Turrax T25 rotor-stator homogeniser and the manufacturer's protocol for extraction of total RNA from animal tissues was followed exactly from this stage (step 4). A mengo virus only reference extraction (600 µl RLT buffer and 10 µl mengo virus) and a negative extraction control (600 µl RLT buffer) were also extracted simultaneously alongside the samples. 80 µl of RNA extract was yielded from the RNA extraction process. The RNA extracts were then frozen at -80°C prior to qRT-PCR for HEV detection.

PCR methods

Actin RNA integrity control

All retail shellfish samples were tested for the presence of the mRNA of a shellfish housekeeping gene*, actin*, using conventional RT-PCR, as a positive control for RNA integrity. The harvesting area shellfish, cetacean and seal samples were not tested for an mRNA integrity control as it was assumed that mRNA would not be present due to the age of some samples. Integrity controls are normally run as soon as a sample is obtained to assess the integrity of host mRNA, and this was not possible for the harvesting area shellfish, seal, or cetacean samples. The primers for the actin mRNA integrity control assay were designed by aligning a Pacific oyster actin mRNA sequence (accession number NM_001308859.1) and a Pacific oyster actin DNA sequence (accession number NW_011936474.1) to identify the introns and exons within the gene. The primers were designed to anneal to an intron exon boundary, with the sense primer at the position 37 – 55 on the actin mRNA and the antisense primer on the boundary at position 120 – 142, meaning the 106 bp amplicon could only possibly be yielded from amplification of the mRNA and not the actin gene (nuclear DNA). The genomic DNA would have yielded an amplicon of 748 bp, had it amplified. The primers were checked for self-complementarity, primer dimer possibilities and the ideal annealing temperature using the ThermoFisher Multiple Primer Analyzer and Oligo Calc from Northwestern University. The primers were tested by RT-PCR at a concentration of 1 µM on a synthetic DNA control string which

was ordered from ThermoFisher, Geneart, designed from the actin mRNA sequence. The details of the control DNA sequence can be seen in supplementary data 5.1. The assay was shown to work to amplify an amplicon of the expected size (106 bp), visualised using agarose gel electrophoresis (2% agarose with ethidium bromide). The cycling parameters for the actin RT-PCR are described below, in Table 5.2.

Two technical replicates (duplicate assay for one sample) of each retail shellfish sample RNA extract were tested using the actin primers and the Promega Access RT-PCR System according to the manufacturer's protocol, alongside the DNA control, and the resulting PCR products were visualised by gel electrophoresis using a 2% agarose gel with ethidium bromide. Results for the target viruses were only accepted for samples which tested positive for the actin RNA integrity assay.

Following the actin RT-PCR testing, if applied, samples were tested for the mengo virus extraction control. Retail shellfish samples were then tested for the presence of HEV, HAV, norovirus, and sapovirus, and cetaceans and seal samples were tested for HEV only. The primer and probe sets for mengo virus, HEV and norovirus can be seen in chapter 4, Table 4.2. The primer and probes sets and cycling parameters for Actin, Sapovirus and HAV are shown in Table 5.2. Final primer concentrations were 0.625 µM for the forward primer and 1.125 µM for the reverse primer. The mengo virus and norovirus assays are the same as those used within the international standard ISO 15216- 1:2017. Two technical replicates of the same RNA extract were tested for each target virus assay, and one technical replicate was tested for detection of the extraction control (mengo virus). A mastermix of 20 µl, prepared using Invitrogen RNA UltraSense™ One-Step Quantitative RT-PCR System reagents, with 5 µl of RNA extract was used for each technical replicate in each assay. PCR was carried out using the QuantStudio 3 Real-Time PCR System as the detection platform.

Table 5.2 Primer and probe sequences and details for all PCR methods

Target	PCR method	Primer and Probe Sequences	Cycling parameters	Reference
Actin	RT-PCR	FWD: 5'-AATGGATCCGGAATGTGCA-3' REV: 5'-TACCAACCATCACACCCTGATGT-3'	45°C 45min 94°C 2min 40 cycles of: 94°C 30sec 61°C 1min 68°C 2min Then: 68°C 7min 4°C hold	This study
HAV	qRT-PCR	FWD: 5'-TCACCGCCGTTTGCCTA -3' REV: 5'-GGAGAGCCCTGGAAGAAAG-3' PROBE: 5'-CCTGAACCTGCAGGAATTAA-3' 5'-FAM 3'-MGB-NFQ	55°C 60 min 95°C 5 minutes 45 cycles of: 95°C 15 seconds 60°C 1 minute 65°C 1 minute	Costafreda, Bosch and Pintó, (2006)
Sapovirus	RT-PCR	FWD: 5'-GAYCASGCTCTCGCYACCTAC-3' REV: 5'-CCCTCCATYTCAAACACTA-3' PROBE: 5'- CCCCTATRAACCA-3' 5'-FAM 3'-MGB-NFQ	55°C 60 minutes 95°C 15 minutes 40 cycles of: 94°C 15 seconds 62°C for 1 min	Oka *et al.*, (2006)
Mengo virus	qRT-PCR	FWD: 5'-GCGGGTCCTGCCGAAAGT-3' REV: 5'-GAAGTAACATATAGACAGACGCACAC-3' PROBE: 5'-ATCACATTACTGGCCGAAGC-3' 5'-FAM 3'-MGB-NFQ	55°C 60 min 95°C 5 minutes 45 cycles of: 95°C 15 seconds 60°C 1 minute 65°C 1 minute	Pintó, Costafreda and Bosch, (2009)

Several controls were included for the qRT-PCR assays. Mengo virus was used as an extraction control for all samples, using a relative quantification method. RNA from the reference extraction was serially diluted by a factor of 10, to 10^{-3}, and used as a standard curve to assess the extraction efficiency of samples. The extraction efficiency threshold was 0.5%, which is less than the 1% threshold set in ISO 15216- 1:2017. Under the guidelines in ISO 15216-1:2017, samples under the 0.5% extraction efficiency threshold that gave positive results for one or more target virus were considered valid positive results but were non-quantifiable.

Standard curves were also included for the norovirus, HAV and HEV virus assays, using synthetic control DNA preparations which acted as positive PCR controls for quantification of viral RNA. The process by which the HEV control DNA was created was described previously (see chapter 3). The synthetic DNA controls for norovirus (GI and GII) were created following the ISO 15216-1:2017 guidelines. The sequence composition for the HEV, norovirus (GI and GII) and HAV control material are detailed in supplementary data files 3.9, 4.1, and 5.3 respectively. For Sapovirus, a modified plasmid vector, pMA-T, containing a Sapovirus control insert was used as a positive DNA control for the assay due to time restrictions caused by the SARS-CoV-2 pandemic. The Sapovirus assay was therefore used as a non-quantitative assay, without an inhibition control. The details of the Sapovirus insert and plasmid can be seen in supplementary data 5.3 and 5.4 respectively.

For the norovirus, HAV and HEV standard curves, control DNA of concentration 10^5 copies/µl was diluted serially (by a factor of 10) to 10 copies/µl. Two technical replicates for each of the five dilutions were tested, utilising 20 µl of mastermix with 5 µl of standard DNA. Standard curves had to conform to an r^2 value of 0.99 and a slope of between -3.1 and -3.6 for sample test results to be accepted; values outside of these ranges were deemed to be caused by human error in preparation of the qRT-PCR mastermixes and the assays were repeated.

For the shellfish samples, calculation of sample concentrations in copies/g from the C_T values utilised the slope and intercept of the standard curve, in addition to the volume (ml) of gland homogenate (yielded by the proteinase K digestion) and the grams of glands tested (normally 2g). The calculation can be seen in supplementary data 5.5. For the cetacean samples, calculation of sample concentrations in copies/mg from the C_T values utilised the slope and intercept of the standard curve, in addition to the

number of mg (30mg) of cetacean liver used in the RNA extraction. The calculation can be seen in supplementary data 5.6.

Controls for measuring inhibition of the sample matrix to the qRT-PCR were also included for norovirus, HAV and HEV, using synthetic RNA (the process for creating the HEV RNA control is described in chapter 3 using the same method from ISO 15216-1:2017 for creation of norovirus and HAV inhibition controls). Additional reaction wells were prepared, separate to those for virus detection, containing 5 µl of sample RNA extract, 20 µl of mastermix, and 1 µl of the inhibition RNA control. The C_T values for these reactions were compared to a C_T value obtained from a well containing 5 µl of molecular grade water, 20 µl of mastermix, and 1 µl of the inhibition RNA control. The difference in C_T values was converted into an inhibition percentage by reference to the standard curve. Samples had to have an RT-PCR inhibition of ≤75% (approximately two C_T values difference depending on standard curve) to be considered reliable quantification. Under the guidelines in ISO 15216-1:2017, samples over the 75% inhibition threshold that gave positive results for one or more target virus would be regarded as a valid positive detection, but non-quantifiable. This threshold is used because it is considered that if the qRT-PCR inhibition is above 75%, there is a high likelihood of false negative results, and poor amplification of the virus, leading to inaccurate quantification.

In terms of negative controls, an extracted negative control was tested for both mengo virus and the target viruses (acting as a control for cross contamination during RNA extraction), and a negative qRT-PCR control (molecular grade water only) was also tested for mengo virus and the target viruses (acting as a control for cross contamination during plate preparation). Sample preparation, RNA extraction and qRT-PCR plate preparation, and the qRT-PCR itself all occurred in separate laboratory rooms to minimise contamination risk.

Statistical Analysis

All statistical analysis and graphs were produced in R version 3.4.3 (R Core Team, 2018). Norovirus and HEV data within shellfish and cetacean samples were initially tested for normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965). The data were found not to be normally distributed when $\log_{10}$ transformed, or when untransformed, and as zeroes were meaningful, the data were analysed using non-parametric tests.

Mean virus copies/g digestive glands and copies/mg of liver were calculated using the quantification values obtained for all samples; negative samples were held to contain 0 copies/g or copies/mg. Limits of quantification were not applied. Total norovirus levels where used were calculated by addition of GI and GII copies/g. The prevalence of virus in individual oysters was calculated using R code from Webster *et al.*, (2015), and utilised 95% confidence intervals (95% CI).

Spearman's rank correlation was used to check for correlation between levels of GI and GII norovirus in shellfish samples (Jerrold, 2005). The Fisher's Exact test was used to check for an association between the level of total norovirus in a sample and the suppliers which the samples had originated from Fisher, (1934). Fisher's exact tests were also used to assess whether norovirus presence was an indicator of HEV presence in the archived shellfish samples from harvesting areas, and to assess whether cetacean sex or life stage at death (as age could not be ascertained) could be important risk factors for contracting HEV in cetaceans. Life stage was defined into one of five categories: Neonate, Calf, Juvenile, Sub-adult, Adult. A Kruskal-Wallis test was used to identify if there were significant differences in the distribution of total Norovirus levels between the shellfish suppliers (Kruskal and Wallis, 1952), and was followed by post-hoc pairwise Wilcoxon tests with Holm correction (Wilcoxon, 1992; Holm, 1979).

Results

Actin

The retail shellfish samples were all expected to yield a positive detection result for the actin gene as they should have been sold alive and in good condition for consumption purposes. The expected amplicon size was 106 bp. Figure 5.1 shows one gel electrophoresis containing samples with both positive and negative detections. One sample of the 85 shellfish samples (sample 19-499, seen in Figure 5.1) failed this assay and was subsequently excluded from data analysis. Table 5.3 shows the positive or negative detection results for each sample.

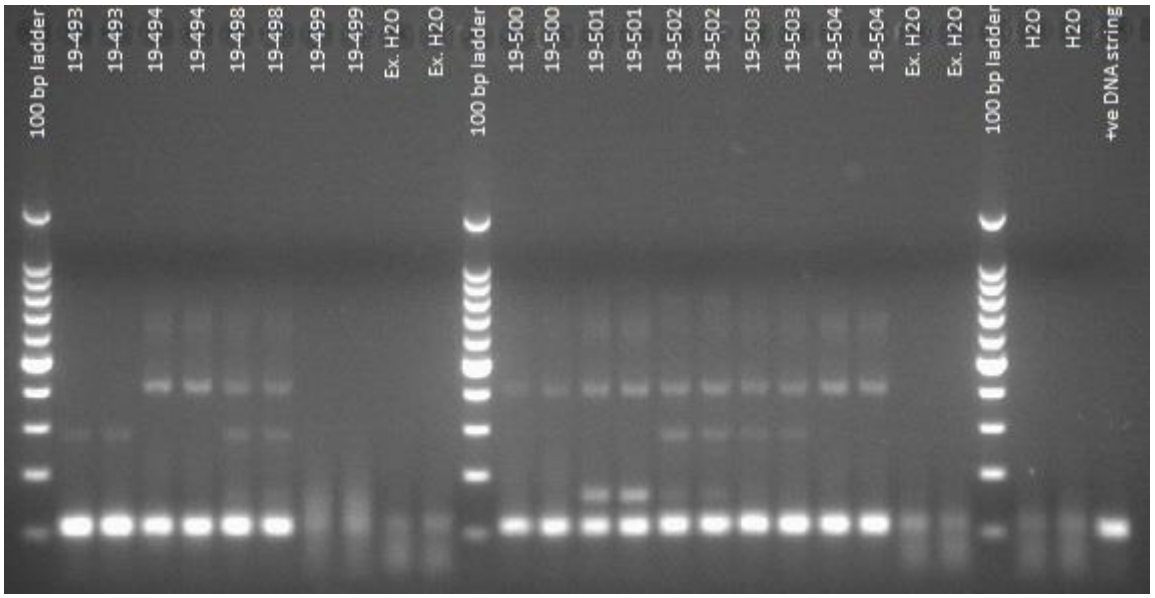


Figure 5.1 Example gel electrophoresis for nine retail shellfish samples

Retail shellfish sample RNA extracts were tested in duplicate (two technical replicates of one extract) to determine whether actin mRNA was present within the shellfish samples. Negative results indicated that the mRNA had degraded and that the sample may not be fit for viral testing. From left to right, well 1 is a 100 base pair DNA ladder, wells 2 and 3 correspond to sample 19-493, wells 3 and 4 correspond to sample 19-494, wells 5 and 6 correspond to sample 19-498, wells 7 and 8 correspond to sample 19-499, and wells 9 and 10 correspond to a negative extraction control containing water instead of sample. Well 11 is another 100 bp DNA ladder. Wells 12 and 13 correspond to sample 19-500, wells 14 and 15 correspond to sample 19-501, wells 16 and 17 correspond to sample 19-502, wells 18 and 19 correspond to sample 19-503, wells 20 and 21 correspond to sample 19-504, and wells 22 and 23 are another negative extraction control. Well 24 is a 100 bp ladder. Wells 25 and 26 are PCR negative controls (water and PCR mastermix instead of sample). Well 27 is a positive control from a synthetic Actin DNA.

Table 5.3 Presence or absence of actin mRNA in the retail shellfish samples

Sample number	Actin +/-	Sample number	Actin +/-	Sample number	Actin +/-
19-369	+	19-440	+	19-500	+
19-370	+	19-441	+	19-501	+
19-373	+	19-442	+	19-502	+
19-374	+	19-443	+	19-503	+
19-375	+	19-444	+	19-504	+
19-376	+	19-445	+	19-505	+
19-377	+	19-446	+	19-506	+
19-378	+	19-455	+	19-507	+
19-379	+	19-456	+	19-508	+
19-380	+	19-457	+	19-510	+
19-381	+	19-458	+	19-511	+
19-382	+	19-459	+	19-512	+
19-383	+	19-460	+	19-513	+
19-384	+	19-461	+	19-514	+
19-385	+	19-462	+	19-515	+
19-386	+	19-483	+	19-516	+
19-411	+	19-484	+	19-517	+
19-412	+	19-485	+	19-518	+
19-413	+	19-486	+	19-519	+
19-414	+	19-487	+	19-520	+
19-415	+	19-488	+	19-521	+
19-416	+	19-489	+	19-522	+
19-417	+	19-490	+	19-523	+
19-418	+	19-491	+	19-524	+
19-435	+	19-492	+	19-525	+
19-436	+	19-493	+	19-526	+
19-437	+	19-494	+	19-527	+
19-438	+	19-498	+		
19-439	+	19-499	-		

HEV in shellfish from harvesting areas

Raw data for the shellfish harvesting area pilot study can be seen in Table 5.5. Seven (8%) of the 91 shellfish samples from harvesting areas produced extraction efficencies lower than the 0.5% threshold. One of these seven samples exceeded the 75% threshold for inhibition, but was the only sample of this dataset to do so. Another of the seven samples was positive for HEV RNA, but non-quantifiable due to the extraction efficiency being below the 0.5% threshold.

Extraction efficiencies ranged from 0.9% to 48.2%, and percentage inhibition ranged from 0.0% to 43.5% in the remaining 84 samples. The seven samples which failed the extraction efficiency were excluded from further data analysis. A subset of 19 samples were re-tested (in 2019) for norovirus and the results were compared to the historical norovirus results (from 2009-2018). There was no significant difference in the levels of norovirus detected in the samples (p= 0.260) using a paired T-test, suggesting little degradation of norovirus RNA levels.

Of the 91 samples selected for HEV testing, five (6%) gave HEV positive results by qRT-PCR, but only two of these gave a positive result in both technical replicates, and only four were quantifiable, the fifth was non-quantifiable due to low extraction efficiency. Within the positive samples, the minimum HEV genome copies/g of digestive glands was 16.3 genome copies/g, and the maximum was 121.3 genome copies/g (C_T value range 37 – 40). The HEV-positive samples originated from five different locations, and three different years (two in 2009, one in 2010 and two in 2018). The two 2018 samples were collected at different locations within the same estuary, on the same day. The positive samples were collected in March, July, and December. Norovirus presence in the shellfish was determined not to be an indicator of HEV presence (Fisher's Exact test, p= 0.58). The contingency table for this analysis can be seen in Table 5.4.

Table 5.4 Contingency table to show the lack of association between norovirus and HEV in shellfish from harvesting areas

	Norovirus positiive	**Norovirus negative**
HEV positive	5	0
HEV negative	67	19

In terms of norovirus results for the HEV-positive samples, which were obtained the year the shellfish were harvested by CEFAS colleagues, the range of total norovirus was from 85 copies/g to 6,048 copies/g of digestive glands, and all the HEV-positive samples were also norovirus positive. The data can be seen in Table 5.5.

Table 5.5 Raw data for the shellfish harvesting area pilot study

Harvesting Area	Classification at time of harvest	Years of harvest	Number of samples	Number of GI positives	Number of GII positives	Number of GI and GII positives	Geomean norovirus copies/g	Range of norovirus copies/g*	Number of HEV positive samples
A	B	2009-2010	9	8/9	9/9	8/9	2243	16 – 14160	0
B	B	2009-2010	8	8/8	5/8	5/8	462	7 – 4144	0
C	B	2018	4	4/4	4/4	4/4	2813	635 – 6048	2
D	B	2009-2010	10	7/10	9/10	7/10	291	1 – 4059	1
E	B	2009	8	3/8	3/8	2/2	102	5 – 2042	1
F	B	2009-2010	8	2/8	3/8	2/8	180	43 – 2560	0
G	B	2009-2010	8	7/8	7/8	7/8	537	20 – 6114	0
H	C	2009-2010	9	5/9	4/9	3/9	174	11 – 9439	1
I	B	2009-2010	8	7/8	7/8	8/8	393	15 – 5250	0
J	B	2009-2010	8	5/8	5/8	3/8	272	1-2653	0
K	B	2009-2010	11	11/11	8/11	8/11	755	17 – 11961	0

*In samples positive for norovirus RNA only

Norovirus in retail shellfish

Of the 85 shellfish samples obtained in March 2019, two were invalid due to low extraction efficiency. One of these had also failed the actin integrity control assay, but was positive for both GI and GII norovirus. The two invalid samples were not included in quantitative data analysis. Extraction efficiencies ranged from 0.55 – 19.2% for the remaining retail shellfish samples. Low extraction efficiency is typical of shellfish samples, but is used as a crude measurement of extraction efficiency as it often does not accurately reflect the recovery of the target virus. The extraction efficiency and concentration of GI and GII norovirus (in copies/g) did not correlate (Spearman's rank correlations, GI norovirus *p* value= 0.182, Rho= -0.17; GII norovirus *p* value = 0.566, Rho= 0.08). Virus levels were therefore not corrected by extraction efficiency. All of these samples were within the 75% RT-PCR inhibition threshold, and the inhibition percentage ranged from 0.0-65.6%.

Of all samples, 67/85 (79%) were positive for noroviruses from either or both genogroups. Of these, 61/85 (72%) were positive for GI, and 58/85 (68%) were positive for GII norovirus. Both genogroups were detected in 52/85 (61%) of these shellfish samples. Of the 83 valid samples, 66 (80%) were found to contain norovirus, of either or both genogroups I and II. GI norovirus was found in 60/83 (72%) of the shellfish samples and GII norovirus was found in 58/83 (70%) of samples. Both genogroups were detected in 51/83 (61%). The mean level of GI norovirus was 258 genome copies/g of digestive glands, and the mean level of GII norovirus was 122 genome copies/g of digestive glands. The mean total norovirus was 272 genome copies/g of digestive glands. Analysis using Spearman's Rank correlation showed a positive correlation between the level of GI and GII norovirus in samples (correlation coefficient Rho= 0.76; $p<0.0001$). Figure 5.2 shows a comparison of the GI and GII quantities in the shellfish sample population.

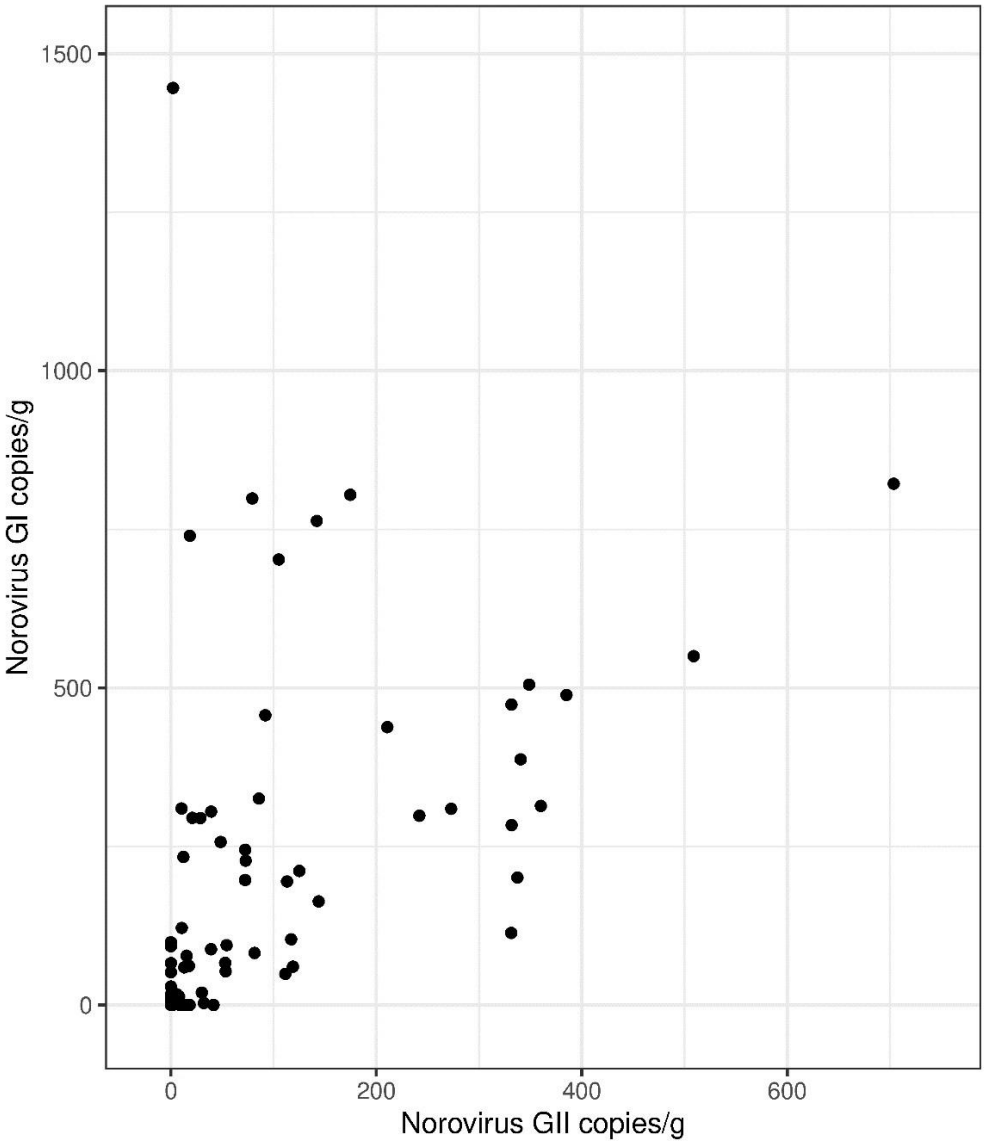


Figure 5.2 Comparison of GI and GII norovirus levels in oyster samples

Comparison of the genome copies/g of GI and GII norovirus in shellfish digestive glands from individual shellfish samples.

The minimum level of total norovirus found in any positive shellfish sample was 1 copy/g digestive glands, and the maximum level was 1,525 copies/g digestive glands. The minimum and maximum levels of GI norovirus in positive samples were 3 and 1,446 copies/g digestive glands respectively (C_T value range 33-40). For GII, the minimum and maximum levels were 1 and 703 copies/g digestive glands respectively (C_T value range 34 – 42). Figure 5.3 shows a frequency distribution of the samples which tested positive for norovirus.

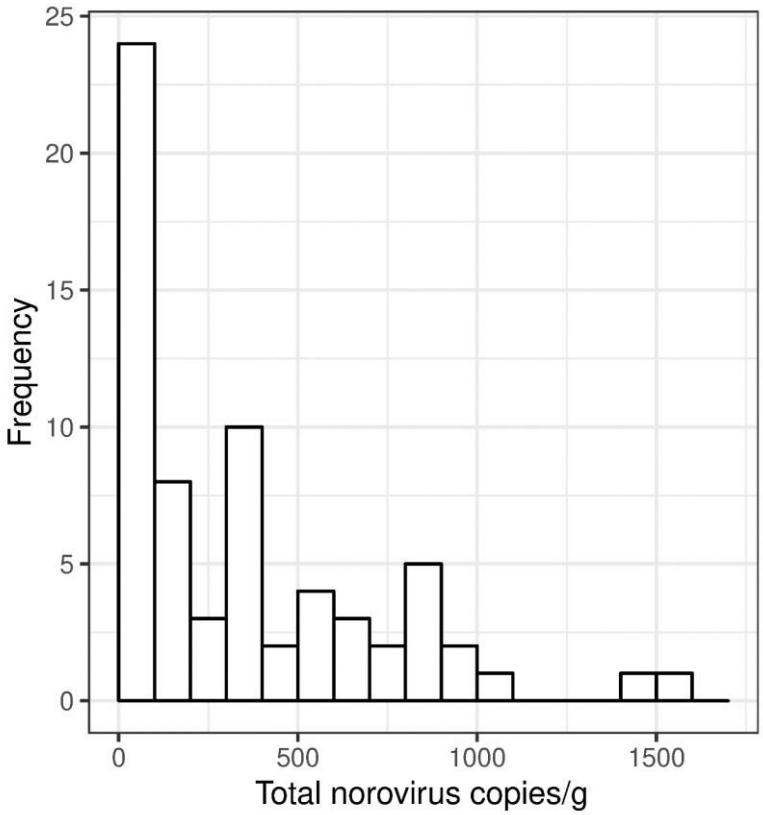


Figure 5.3 The frequency distribution of the total norovirus copies/g digestive glands

A histogram showing the frequency of groups of norovirus quantities in shellfish samples. Shellfish samples are grouped by 100 copies/g. Samples which did not test positive for either genogroup using qRT-PCR were not included in the histogram.

All shellfish suppliers provided at least one sample which tested positive for norovirus. Supplementary data 5.7 shows a raw data table with norovirus results by supplier. 19/83 (23%) of the oyster samples contained total norovirus levels of over 500 copies/g digestive glands. This threshold was chosen due to draft European Commission legislation suggesting a 500 copies/g norovirus limit in shellfish samples (Younger, A. Personal Communication, 2021). Eight of eleven suppliers provided at least one sample which gave a result of over 500 copies/g of total norovirus. The supplier providing most of the samples with over 500 copies/g of total norovirus was supplier 5, which also gave the highest average total norovirus of all the suppliers. All samples over 500 copies/g originated from locations in Southern England except for two with unknown locations (Table 5.6).

Table 5.6 GI, GII and total norovirus copies/g for samples with over 500 copies/g total norovirus by supplier

Supplier number	Sample number	Harvesting Locations	Norovirus GI copies/g	Norovirus GII copies/g	Total Norovirus copies/g
1	19-458	Southeast England	309	273	582
2	19-376	Southern England	457	92	549
2	19-461	Southern England	550	509	1059
2	19-462	Southwest England	474	332	805
3	19-520	Southwest England	1,446	2	1,448
4	19-378	Southwest England	284	332	615
4	19-442	Southwest England	805	175	979
4	19-486	Southwest England	799	79	878
5	19-418	Southeast England	299	242	540
5	19-459	Southeast England	822	704	1,526
5	19-502	Southeast England	314	360	674
5	19-503	Southeast England	489	385	874
5	19-517	Southeast England	763	142	905
9	19-440	Southeast England	387	341	728
9	19-441	Southeast England	201	337	538
9	19-460	Southeast England	505	349	854
10	19-373	Southeast England	438	211	649
11	19-483	Unknown	703	105	808
11	19-498	Unknown	740	18	758

We then examined the average level of total norovirus from samples by supplier (Figure 5.4). We found limited evidence that certain suppliers were more likely to provide positive samples, (Table 5.7, Fisher's exact test, p= 0.0507). We found no

association between harvesting location and norovirus positivity (Table 5.7, Fisher's exact test, p= 0.429).

Table 5.7 Contingency table for testing whether certain suppliers were more likely to provide shellfish samples containing norovirus

Supplier	Negative	Positive
1	0	8
2	0	6
3	0	8
4	0	8
5	0	8
6	6	2
7	2	5
8	7	1
9	0	7
10	1	6
11	1	7

Table 5.8 Contingency table for Fisher's exact test of norovirus positivity between known harvesting locations

Location number	Norovirus positive	Norovirus negative
1	8	0
2	8	0
3	8	0
5	5	2
7	8	0
9	6	0
11	9	0

Only locations with more than 6 samples were included in this analysis

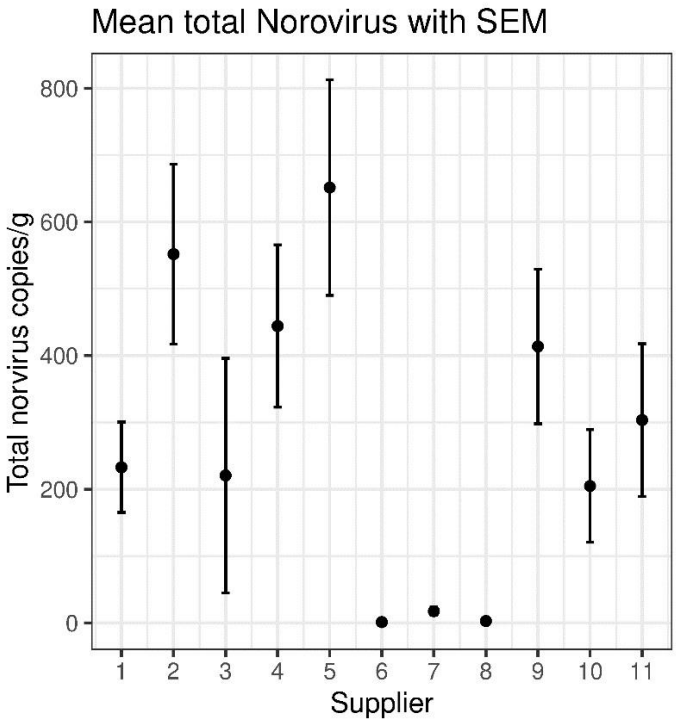


Figure 5.4 Average norovirus levels for each supplier

The average levels of norovirus identified in samples from each supplier, alongside error bars showing the standard error.

We determined that there was significant variation in total norovirus levels between suppliers (Kruskal-Wallis test, $p<0.0001$) and that this variation was mostly driven by the low levels of norovirus from suppliers 6, 7 and 8. Significant *p* values are displayed in Table 5.9.

Table 5.9 *p* values to identify which suppliers have significantly different total norovirus variation between their samples

Supplier	**1**	**2**	**3**	**4**	**5**	**9**
6	0.035	0.065*	0.035	0.035	0.035	0.049
7	0.064*			0.064*	0.064*	
8	0.030	0.056*	0.056*	0.030	0.030	0.056*

* Significant at a 0.1 significance level.

We also found norovirus positive shellfish were more likely to come from certain regional locations (Table 5.10, Fisher's exact test, $p= 2.01 \times 10^{-9}$).

Table 5.10 Contingency table for Fisher's exact test of differences in norovirus positivity between regional locations

Region	Norovirus positive	Norovirus negative
Northeast England	5	2
Southeast England	27	0
Southwest England	24	0
Channel Islands	0	1
Scotland	1	7

We identified significant variation in total norovirus between regions in the South of England and the North of England and Scotland (Kruskal Wallis test followed by Wilcoxon pairwise post-hoc test with Holm correction). The significant results can be seen in Table 5.11, and raw data by region can be seen in supplementary data 5.8.

Table 5.11 Significant *p* values from the post-hoc test of differences in norovirus variation between regions

Region	Northeast England	Scotland
Southeast England	9.96×10^{-4}	1.90×10^{-4}
Southwest England	1.55×10^{-3}	2.16×10^{-4}

HEV, HAV and sapovirus in retail shellfish

Raw retail shellfish samples were tested for the presence of HEV, HAV and sapovirus by qRT-PCR. None of the 85 shellfish samples gave a positive result for any of these virus assays. Positive control results indicated no issues with the assays. The prevalence of these viruses within individual oysters was calculated to be 0% (95% CI: 0.000 – 0.002). The data can be seen in Table 5.12.

Table 5.12 Raw data showing the results of the sapovirus and HAV testing

Sample number	Sapovirus	HAV	Sample number	Sapovirus	HAV	Sample number	Sapovirus	HAV
19-369	ND	ND	19-440	ND	ND	19-500	ND	ND
19-370	ND	ND	19-441	ND	ND	19-501	ND	ND
19-373	ND	ND	19-442	ND	ND	19-502	ND	ND
19-374	ND	ND	19-443	ND	ND	19-503	ND	ND
19-375	ND	ND	19-444	ND	ND	19-504	ND	ND
19-376	ND	ND	19-445	ND	ND	19-505	ND	ND
19-377	ND	ND	19-446	ND	ND	19-506	ND	ND
19-378	ND	ND	19-455	ND	ND	19-507	ND	ND
19-379	ND	ND	19-456	ND	ND	19-508	ND	ND
19-380	ND	ND	19-457	ND	ND	19-510	ND	ND
19-381	ND	ND	19-458	ND	ND	19-511	ND	ND
19-382	ND	ND	19-459	ND	ND	19-512	ND	ND
19-383	ND	ND	19-460	ND	ND	19-513	ND	ND
19-384	ND	ND	19-461	ND	ND	19-514	ND	ND
19-385	ND	ND	19-462	ND	ND	19-515	ND	ND
19-386	ND	ND	19-483	ND	ND	19-516	ND	ND
19-411	ND	ND	19-484	ND	ND	19-517	ND	ND
19-412	ND	ND	19-485	ND	ND	19-518	ND	ND
19-413	ND	ND	19-486	ND	ND	19-519	ND	ND
19-414	ND	ND	19-487	ND	ND	19-520	ND	ND
19-415	ND	ND	19-488	ND	ND	19-521	ND	ND
19-416	ND	ND	19-489	ND	ND	19-522	ND	ND
19-417	ND	ND	19-490	ND	ND	19-523	ND	ND
19-418	ND	ND	19-491	ND	ND	19-524	ND	ND
19-435	ND	ND	19-492	ND	ND	19-525	ND	ND
19-436	ND	ND	19-493	ND	ND	19-526	ND	ND
19-437	ND	ND	19-494	ND	ND	19-527	ND	ND
19-438	ND	ND	19-498	ND	ND			
19-439	ND	ND	19-499	ND	ND			

ND = Not Detected

HEV in Cetaceans and Seals

For the seal samples, 65 of 66 seal liver samples gave results within the quality control parameters, with all passing the extraction efficiency threshold, but one giving invalid inhibition results. This one sample was excluded from further data analysis. Extraction efficiencies ranged from 1.4 – 205.9%, and inhibition levels ranged from 0.0 – 68.1%. For the cetacean liver samples, 102 of 105 samples gave

acceptable results, with all passing the extraction efficiency threshold, and three giving invalid inhibition results. These three samples were excluded from further quantitative analysis and belonged to a harbour porpoise and two common dolphins. Two of these samples had provided a positive result for the presence of HEV RNA. Extraction efficiencies ranged from 2.5 – 606.6% for the valid cetacean samples, and the inhibition percentages ranged from 0.0 – 71.9%.

None of the 66 valid seal liver samples tested positive for the presence of HEV RNA (0% CI: 0.0 – 6.8%). Positive control results indicated no issues with the HEV assay. An RNA integrity control was not used due to the storage conditions (liver samples kept at -80°C from 2013 onwards). Of the 105 cetacean liver samples, 34 (32%) tested positive for the presence of HEV RNA. Of the HEV positive samples, only 32 of 34 gave valid quantification results, the remaining two were classed as positive but non-quantifiable and so were not analysed further. Results by species can be seen in Table 5.13 and more detailed raw data can be seen in supplementary data 5.9.

Table 5.13 HEV positive samples from each marine mammal species

Common name	Positive/Total	HEV prevalence (CI range)
Common dolphin	29/73	39.7% (28.7 – 51.9%)
Striped dolphin	0/3	0.0%*
Risso's dolphin	0/1	0.0%*
Harbour porpoise	5/27	18.5% (7.0 – 38.7%)
Short-finned pilot whale	0/1	0.0%*
Grey seal	0/66	0.0% (0.0 – 6.4%)

*Sample size too low to calculate prevalence estimate

Of the valid quantitative data, the mean HEV level was 15 copies/mg of cetacean liver. The minimum HEV level within the positive samples was 1 copy/mg of cetacean liver. The maximum HEV level was 210 copies/mg (C_T range 29 – 39).
Mean levels of HEV differed between species with positive detections (common dolphin and harbour porpoise), but this was not tested statistically for significance due to the differences in sample size for each species. Figure 5.5 shows the average HEV copies/mg observed from each species of cetacean within the study, where data was applicable.

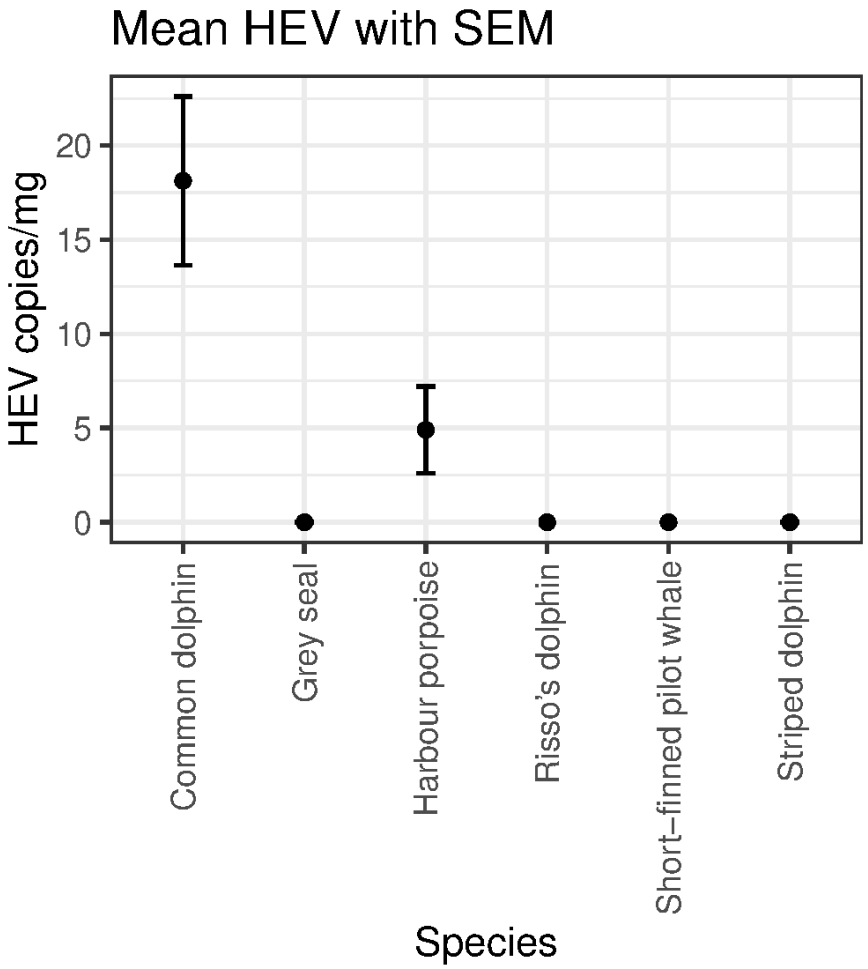


Figure 5.5 The mean HEV copies/mg observed from the six different species involved in the study

The mean HEV copies/mg of liver observed for each of the species of cetaceans and seals included in the study. Low sample sizes for the Risso's dolphin, short-finned pilot whale, and striped dolphin were a result of very few of these animals beaching between 2013 and 2019 in Southwest England, and therefore it is not possible to identify a baseline level of HEV prevalence in these species.

Due to the uneven sample sizes for each species, it was not possible to accurately determine if HEV was more likely to occur in a certain species of cetacean compared to the others. However, we did identify that that there was a higher chance of HEV infection in male cetaceans than females (one sided (greater) Fisher's exact test, p= 0.001). The contingency table for this analysis can be seen in Table 5.14.

Table 5.14 Contingency table for the comparison of HEV infection in male vs female cetaceans

	Positive	Negative
Male	23	26
Female	9	44

No significant relationship was identified between HEV infection and life stage of the cetaceans (Fisher's exact test, p= 0.074; the contingency table for this can be seen in Table 5.15. The causes of death were too numerous to test whether HEV infection was associated with a specific cause.

Table 5.15 Contingency table for Fisher's test of relationship between life stage at death and HEV presence

Life stage at death	Positive	Negative
Neonatal	0	1
Calf	3	2
Juvenile	7	19
Sub-adult	8	6
Adult	14	42

Discussion

In this study, we examined the presence of norovirus and HEV in UK shellfish, and HEV in cetaceans, to assess contamination of the aquatic environment. We found that 6% of archived shellfish samples from harvesting areas were positive for HEV, but no shellfish samples from the retail study were positive for HEV. The retail shellfish samples also showed no sign of contamination with sapovirus or HAV. However, 80% of them were positive for norovirus. We also identified HEV within 32% of the cetacean samples studied, but in none of the seal samples. This is the first study in the UK to assess the presence of HEV, HAV and sapovirus within UK shellfish, and the first to assess the presence of HEV within wild cetaceans and seals.

HEV in shellfish from harvesting areas

Five out of 91 archived shellfish samples from harvesting areas in England were identified to be positive for HEV RNA, with no pattern of seasonality able to be ascertained (due to a low number of positive results). Only two of these samples contained HEV in both technical replicates, however this is a common observation in

food samples with low virus abundance and may indicate that the result is at or below the limit of detection. Low levels of HEV were observed in all positive samples, which may be indicative of low abundance within the aquatic environment. Though only one study has provided a tentative link between shellfish consumption and an outbreak of HEV (Said *et al.*, 2009), this finding reinforces that possibility. We determined that norovirus was not an indicator of HEV presence statistically, most likely because there were many samples containing norovirus RNA but not HEV RNA. However, all five of the HEV-positive samples were also positive for norovirus, which was expected as norovirus presence suggests faecal contamination. HEV presence was much less common than norovirus presence, with 72/91 (79%) of these selected samples norovirus positive, and just 5/91 (5%) HEV-positive, which is likely due to the contrast in norovirus and HEV cases in the community. For example, from June 2017 to June 2018, reported norovirus cases reached 6,719 in England and Wales (Public Health England, 2018a), whereas in 2018 HEV cases reached 1002 (Public Health England, 2019). However, underreporting of norovirus illness is a well-known phenomenon and the Food Standards Agency estimates that 3 million norovirus cases occur yearly in the UK, of which 380,000 are from foodborne sources (Gherman *et al.*, unknown). True case numbers for HEV in the UK are unknown but due to the severity of clinical symptoms the scale of underreporting is likely to be less than for norovirus, emphasising that HEV is indeed likely to be much rarer in the population. The HEV viral load within the shellfish samples may have been influenced by degradation during their storage at -20°C for up to nine years and therefore prevalence and quantities of the virus in the samples may be underestimated. No further data analysis was performed on this dataset for these reasons. However, to the best of our knowledge, this represents the first detection of HEV RNA in English shellfish from harvesting areas. The percentage of positive samples detected in this study was obtained from a biased sample population, and therefore should not be compared to the prevalence of HEV detected in other studies. However, the result is similar to the study by O'Hara et al (2018), which showed that retail shellfish purchased in Scotland had a HEV prevalence of 2.9%.

These results indicate that the aquatic environment is becoming contaminated by HEV, but to an unknown extent. The fact that these shellfish were collected directly from harvesting areas and were not subjected to further processing by depuration, which was and is currently required of all class B and C shellfish harvesting areas in

the UK (which make up the majority of UK harvesting areas) (UK Government, 2021), may also mean that HEV estimates in these samples may be higher than those in retail shellfish. However, the effect of depuration on HEV in shellfish is yet to be determined, though viruses such as norovirus are not easily removed from shellfish due to their ability to bind to histoblood group antigen-like molecules, and sialic acids within shellfish tissues (Almand et al., 2017; Maalouf *et al.*, 2010).

HEV, HAV and sapovirus in shellfish from retail suppliers

None of the retail shellfish within this study provided positive results for the presence of HEV, HAV or sapovirus. This is possibly due to low virus abundance in the aquatic environment or effectiveness of depuration methods against these viruses (the majority, if not all samples would have been subjected to depuration prior to sale). However it could also be because the samples were not representative enough of all harvesting areas in the UK. This study was designed to identify a baseline of viral presence, and the risk that this may have to the public, not as an estimate of UK-wide prevalence, due to the lack of representation of some areas of the UK and some shellfish suppliers. As it is unknown whether seasonality effects HEV abundance within shellfish, it is difficult to know if the date of the study could have influenced the results, as all samples were collected in March. It is known that norovirus has a pattern of seasonality, where the average quantity of norovirus in retail shellfish samples increases during winter months (October – March) (Lowther *et al.*, 2018). To reliably identify the prevalence and seasonality of HEV, HAV and sapovirus in the UK aquatic environment, a more systematic study would be required.

Despite the lack of contamination of ready to eat shellfish with HEV, the presence of norovirus in such a large proportion of samples indicates ongoing faecal pollution affecting shellfish, and this could therefore mean HEV contamination which perhaps has not been detected during this limited study. Though it appears there was little to no risk of contracting HEV from the shellfish tested during this retail study, it should be noted that HEV has been detected in English shellfish from harvesting areas, from wild shellfish in Scotland (Crossan *et al.*, 2012), and also from ready to eat shellfish purchased in a Scottish supermarket (O'Hara *et al.*, 2018). Due to higher levels and prevalence of HEV in shellfish which are wild, or have been tested straight from harvesting areas (Crossan *et al.*, 2012), this may constitute more of a risk of HEV transmission for people who collect their own shellfish in the UK, rather than

purchasing from suppliers.

Norovirus in Shellfish

This study identified that 79% of the retail shellfish were positive for the RNA of at least one genogroup of norovirus. We also identified that GI norovirus had a slightly higher prevalence, average, and maximum quantity than GII norovirus. This is in contrast to Lowther *et al.*, (2018), where GII had a higher maximum level than GI. Circulation of noroviruses in reported cases in March 2019 were dominated by GII noroviruses (Public Health England, 2020), which is an interesting contrast to our norovirus data, and may indicate that the GI contamination of shellfish in our study originates from shedding of virus resulting from unreported cases or asymptomatic infections, or better binding of GI noroviruses to shellfish tissues than GII noroviruses. Indeed, Maalouf *et al.*, (2010) showed that GI noroviruses have an increased binding capacity to shellfish digestive tissues in winter months (January to May), but this pattern was not observed for GII noroviruses. We also observed a significant positive correlation between levels of GI and GII norovirus in the retail shellfish samples, suggesting that contamination of shellfish is often being caused by sewage containing similarly varying levels of GI and GII noroviruses.

We also identified that 23% of the shellfish samples contained a total norovirus quantity over 500 copies/g of digestive glands, and that these samples originated from eight of the eleven suppliers. All of these samples except two were from Southern England, with the remaining two from an unknown location. We also found that there was a significant difference in the variation of levels of norovirus between regions in southern England and northern England or Scotland. This suggests that faecal contamination of shellfish harvesting areas is an ongoing problem affecting many shellfish producers in the UK, particularly in Southern England. The observation of lower levels of norovirus in Scottish shellfish has been made before and suggests that Scottish waters are less polluted (Lowther, 2011; Williams and O'Brien, 2019). However, the data may have been skewed as only eight samples originated from Scotland, and seven from Northern England, in comparison to 51 samples from Southern England. Following the European baseline survey for norovirus in oysters (EFSA, 2019), an end product standard in oysters of 500 copies/g has been discussed by the European Commission and representatives of EU Member States (Younger, A. Personal Communication, 2021). If regulations are introduced which limit the

acceptable quantity of norovirus to 500 copies/g, this could be damaging to many shellfish harvesters without the availability or support to move their businesses to cleaner waters. However, between the years 2000 and 2007, 17.5% of international food and waterborne outbreaks of norovirus originated from the consumption of shellfish (Baert *et al.*, 2009), and this is therefore an important route of transmission for the virus. With aquatic faecal pollution being so prominent within the UK, it is unsurprising that certain suppliers were more likely to provide shellfish containing norovirus than others; this is most likely due to sewage release impacting the locations from which the shellfish were harvested. As the study took place in March, and norovirus seasonality tends to lead to higher levels of norovirus in colder months (October to March) (Lowther et al., 2012), this may also explain why norovirus prevalence in this study was high. Unfortunately, the use of non-parametric testing may mean the data has low statistical power. Larger sample sizes were unfortunately not possible to obtain due to resource and time limitations.

In comparison to the data on norovirus in sewage (see chapter 4), the shellfish samples had generally lower levels of norovirus than the sewage samples (maximum total norovirus in sewage samples 9,034 copies/ml of sewage, maximum total norovirus in shellfish samples 1,525 copies/g digestive glands), this may be because the geographical locations of the shellfish and sewage study sites were not the same and therefore cannot be directly compared. In addition, effluent, and raw sewage from CSOs is normally diluted by release into surface waters before viruses then bioaccumulate within the shellfish, therefore levels in shellfish would be expected to be lower. An interesting observation when comparing the norovirus data from the sewage and shellfish is that GI was more common and found at higher levels in shellfish, whereas GII was more common and found at higher levels in sewage. This could potentially be explained again by geographical location or because the sewage and shellfish studies were performed seven months apart, and therefore different norovirus strains may have been more dominant in the local populations at the times of the studies. However, reported case data from UK Health Security Agency suggests that GII strains were dominant within the population at the times of both studies (Public Health England, 2020). Previous studies have shown that while GI noroviruses are as prevalent as GII noroviruses in UK retail shellfish (Lowther *et al.*, 2018), GI norovirus is rarely reported in clinical cases (Public Health England, 2020), possibly suggesting that GI may cause asymptomatic infections more commonly.

Another explanation could be that GI bioaccumulates within shellfish more easily than GII, considering that GI noroviruses bind more efficiently to shellfish digestive tissues in winter months (Maalouf *et al.*, 2010).

Our data suggests that aquatic faecal pollution is an ongoing issue in the UK, and that shellfish still pose a risk of foodborne transmission of norovirus. However, it is important to note that viral RNA may derive from non-viable virus particles, which would be unable to cause infection. Limited culturing of norovirus has become possible in recent years (Costantini *et al.*, 2018), and estimates have been made for norovirus viability using surrogate viruses. CrAssphage (a bacteriophage which is almost ubiquitous in human sewage) was studied within wastewater and influent and determined to be ubiquitous in wastewater, however it did not correlate with norovirus presence or show the classic norovirus winter seasonality in shellfish (Farkas *et al.*, 2019). F-specific bacteriophages (genogroup II) have also been investigated as an indicator of norovirus viability (Lowther *et al.*, 2019). These viruses show a similar seasonality to norovirus and a significant positive correlation of virus levels, however the GII phages were not present in all shellfish which contained norovirus, and the techniques through which viability of genogroup II phage was determined are time consuming. Porcine gastric mucin beads have also been used in an attempt to assess viability of norovirus, assuming that virus particles without intact capsids will not bind to the beads (Dancho et al., 2012). However, viruses may have intact capsids but degraded RNA, and therefore these estimates may also be biased. A satisfactory viability assay of norovirus in shellfish has not yet been established, so it is currently impossible to determine the levels of viable norovirus within shellfish samples to determine the true risk that contaminated shellfish may pose.

HEV in Cetaceans and Seals

No HEV was discovered in grey seals, which indicates either; i) this species is not subject to HEV infection ii) the sample size was too low to detect prevalence or iii) that cetaceans are infected with a HEV-like virus which is unrelated to strains which circulate in humans, and this is incapable of infecting grey seals. As grey seals live in shallower waters closer to shores (Jefferson *et al.*, 2011), it is unlikely that their habitats would be less polluted than those of cetaceans, which generally live further from land, however some cetaceans inhabit near shore waters (Jefferson *et al.*, 2011). Contrary to the seal data, 32% of the cetacean liver samples tested positive for the

presence of HEV RNA, the majority of which (29/34) occurred within common dolphins (*Delphinus delphis*). HEV was also detected in four harbour porpoises (*Phocoena phocoena*), but in none of the remaining cetacean species. The detection in the common dolphins and harbour porpoises is likely to be a result of larger sample sizes for these animals, in comparison to the other species which had much more limited numbers, and therefore results are inconclusive in terms of comparing HEV prevalence across different cetacean species. The studies were limited to the animals which had been collected by CSIP after their beaching and deaths within southwest England. This is the first recorded presence of HEV RNA presence in wild cetaceans to our knowledge. One previous study identified and sequenced HEV RNA from dolphins in captivity in Cuba which were experiencing clinical signs of infection prior to HEV testing (Villalba *et al.*, 2017). Therefore, it is possible that, with the results of this study, dolphins and other cetaceans are becoming infected with HEV within the marine environment. An investigation into the source of these infections would be required to ascertain the transmission route of HEV to dolphins, but it could be theorised to originate from the fish diet of these animals, from sewage outfalls near to land, from animal farm run-off, or that a HEV-related virus is endemic within cetaceans.

The mean HEV level of all the cetacean samples was 15 copies/mg of liver, and the highest level observed was 210 copies/mg. If we were to extrapolate this maximum result to copies/g of liver, we would have $2.1x10^5$ copies/g of cetacean liver. In comparison, pig livers have been reported to contain $1x10^3$ – $4.7x10^6$ copies/g (Wilhelm *et al.*, 2014). Cetacean liver samples with lower levels of HEV RNA than this may be suggestive of early stages of infection. The levels of HEV contamination in the cetaceans are higher than levels seen in many of the sewage samples previously (see chapter 4), where the mean HEV in influent samples was 4.4 copies/ml, and the maximum was 113 copies/ml. Whether these quantities of virus would be enough to cause clinical infection in cetaceans is unknown, as Villalba *et al.*, (2017) identified some HEV viral loads of over 10^4 copies/ml of dolphin serum in symptomatic dolphins. However, it should be noted that wastewater treatment companies in the UK collectively released raw sewage into surface waters for over 3 million hours in 2020, and this would constitute millions of litres of raw sewage entering the habitats of aquatic organisms (Environment Agency, 2020a). In addition, farm run-off from animals which are known reservoirs of the virus (e.g., pigs and deer) could also be

contributing to this faecal pollution, which may also present an infection risk to marine mammals.

An interesting finding was that male cetaceans were significantly more likely to be HEV-positive than females. This observation has also been made within humans in some countries of the world, including the UK (Smith *et al.*, 2021). The reason for the enhanced susceptibility to infection of male humans has not yet been elucidated and it is unknown whether this is a genuine risk factor for cetaceans or whether it may be a coincidental artifact of the study. No significant relationship was observed between the life stage of the cetaceans and HEV infection, which may be due to some life stages being rarer in the sample population than others, and uneven numbers between life stage groups. It is also acknowledged that “life stage at death” is not a precise representation of the age of the cetaceans.

Limitations

A significant limitation was the observation of some cetacean samples with over 100% extraction efficiency. This is likely because the reference extraction did not extract as well as some samples and suggests that the RNeasy Mini kit used may not be the most ideal extraction method. An extraction efficiency of 0.5% was used instead of the 1% utilised in ISO 15216-1:2017 due to issues with the mengo virus culture batch compared to previous batches, as explained in chapter 3 (International Organization for Standardization, 2017).

Additionally, an integrity control was not utilised for the cetacean samples or archived shellfish samples directly from harvesting areas due to the age of some samples. The longterm storage of the samples at -80°C may have caused some HEV to degrade. An mRNA integrity control was not used because decay of mRNA typically occurs within hours of mRNA production (Friedel *et al.*, 2009), and therefore testing for it when a sample is fresh is essential. However, virus RNA can persist within samples stored at ambient temperature for days (Weesendorp et al., 2010), and HEV RNA has been detected up to 21 days at 37°C in cell culture (Johne *et al.*, 2016). Therefore freezing samples whilst they are still fresh can enable retention of viral RNA for later testing. We therefore felt that the samples which had been frozen for long periods of time may still provide valuable data. For the cetaceans, new samples could not be obtained as they were provided as and when cetaceans beached and died. The results from the older cetacean samples therefore bear the caveat that viral RNA

within the samples may have degraded to the point of being undetectable due to their storage conditions; indeed, HEV positive samples were only obtained from cetaceans which had beached between 2016 and 2019.

The study of archived shellfish from harvesting areas utilised a biased set of samples, selecting shellfish samples from harvesting areas which typically had high norovirus levels in winter months, with the hypothesis that samples from harvesting areas with a high norovirus geomean would be more likely to contain HEV. In addition, these samples had been harvested, processed and frozen at -20°C from 2009 onwards, meaning HEV RNA present at the time of sampling may have degraded to become undetectable in some samples. Therefore, HEV presence in these samples may be underestimated and should not be used as a prevalence measure. Additionally, the retail study shellfish results may have been affected by the storage of the shellfish glands at 4°C for 4 to 12 hours; however Johne et al., (2016) showed that HEV RNA was detectable up to 56 days at 4°C in cell culture, and therefore the virus was not expected to degrade significantly in that time. The retail study was also limited by its length, being conducted over two weeks in March. Though no seasonality of HEV has been established yet, it is possible that HEV levels in March are low within shellfish. The study was also limited by the number of shellfish suppliers available to purchase from, which prevented involvement of suppliers from Wales and Northern Ireland and limited the number of harvesting locations and samples which could be obtained from different regions. A more systematic study across the space of a year with more shellfish harvesting areas, regions and suppliers involved would be required to make an accurate estimate of HEV prevalence in shellfish. In addition, though one shellfish sample has been successfully sequenced (see chapter 6), HEV could not be sequenced from any of eighteen cetacean samples with the highest C_T values (31 – 33) using a nested PCR assay and amplicon sequencing which was specific for genotype 3 HEV (due to the findings of Villalba *et al.*, (2017), which suggests either that a different HEV genotype is infecting cetaceans, or perhaps the assay has detected a HEV-like virus which persists naturally within the cetacean populations.

Conclusion

This study has shown that norovirus is present in retail shellfish samples in moderate to high levels and in a large proportion of shellfish samples. It also demonstrates that

UK shellfish can become contaminated with HEV, though at a much lower prevalence and concentration. I have also demonstrated that HAV and sapovirus contamination is likely to be rare in shellfish from retailers, but more research must be conducted to better represent all harvesting areas and retailers in the UK. I have shown that shellfish which are harvested from areas within southern England are more likely to contain norovirus than those in Northern England or Scotland. HEV has not been detected in English shellfish until now, and though HEV in UK shellfish appears to be rare, increasing HEV cases within the UK population yearly mean that it could be an emerging public health risk. A more comprehensive study of freshly harvested and ready to eat shellfish in the UK is required to fully assess the risk which HEV may pose to humans through the consumption of shellfish, and a study investigating the effect of depuration of shellfish contaminated with HEV would also be beneficial. High prevalence of norovirus in retail shellfish also indicates a risk of norovirus transmission from English shellfish to consumers, much like previous studies (Lowther *et al.*, 2012; Lowther *et al.*, 2018); whereas the risk from HEV, HAV and sapovirus appears to be significantly lower, given little presence or total absence of detection in this study.

Significantly, within this study I have identified, for the first time, the presence of HEV RNA within wild common dolphins and harbour porpoises, and that male cetaceans may be more susceptible to HEV infection than females. This detection brings new questions, regarding how the virus is transmitted to cetaceans, whether human faecal pollution may be a contributing factor to disease within marine mammals, and whether the detection of this viral RNA originates from a virus which circulates within humans, or whether perhaps it is a newly discovered cetacean strain. It also brings the question of whether the observation of higher counts within males than females is reflective of a genuine risk factor within cetaceans, or a coincidental artifact of the study; and whether the results were caused by HEV or a HEV-like virus. Further investigations will be required to address these questions.

The contamination of the aquatic environment with faecal pollution is damaging to not only shellfish harvesting practises, but also potentially to marine and aquatic life, and must be addressed with improvements to sewage treatment plants and reduced release of raw sewage. However, it is important to remember that presence of viral RNA may not indicate presence of viable virus, and until these viruses can be cultured effectively from food and environmental samples, it is difficult to accurately estimate

the risk that aquatic contamination poses.

References

Almand, E. A., Moore, M. D. and Jaykus, L.-A. (2017) 'Norovirus binding to ligands beyond histo-blood group antigens', *Frontiers in microbiology,* 8, pp. 2549.

Baert, L., Uyttendaele, M., Stals, A., Van Coillie, E., Dierick, K., Debevere, J. and Botteldoorn, N. (2009) 'Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context', *Epidemiology & Infection,* 137(3), pp. 316-325.

Boxman, I. L., Verhoef, L., Vennema, H., Ngui, S.-L., Friesema, I. H., Whiteside, C., Lees, D. and Koopmans, M. (2016) 'International linkage of two food-borne hepatitis A clusters through traceback of mussels, the Netherlands, 2012', *Eurosurveillance,* 21(3), pp. 30113.

Campos, C. J., Avant, J., Gustar, N., Lowther, J., Powell, A., Stockley, L. and Lees, D.N. (2015) 'Fate of human noroviruses in shellfish and water impacted by frequent sewage pollution events', *Environmental science & technology,* 49(14), pp. 8377-8385.

Choi, C. and Kingsley, D. H. (2016) 'Temperature-dependent persistence of human norovirus within oysters (Crassostrea virginica)', *Food and environmental virology,* 8(2), pp. 141-147.

Costafreda, M. I., Bosch, A. and Pintó, R. M. (2006) 'Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples', *Appl. Environ. Microbiol.,* 72(6), pp. 3846-3855.

Costantini, V., Morantz, E. K., Browne, H., Ettayebi, K., Zeng, X.-L., Atmar, R. L., Estes, M. K. and Vinjé, J. (2018) 'Human norovirus replication in human intestinal enteroids as model to evaluate virus inactivation', *Emerging infectious diseases,* 24(8), pp. 1453.

Crossan, C., Baker, P. J., Craft, J., Takeuchi, Y., Dalton, H. R. and Scobie, L. (2012) 'Hepatitis E virus genotype 3 in shellfish, United Kingdom', *Emerging infectious diseases,* 18(12), pp. 2085.

da Silva, A. K., Le Saux, J.-C., Parnaudeau, S., Pommepuy, M., Elimelech, M. and Le Guyader, F. S. (2007) 'Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II', *Appl. Environ. Microbiol.,* 73(24), pp. 7891-7897.

Dancho, B. A., Chen, H. and Kingsley, D. H. (2012) 'Discrimination between infectious and non-infectious human norovirus using porcine gastric mucin', *International Journal of Food Microbiology,* 155(3), pp. 222-226.

Environment Agency. (2020a) 'Event Duration Monitoring - Storm Overflows - 2020' [Database of CSO use throughout UK in 2020]. Available at: https://environment.data.gov.uk/dataset/21e15f12-0df8-4bfc-b763-45226c16a8ac (Accessed.

Environment Agency. (2020b) *Water and sewerage companies in England: environmental performance report for 2019*: Gov.UK. Available at: https://www.gov.uk/government/publications/water-and-sewerage-companies-in-england-environmental-performance-report-2019/water-and-sewerage-

companies-in- england-environmental-performance-report-for-2019.

European Food Safety Authority. (2019) 'Analysis of the European baseline survey of norovirus in oysters', *EFSA Journal,* 17(7), pp. e05762.

Farkas, K., Adriaenssens, E. M., Walker, D. I., McDonald, J. E., Malham, S. K. and Jones, D. L. (2019) 'Critical Evaluation of CrAssphage as a Molecular Marker for Human-Derived Wastewater Contamination in the Aquatic Environment', *Food and environmental virology,* 11(2), pp. 113-119.

Fisher, R. A. (1934) 'Statistical methods for research workers', *Statistical methods for research workers.,* (5th Ed).

Formiga-Cruz, M., Tofino-Quesada, G., Bofill-Mas, S., Lees, D., Henshilwood, K., Allard, A., Conden-Hansson, A.-C., Hernroth, B., Vantarakis, A. and Tsibouxi, A. (2002) 'Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden, and the United Kingdom', *Applied and Environmental Microbiology,* 68(12), pp. 5990-5998.

Fusco, G., Anastasio, A., Kingsley, D. H., Amoroso, M. G., Pepe, T., Fratamico, P. M., Cioffi, B., Rossi, R., La Rosa, G. and Boccia, F. (2019) 'Detection of hepatitis A virus and other enteric viruses in shellfish collected in the Gulf of Naples, Italy', *International journal of environmental research and public health,* 16(14), pp. 2588.

Gao, S., Li, D., Zha, E., Zhou, T., Wang, S. and Yue, X. (2015) 'Surveillance of hepatitis E virus contamination in shellfish in China', *International journal of environmental research and public health,* 12(2), pp. 2026-2036.

Garson, J. A., Ferns, R. B., Grant, P. R., Ijaz, S., Nastouli, E., Szypulska, R. and Tedder, R. S. (2012) 'Minor groove binder modification of widely used TaqMan probe for hepatitis E virus reduces risk of false negative real-time PCR results', *Journal of virological methods,* 186(1), pp. 157-160.

Gherman, I., Holland, D., Clifford, R., Mahmoudzadeh, N., Uy, F., Adkin, A., Kainth, B., Cook, P., Day, A. and Wilson, A. 'Technical Report: Review of Quantitative Risk Assessment of foodborne norovirus transmission', *Science,* 1, pp. 1.

Gibson, K. E., Opryszko, M. C., Schissler, J. T., Guo, Y. and Schwab, K. J. (2011) 'Evaluation of human enteric viruses in surface water and drinking water resources in southern Ghana', The American journal of tropical medicine and hygiene, 84(1), pp. 20-29.

Grierson, S., Heaney, J., Cheney, T., Morgan, D., Wyllie, S., Powell, L., Smith, D., Ijaz, S., Steinbach, F. and Choudhury, B. (2015) 'Prevalence of hepatitis E virus infection in pigs at the time of slaughter, United Kingdom, 2013', *Emerging infectious diseases,* 21(8), pp. 1396.

Guillois-Bécel, Y., Couturier, E., Le Saux, J.-C., Roque-Afonso, A., Le Guyader, F., Le Goas, A., Pernes, J., Le Bechec, S., Briand, A. and Robert, C. (2009) 'An oyster- associated hepatitis A outbreak in France in 2007', *Eurosurveillance,* 14(10), pp. 19144.

Hardstaff, J., Clough, H., Harris, J., Lowther, J., Lees, D. and O'Brien, S. (2019) 'The use of capture-recapture methods to provide better estimates of the burden of norovirus outbreaks from seafood in England, 2004–2011', *Epidemiology & Infection,* 147.

Hassard, F., Sharp, J. H., Taft, H., LeVay, L., Harris, J. P., McDonald, J. E., Tuson, K., Wilson, J., Jones, D. L. and Malham, S. K. (2017) 'Critical review on the public health impact of norovirus contamination in shellfish and the environment: a UK perspective', *Food and environmental virology,* 9(2), pp. 123-141.

Hakze-van der Honing, R. W., van Coillie, E., Antonis, A. F. and van der Poel, W. H. (2011) 'First isolation of hepatitis E virus genotype 4 in Europe through swine surveillance in the Netherlands and Belgium', PLoS One, 6(8), pp. e22673.

Hoehne, M. and Schreier, E. (2006) 'Detection of Norovirus genogroup I and II by multiplex real-time RT-PCR using a 3'-minor groove binder-DNA probe', *BMC infectious diseases,* 6(1), pp. 69.

Holm, S. (1979) 'A simple sequentially rejective multiple test procedure', *Scandinavian journal of statistics*, pp. 65-70.

Iizuka, S., Oka, T., Tabara, K., Omura, T., Katayama, K., Takeda, N. and Noda, M. (2010) 'Detection of sapoviruses and noroviruses in an outbreak of gastroenteritis linked genetically to shellfish', *Journal of medical virology,* 82(7), pp. 1247-1254.

International Organization for Standardization (2017)*: ISO 15216-1: 2017: Microbiology of food and animal feed—Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR.* Geneva.

Jefferson, T. A., Webber, M. A., Pitman, R. L. and Jarrett, B. (2011) *Marine Mammals of the World: A Comprehensive Guide to Their Identification.* Elsevier Science.

Jerrold, Z. (2005) 'Spearman rank correlation', *Encyclopedia of Biostatistics,* 7.

Johne, R., Trojnar, E., Filter, M. and Hofmann, J. (2016) 'Thermal stability of hepatitis E virus as estimated by a cell culture method', Applied and environmental microbiology, 82(14), pp. 4225-4231.

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. and Hill, V. R. (2006) 'A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus', *Journal of virological methods,* 131(1), pp. 65-71.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., Takeda, N. and Katayama, K. (2003) 'Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR', *Journal of clinical microbiology,* 41(4), pp. 1548-1557.

Kitaura, S., Wakabayashi, Y., Okazaki, A., Okada, Y., Okamoto, K., Ikeda, M., Okugawa, S. and Moriya, K. (2020) 'The First Case Report of Acute Symptomatic HEV Genotype 4 Infection in an HIV-positive Patient in Japan', Intern Med, 59(13), pp. 1655-1658.

Kruskal, W. H. and Wallis, W. A. (1952) 'Use of ranks in one-criterion variance analysis', *Journal of the American statistical Association,* 47(260), pp. 583-621.

La Bella, G., Martella, V., Basanisi, M., Nobili, G., Terio, V. and La Salandra, G. (2017) 'Food-borne viruses in shellfish: investigation on norovirus and HAV presence in Apulia (SE Italy)', *Food and environmental virology,* 9(2), pp. 179-186.

La Rosa, G., Proroga, Y., De Medici, D., Capuano, F., Iaconelli, M., Della Libera, S. and Suffredini, E. (2018) 'First detection of hepatitis E virus in shellfish and in seawater from production areas in Southern Italy', Food and environmental

virology, 10(1), pp. 127-131.

Lees, D. (2000) 'Viruses and bivalve shellfish', *International journal of food microbiology,* 59(1-2), pp. 81-116.

Li, T.-C., Miyamura, T. and Takeda, N. (2007) 'Detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (Corbicula japonica) in Japan', The American journal of tropical medicine and hygiene, 76(1), pp. 170-172.

Lodder, W. J. and de Roda Husman, A. M. (2005) 'Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands', Applied and environmental microbiology, 71(3), pp. 1453-1461.

Loisy, F., Atmar, R., Guillon, P., Le Cann, P., Pommepuy, M. and Le Guyader, F. (2005) 'Real-time RT-PCR for norovirus screening in shellfish', *Journal of virological methods,* 123(1), pp. 1-7.

Lowther, J. (2011) 'Investigation into the prevalence, distribution and levels of norovirus titre in oyster harvesting areas in the UK', *Weymouth: CEFAS Weymouth laboratory*.

Lowther, J., Gustar, N., Powell, A., O'Brien, S. and Lees, D. (2018) 'A one-year survey of norovirus in UK oysters collected at the point of sale', *Food and environmental virology,* 10(3), pp. 278-287.

Lowther, J. A., Cross, L., Stapleton, T., Gustar, N. E., Walker, D. I., Sills, M., Treagus, S., Pollington, V. and Lees, D. N. (2019) 'Use of F-Specific RNA Bacteriophage to Estimate Infectious Norovirus Levels in Oysters', *Food and Environmental Virology,* 11(3), pp. 247-258.

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E. and Lees, D. N. (2012) 'Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom', *Applied and environmental microbiology,* 78(16), pp. 5812-5817.

Ma, L. P., Zhao, F., Yao, L., Li, X. G., Zhou, D. Q. and Zhang, R. L. (2013) 'The presence of genogroup II norovirus in retail shellfish from seven coastal cities in China', Food Environ Virol, 5(2), pp. 81-6.

Maalouf, H., Zakhour, M., Le Pendu, J., Le Saux, J.-C., Atmar, R. and Le Guyader, F. (2010) 'Distribution in Tissue and Seasonal Variation of Norovirus Genogroup I and II Ligands in Oysters', *Applied and Environmental Microbiology*, 76(16), pp. 5621-5630, *Applied and Environmental Microbiology* Available: American Society for Microbiology.

Manso, C., Polo, D., Vilariño, M. and Romalde, J. (2010) 'Genotyping of hepatitis A virus detected in bivalve shellfish in Galicia (NW Spain)', *Water Science and Technology,* 61(1), pp. 15-24.

Marcheggiani, S., D'Ugo, E., Puccinelli, C., Giuseppetti, R., D'Angelo, A. M., Gualerzi, C. O., Spurio, R., Medlin, L. K., Guillebault, D. and Weigel, W. (2015) 'Detection of emerging and re-emerging pathogens in surface waters close to an urban area', *International journal of environmental research and public health,* 12(5), pp. 5505-5527.

Mesquita, J. R., Oliveira, D., Rivadulla, E., Abreu-Silva, J., Varela, M. F., Romalde, J. L. and Nascimento, M. S. (2016) 'Hepatitis E virus genotype 3 in mussels (Mytilus galloprovinciallis), Spain', *Food microbiology,* 58, pp. 13-15.

Oeser, C., Vaughan, A., Said, B., Ijaz, S., Tedder, R., Haywood, B., Warburton, F., Charlett, A., Elson, R. and Morgan, D. (2019) 'Epidemiology of Hepatitis E in England and Wales: A 10-Year Retrospective Surveillance Study, 2008–2017', The Journal of Infectious Diseases, 220(5), pp. 802-810.

Oka, T., Katayama, K., Hansman, G. S., Kageyama, T., Ogawa, S., Wu, F. T., White, P. A. and Takeda, N. (2006) 'Detection of human sapovirus by real-time reverse transcription-polymerase chain reaction', *Journal of medical virology,* 78(10), pp. 1347-1353.

O'Hara, Z., Crossan, C., Craft, J. and Scobie, L. (2018) 'First Report of the Presence of Hepatitis E Virus in Scottish-Harvested Shellfish Purchased at Retail Level', *Food and environmental virology*, pp. 1-5.

Pintó, R. M., Costafreda, M. I. and Bosch, A. (2009) 'Risk assessment in shellfish-borne outbreaks of hepatitis A', *Appl. Environ. Microbiol.,* 75(23), pp. 7350-7355.

Potasman, I., Paz, A. and Odeh, M. (2002) 'Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective', *Clinical Infectious Diseases,* 35(8), pp. 921-928.

Public Health England . (2018) *PHE National norovirus and rotavirus Report*: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/724329/Norovirus_update_2018_weeks_22_to_26.pdf.

Public Health England. (2019) *Hepatitis E: symptoms, transmission, treatment and prevention*. gov.uk: Public Health England. Available at: https://www.gov.uk/government/publications/hepatitis-e-symptoms-transmission- prevention-treatment/hepatitis-e-symptoms-transmission-treatment-and-prevention (Accessed: 27 August 2019).

Public Health England. (2020) *PHE National norovirus and rotavirus Report*, GOV.UK: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/871753/Norovirus_update_2020_weeks_08_to_09.pdf.

Razafimahefa, R. M., Ludwig-Begall, L. F. and Thiry, E. (2020) 'Cockles and mussels, alive, alive, oh—The role of bivalve molluscs as transmission vehicles for human norovirus infections', *Transboundary and emerging diseases,* 67, pp. 9-25.

R Core Team (2018) *R: A Language and Environment for Statistical Computing* (Version 1.1.383). Available at: https://www.R-project.org/.

Rivadulla, E., Varela, M. F., Mesquita, J. R., Nascimento, M. S. and Romalde, J. L. (2019) 'Detection of hepatitis E virus in shellfish harvesting areas from Galicia (Northwestern Spain)', *Viruses,* 11(7), pp. 618.

Said, B., Ijaz, S., Kafatos, G., Booth, L., Thomas, H. L., Walsh, A., Ramsay, M. and Morgan, D. (2009) 'Hepatitis E outbreak on cruise ship', *Emerging infectious diseases,* 15(11), pp. 1738.

Shapiro, S. S. and Wilk, M. B. (1965) 'An analysis of variance test for normality (complete samples)', *Biometrika,* 52(3/4), pp. 591-611.

Smith, D. B., Paddy, J. O. and Simmonds, P. (2016) 'The use of human sewage screening for community surveillance of hepatitis E virus in the UK', *Journal of medical virology,* 88(5), pp. 915-918.

Smith, I., Said, B., Vaughan, A., Haywood, B., Ijaz, S., Reynolds, C., Brailsford, S., Russell, K. and Morgan, D. (2021) 'Case–Control Study of Risk Factors for Acquired Hepatitis E Virus Infections in Blood Donors, United Kingdom, 2018–2019', *Emerging Infectious Diseases,* 27(6), pp. 1654-1661.

Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B. and Koopmans, M. (2007) 'Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005', *Journal of clinical microbiology,* 45(5), pp. 1389-1394.

Sánchez, G., Pintó, R. M., Vanaclocha, H. and Bosch, A. (2002) 'Molecular characterization of hepatitis A virus isolates from a transcontinental shellfish-borne outbreak', *Journal of Clinical Microbiology,* 40(11), pp. 4148-4155.

Tang, Y., Wang, J., Xu, Z., Guo, Y., Qian, W. and Xu, J. (1991) 'A serologically confirmed, case-control study, of a large outbreak of hepatitis A in China, associated with consumption of clams', *Epidemiology & Infection,* 107(3), pp. 651-657.

Ueki, Y., Shoji, M., Suto, A., Tanabe, T., Okimura, Y., Kikuchi, Y., Saito, N., Sano, D. and Omura, T. (2007) 'Persistence of caliciviruses in artificially contaminated oysters during depuration', *Applied and environmental microbiology,* 73(17), pp. 5698-5701.

Varela, M. F., Polo, D. and Romalde, J. L. (2016) 'Prevalence and genetic diversity of human sapoviruses in shellfish from commercial production areas in Galicia, Spain', *Applied and environmental microbiology,* 82(4), pp. 1167-1172.

Villalba, M. C. M., Martínez, D. C., Ahmad, I., Lay, L. A. R., Corredor, M. B., March, C. G., Martínez, L. S., Martínez-Campo, L. S. and Jameel, S. (2017) 'Hepatitis E virus in bottlenose dolphins Tursiops truncatus', *Diseases of aquatic organisms,* 123(1), pp.13-18.

Webster, C. L., Waldron, F. M., Robertson, S., Crowson, D., Ferrari, G., Quintana, J. F., Brouqui, J.-M., Bayne, E. H., Longdon, B. and Buck, A. H. (2015) 'The discovery, distribution, and evolution of viruses associated with Drosophila melanogaster', *PLoS Biol,* 13(7), pp. e1002210.

Wilcoxon, F. (1992) 'Individual comparisons by ranking methods', *Breakthroughs in statistics*: Springer, pp. 196-202.

Williams, S. and O'Brien, S. J. (2019) *NoVAS: Assessing the contribution made by the food chain to the burden of UK acquired norovirus infection*, UK: Cefas, Fera, Leatherhead Food Research, Liverpool Clinical Laboratories (Royal Liverpool and Broadgreen University Hospital Trust), UK Health Security Agency , University of East Anglia and University of Liverpool (Accessed: 21 July 2021).

Wyn-Jones, A. P., Carducci, A., Cook, N., D'agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R. and Höller, C. (2011) 'Surveillance of adenoviruses and noroviruses in European recreational waters', *Water research,* 45(3), pp. 1025-1038.

Yang, N., Qi, H., Wong, M. M. L., Wu, R. S. S. and Kong, R. Y. C. (2012) 'Prevalence and diversity of norovirus genogroups I and II in Hong Kong marine waters and detection by real-time PCR', Marine Pollution Bulletin, 64(1), pp. 164-168.

Chapter 6

Phylogenetic Analysis of Hepatitis E virus and Norovirus Strains from Sewage and Shellfish in the UK

Abstract

Hepatitis E virus (HEV) and norovirus are enteric pathogens which cause a variety of symptoms, from nausea and sickness to fever and jaundice. Norovirus has been established as one of the largest causes of gastroenteritis worldwide, and HEV is an emerging pathogen which has become the most dominant cause of acute viral hepatitis in recent years. The presence of norovirus and HEV has been established within sewage and shellfish in many countries previously. This study endeavoured to use an amplicon deep sequencing technique ("metabarcoding") to obtain phylogenetic information on norovirus and HEV strains within sewage and shellfish samples from the UK. HEV and norovirus could be sequenced successfully using this approach, the study finding that the HEV sequences most likely originated in humans or swine, and that norovirus diversity was higher than expected, when compared to national data in the community. The confirmed presence of these viruses within treated and untreated sewage and shellfish samples confirms that the aquatic environment is being contaminated with these viruses, which can lead to public health risks through the consumption of shellfish or through recreational water activities.

Introduction

Sequencing is an incredibly useful tool for identifying the epidemiology of a virus. Not only can it be used to trace the mutations of a virus strain over time in order to track new variants, it can also be used for traceability of a viral outbreak, to see geographical patterns of the spread of a virus strain, and help to track the continuation of a pandemic (Martin *et al.*, 2020; Agrawal et al., 2021). There are several methods and platforms through which viruses can be sequenced, which depend on the type of nucleic acid genome the virus has (DNA or RNA), the viral load within the samples, and the desired information from the sequencing data. For studies which require high accuracy for mutational analyses (e.g., SNPs), the Illumina sequencing platform is one of the most used NGS technologies (Malmberg *et al.*, 2019). In comparison, due to the short read lengths used in the Illumina platform, Oxford Nanopore Technologies

(ONT) sequencing is favoured for generating long length reads, and has even been used to sequence whole genomes from single molecules of cDNA (Batista *et al.*, 2020). ONT sequencing is also used in lower resource settings as it tends to be more affordable than Illumina sequencing (Smith *et al.*, 2020a). However, ONT is generally considered to be less accurate than Illumina sequencing due to the differences in basecalling algorithms and nanopore chemistry, and this has been demonstrated in previous studies (McNaughton *et al.*, 2019).

The sequencing of HEV enabled researchers to identify that it is a zoonotic virus with several potential animal reservoirs. In a study by Meng *et al.*, (1997), HEV was sequenced from pigs, and identified to be 77-92% similar (across the three open reading frames) to HEV sequences obtained from human infections. Later studies then identified close phylogenetic relationships between swine and human HEV strains, (with strains from swine and humans sharing ≥97% nucleotide sequence similarity) and led to animal studies identifying that human HEV was capable of infecting pigs, and swine HEV was capable of infecting non-human primates (Meng *et al.*, 1998). Subsequently pigs were established as the main reservoir of HEV, following studies like those by Tamada *et al.*, (2004) which showed HEV infection in five patients to be the likely result of foodborne transmission from consumption of wild boar meat; Ward *et al.*, (2008) which identified 95% nucleotide sequence similarity between pig and human HEV strains; and Grierson *et al.*, (2015) identifying a seroprevalence of 92.8% (584/629), and HEV RNA presence in 21% (129/629) of UK pigs. A summary of the countries in which HEV was detected in pigs is provided in chapter 2 (Treagus *et al.*, 2021). As a consequence of the discovery of the role pigs play in the transmission of HEV to humans, other animals were then investigated, and many possible host species have been subsequently identified (Kenney, 2019). Interestingly, Ijaz *et al.*, (2013) found that HEV sequences from patients in the UK were generally not from the same genotypes as HEV sequences found in UK pigs; though sequencing data from UK pigs was limited; however, another study by Grierson *et al.*, (2015) also identified this pattern, and theorised that pork products from Europe may be the cause of human infections in the UK, however they were unable to rule out other sources of infection. If this is indeed a genuine and ongoing pattern within HEV in UK pigs and humans, it could suggest an alternative HEV reservoir in the UK.

Sequencing has also been used for identifying genogroups, genotypes and strains of norovirus that cause norovirus outbreaks and is capable of being used to track these outbreaks from the source to the infection. A large study by Verhoef *et al.*, (2011) used sequencing data to identify the global sources and relationships between norovirus outbreaks, and was able to conservatively estimate that 7% of norovirus outbreaks had an international geographical distribution (when previously this had been estimated to be 0.4%). Sequencing can also be used for confirmation of an outbreak source. For example, an outbreak of norovirus and sapovirus was traced to the consumption of shellfish in a restaurant through identifying the sequence similarity between patients and shellfish packaging liquid (98.6 – 100% similarity range for both viruses) (Iizuka *et al.*, 2010). Sequencing can also track the evolution of viruses from outbreak to outbreak, and identify the rate of mutation of a viral genome, as well as identifying pre- and post-pandemic variants (Eden *et al.*, 2014). Norovirus GII.4 has typically been the most common genotype to cause outbreaks in recent years (Robilotti et al., 2015), however GII.17 has also become more established of late (Lu *et al.*, 2016; Sakon *et al.*, 2018).

RNA viruses such as norovirus and hepatitis E virus (HEV) in food and environmental samples are often present at very low abundance, which can make their detection and sequencing difficult, especially when culturing the viruses from these matrices may not be possible due to low virus levels, as most cell culture systems for HEV so far require high viral titres (~10^5 – 10^7 genome copies) to establish cell culture infections (Johne *et al.*, 2014; Pellerin et al., 2021), and often these utilize HEV strains which have significant mutations in comparison to the wild types. Additionally, many foods contain lower HEV concentrations than the inoculation titres used in the viral culture (Mesquita *et al.*, 2016; Rivadulla *et al.*, 2019; Kokkinos *et al.*, 2012). Due to this, PCR-based methods are generally used to sequence the genome of these viruses. Sequencing techniques using DNA as a template are preferred for the sequencing of viral RNA within food and environmental samples due to the instability of RNA (and relative stability of DNA) in addition to the presence of RNases. RNA sequencing can also be technically challenging. Sanger sequencing for viral RNA uses DNA amplicons, generated by RT-PCR, to enable maximum sensitivity of the sequencing method. However, unlike Next Generation Sequencing (NGS) techniques, Sanger sequencing is less likely to pick up rare sequences within a sample and may also lead to unresolved bases at certain divergent loci where a sample contains multiple divergent

strains (Mancini *et al.*, 2019; Gao *et al.*, 2016).

This can lead to cloning techniques being required to resolve these sequence ambiguities, which takes time and resources, and may still miss rare strains if present at low abundances. NGS techniques do not have these issues, however. Illumina sequencing has been used previously to trace two divergent sequences of HEV from a blood donor to two recipients (Ledesma *et al.*, 2019). It has also been used to identify mutant variants of HEV in immunocompromised patients with antiviral resistance (Todt *et al.*, 2016). Virus levels in these studies were reportedly high. Despite the higher sensitivity of the nanopore and Illumina sequencing technologies in comparison to Sanger sequencing, it appears that these technologies have not been used in the sequencing of samples with low abundance of HEV, such as food and environmental water samples. Most studies on these types of samples to date have applied PCR based methods to yield small amplicons, followed by Sanger sequencing (Rivadulla *et al.*, 2019; Pallerla *et al.*, 2021). The sequencing of norovirus from environmental samples follows a similar approach to that of HEV, though there are two studies utilising NGS for norovirus analysis. Bartsch *et al.*, (2018) used Illumina sequencing of contaminated strawberries to produce very low read numbers of norovirus, whilst Desdouits *et al.*, (2020) used an Illumina metabarcoding approach to identify genotypes of norovirus within shellfish samples that had been implicated in outbreaks.

With the discovery that HEV variants from humans in the UK may be distinct from HEV variants in UK pigs (Ijaz *et al.*, 2013; Grierson *et al.*, 2015), and that HEV has been detected in vegetarians in the Netherlands (Slot *et al.*, 2017), identifying whether HEV in the aquatic environment could be linked to human infections (whether through recreational water activities or shellfish consumption) may provide insights into other transmission routes of HEV. Sequencing of norovirus from food and environmental samples could also provide a way to trace outbreaks of norovirus to the source (Sliva *et al.*, 2021). However, though sequencing of viruses in samples from food and environmental samples can provide insights into the sources of viral contamination, typically virus sequence databases are dominated by sequences which have originated in humans, and this bias could hinder the identification of potential routes of transmission through foods and the environment (Mulder *et al.*, 2019). Therefore, introducing new methodologies to sequence viruses present at low abundance within such samples could open the door to new discoveries and innovative studies.

Because of the novelty of applying NGS to detect viruses present at low abundance in food or environmental samples, the potential for use in more resource-limited settings (such as less economically developed countries and out in the field), and the general costs associated with Illumina sequencing, this chapter investigated the use of an ONT metabarcoding amplicon-sequencing approach to sequence GII norovirus and HEV sequences within shellfish, sewage and cetacean samples which had previously tested positive by qRT-PCR. This was achieved using nested PCR amplicons from GII norovirus and HEV positive samples. This sequencing data was then used to confirm the presence of GII norovirus and HEV, to phylogenetically analyse the sources of HEV contamination, and to identify the circulating strains of GII norovirus within the community. The aims of this study were to identify if ONT metabarcoding could be used to sequence low levels of virus from food and environmental samples, to find the sources of HEV contamination, and to identify if GII norovirus strains in sewage reflected those from cases reported by the public.

Materials and Methods

Norovirus samples

Forty-two (21 influent and 21 effluent) sewage samples as described in chapter 5 with C_T values between 27 and 39 were selected for sequencing analysis. These were composed of three influent and three effluent samples from each of the seven wastewater treatment plants (WWTPs). These samples were used to compare the GII norovirus strains found in influent and effluent samples, to identify the diversity of GII strains present at each WWTP location and between locations, and to identify previously published sequences which were most closely related to these sequences.

HEV samples

Forty-two sewage samples (C_T range 34 – 44, a different collection of samples from those sequenced for norovirus), five shellfish (C_T range 37 – 40) and eighteen cetacean samples (C_T range 31 – 33) from chapters 4 and 5 were selected for sequencing. All the HEV positive sewage and shellfish samples from chapters 4 and 5 were sequenced, however only 18 cetacean samples were sequenced. These samples were selected to identify if the samples were true positives, and to identify from phylogenetic analysis what the likely source of the HEV strains were.

Semi-nested PCR methods

HEV semi-nested PCR primer design

A first attempt to identify appropriate primers for HEV utilised the Primal Scheme created and published by Quick *et al.*, (2017). However, due to high diversity between sequences, even within subtypes of HEV, the primal scheme was unsuccessful. Therefore, primers were identified manually with the aim to obtain as much sequencing data as possible. Due to high diversity, primers were designed for genotypes 1 (G1) and 3 (G3) separately as these cause most cases in the UK (Oeser *et al.*, 2019). Primers were not designed for G4 due to the rarity of G4 cases in the UK, with only three cases in humans in the UK between 2008 and 2017 (Oeser *et al.*, 2019). The G1 and G3 reference genomes which were classified by Smith *et al.*, (2020b) were subsequently downloaded from GenBank and aligned in order to identify regions which could be used to design a semi-nested PCR assay. Several primer pairs were designed with amplicons of approximately 200 – 300 bases to find working PCR assays which could be used for subsequent barcoding (to allow the sequencing of several samples simultaneously) using a PCR approach. Figure 6.1 shows the process used when designing the primers.

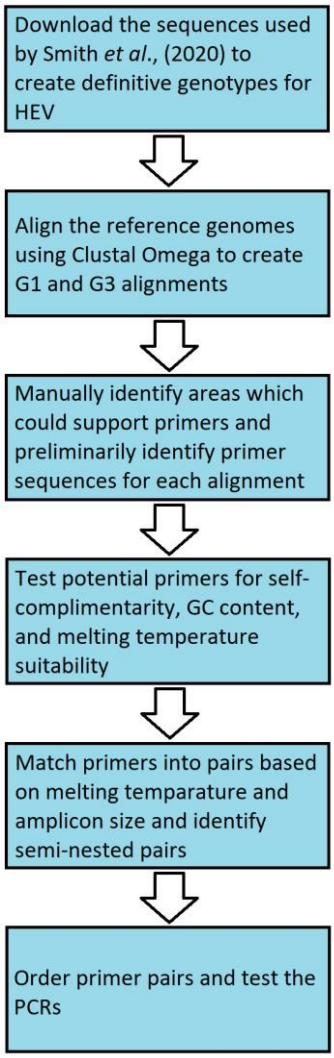


Figure 6.1 Workflow for designing the primers for each HEV genotype and for the two clades of G3

The workflow used to design primers to sequence G1, and G3 HEV. Primers were not designed for other genotypes due to their apparent rare abundance within the UK.

The paper by Smith *et al.*, (2020b) gives an up to date classification of the genotypes and subtypes of HEV. The genome sequences within this paper were downloaded from NCBI and used to create alignments for G1 and G3 using Clustal Omega, which uses CLUSTAL multiple sequence alignment. The alignments of the genomes for G1 and G3 can be found in supplementary data 6.1 and 6.2. Possible primer sequences were then identified manually for each genotype, before then testing these for self-complementarity and melting temperature suitability using the Multiple Primer Analyzer from ThermoFisher Scientific and Oligo Calc from Northwestern University. Primers were considered acceptable if they had a melting temperature of 56-65°C, length of 17-23 bases, GC content between 30-60%, and a maximum of 3 degenerate

bases. Once primer candidates had been found, primer pairs were identified by their melting temperature similarity, the ability to form a semi-nested PCR assay, and the size of the amplicons. Annealing temperatures were calculated using the Thermo Fisher Scientific Multiple Primer Analyzer. The length and position of the semi-nested PCR was dependent on the genotype it had been designed from.

cDNA synthesis and semi-nested PCR

Synthesis of cDNA utilised the Invitrogen SuperScript™ IV First-Strand Synthesis System and random hexamers (final concentration 2.5 µM), per the manufacturers protocol (template volume 10 µl, reaction volume 20 µl). An Eppendorf Mastercycler Nexus was used for cDNA synthesis and the first and second rounds of the semi-nested PCRs (nPCR). The primers for the HEV nPCR were those developed as described above. The primers for the norovirus GII nPCR were described previously; and target the extreme 3’ end of the RdRp polymerase gene (ORF1) and the 5’ start of the VP1 capsid protein gene (ORF2). The primers used in the second round for all assays were modified with 5’ adapter sequences to allow multiplexing by PCR barcoding. The primer sequences and reaction conditions are detailed in Table 6.1. The final concentration of both forward and reverse primers were 0.4 µM.

Assay	Primer sequences	Amplification conditions	Amplicon size	Study
Norovirus GII	First round QNIF2D: 5'-ATGTTCAGRTGGATGAGRTTCTCWGA-3' GIISKR: 5'-CCRCCNGCATRHCCRTTRTACAT-3'	95°C 1 min Then 40 cycles of: 95°C 30 secs 50°C 30 secs 72°C 30 secs Then: 72°C 7 mins	378	Loisy *et al.*, (2005); Kojima *et al.*, (2002)
	Second round GIISKF_T: 5'-**TTTCTGTTGGTGCTGATATTGC**TGTTGCGCAGGTYTGTGT -3' GIISKR_T: 5'-**ACTTGCCTGTCGCTCTATCTTC**CCRCCNGCATRHCCRTTRTACAT-3'		344*	Kojima *et al.*, (2002)
HEV G1	First round FWD: 5'-ATCYTGTGTCGGGTGGAA-3' REV: 5'-TTGGCGAACACGAGGTCC-3'	95°C 1 min Then 40 cycles of: 95°C 30 secs 51°C 30 secs 72°C 30 secs Then: 72°C 7 mins	289	This study
	Second round FWD: 5'-**TTTCTGTTGGTGCTGATATTGC**ATCYTGTGTCGGGTGGAA-3' REV: 5'-**ACTTGCCTGTCGCTCTATCTT**CATTGCGAAGGGCTGAGAATCA-3'		213*	This study
HEV G3	First round FWD: 5'-TGTTGCGCAGGTYTGTGT-3' REV: 5'-GCARCATAGGCARAARCACGA-3'	95°C 1 min Then 40 cycles of: 95°C 30 secs 50°C 30 secs 72°C 30 secs Then: 72°C 7 mins	258	This study
	Second round FWD: 5'-**TTTCTGTTGGTGCTGATATTGC**TGTTGCGCAGGTYTGTGT-3' REV: 5'-**ACTTGCCTGTCGCTCTATCTT**CCATAGGCARAARCACGARGAA-3'		254*	This study

Table 6.1 Sequencing PCR primer sequences and amplification conditions

*The amplicon size does not include the primer adapter, which added 43 more bases. Sequences in bold are the primer adapters.

A negative control was utilised in all PCR reactions (water in place of sample cDNA) which was sequenced alongside any samples providing positive nPCR results. A positive control was used for the first and second round PCRs for the GII norovirus assay in the form of a Norovirus GII LENTICULE (RMNOROG2, UK Health Security Agency). For HEV G3, extracted RNA from cell culture (originating from a patient) was used as a positive control (kindly donated by Eva Trojnar and Reimar Johne of Bundesintitut für Risikobewertung, Berlin, Germany). The positive control amplicons were not sequenced to avoid potential cross contamination at the sequencing stage. Unfortunately, due to resource and time limitations a positive control for the HEV G1 assay was not available.

The nPCR amplicons were visualised using 2% agarose (Scientific Laboratory Supplies) gel electrophoresis with Gel Red (10,000X in water, BIOTIUM) nucleic acid gel stain. Once the PCR products were obtained, and samples were confirmed to have the correct band size by electrophoresis, they were stored at 4°C for up to 48 hours or -20°C for up to 7 days until sequencing.

Sequencing

If samples contained amplicons of the correct size, they were purified using an AMPure XP Reagent for PCR Purification (Beckman Coulter) clean-up; 45 µl of AMPure XP beads were added to 45 µl of amplicons in 1.5 ml DNA Lobind tubes (Eppendorf). The beads were then incubated at room temperature on an Invitrogen Hula Mixer (which rotates to continually invert the samples) for 5 minutes. The tube containing the beads and amplicons were then applied to a magnet to pellet the beads, and the supernatant was removed and discarded. The beads were then washed with 200 µl of 70% ethanol whilst still on the magnet, and the ethanol removed and discarded. The tubes were then centrifuged briefly and opened to allow residual ethanol to evaporate from the beads. The bead pellet was air dried for 2-5 minutes to remove as much ethanol as possible. The tube was then removed from the magnet and 50 µl of nuclease free water was then added to the beads and left to incubate at room temperature for 2 minutes. The beads were then pelleted on the magnet again, and the supernatant recovered and used for subsequent PCR barcoding, where a unique barcode was assigned to each sample prior to later pooling of the samples for sequencing. The rest of the procedure follows the Oxford Nanopore Technologies protocol “PCR barcoding (96) amplicons (SQK- LSK109)”. The ligation

sequencing kit (SQK-LSK109) and PCR barcodes (EXP-PBC096) were used with a companion module (E7180S) and flow cell priming kit (EXP-FLP002) per the manufacturer's instructions. This enabled preparation of DNA libraries for sequencing on a MinION MK1C machine. Sequencing runs took between 8 and 48 hours to generate a minimum of 20,000 reads per sample.

Once the MinION had generated 20,000 reads for each sample, the sequencing runs were stopped and the fast5 files were processed using the high accuracy basecalling program on the MinKnow software to generate high accuracy fastq files for each barcode (using a minimum quality score of 7). The fastq files were then processed using a bioinformatics pipeline. Firstly, reads were trimmed using the program Cutadapt (version 3.2) to remove adapters, barcodes, and primer sequences. Trimmed reads were then aligned to reference genome sequences from Smith *et al.*, (2020b) using Minimap (version 2.17) and Samtools (version 1.1). For each group of sequences that have aligned to the same reference sequence, reads were error corrected using Canu (version 2.1.1), and initial consensus sequences were generated using a minimum of 1,000 reads. Randomly selected consensus sequences of the correct size were then saved into individual files for each barcode using Seqtk (version 1.3), and the consensus sequences from each barcode were then aligned using MAFFT (version 7.475). These alignments were then manually checked, and any duplicate consensus sequences were removed (using a distance matrix to identify duplicates) using UGENE software (windows version 40). Sequence reads were then aligned against the consensus sequences, and information such as coverage, alignment quality and the proportion of reads which aligned to a consensus were recorded, using Minimap and Samtools. The CPU version of Medaka (version 1.2.3) was then used to polish the consensus sequences. The HEV and GII norovirus processing pipelines were very similar, differing only by the amplicon length, primer sequences and database (reference sequences). The code for these pipeline processes can be seen in supplementary data 6.3 and 6.4, alongside brief explanations of the process. The alignment files used can be seen in supplementary data 6.5 and 6.6. A minimum of 1,000 supporting reads was required to determine that the sequence was not due to imperfect barcoding or cross-contamination. All negative controls had less than 100 reads.

Statistical Analysis

All statistical analysis was performed using R (version 4.04) (R Core Team, 2018). As the read data did not follow normal distributions (tested by Shapiro-Wilk test), Spearman's rank correlations were used to identify if the number of sequence reads correlated with the C_T values obtained from qRT-PCR of the samples. We also used a Spearman's rank correlation to determine if the total supporting reads from all sequences in a sample correlated with C_T values from the samples. A one-sided Wilcoxon test was used to determine whether the median of C_T values from samples which failed to sequence (either produced no PCR amplicon or no sequencing data) was significantly higher than the median of C_T values from samples which sequenced successfully.

Phylogenetic Analysis

Reference sequences used for the phylogenetic analysis of the sequencing data were retrieved from NCBI; 514 sequences for HEV genotypes 1-8, and 96 sequences for norovirus GII were downloaded and merged into two separate FASTA files, using BioEdit. The polished HEV and GII norovirus sample sequence reads were then added to the appropriate FASTA files before alignment using Clustal Omega (CLUSTAL multiple sequence alignment). The sequences were then trimmed to be the same length as the sequenced amplicons and alignments were curated by eye to remove gaps. Sequences with a Hamming Dissimilarity percentage (calculated within Unipro UGENE (Okonechnikov *et al.*, 2012)) greater than 10% were classed as different sequences. The HEV and GII norovirus alignments were processed by IQTREE, where the most suitable evolutionary model was automatically chosen based on Bayesian Information Criteria scores (Nguyen *et al.*, 2015). The substitution model selected was TIM2 with gamma distribution (TIM2 + G) for GII norovirus and HEV. For the norovirus phylogenetic tree, the full amplicon was 302 bp but 20 bp of RNA-dependent RNA polymerase gene sequence was trimmed from the beginning of the sequences to enable comparison to more capsid sequences obtained from NCBI, and because inclusion of this fragment may have distorted the phylogenetic analysis due to the presence of polymerase/capsid recombinants in the database.

To identify how closely related the sequence from the shellfish sample (Shellfish_seq1) was to genotype 3 and subtype 3f, the reference sequences for G3 from Smith *et al.*, (2020b) and 44 G3f strains with a complete or mostly complete

genome were downloaded from GenBank (Benson *et al.*, 2013) and aligned with Shellfish_seq1 and Hamming Dissimilarity matrices were calculated within Unipro UGENE to identify whether the shellfish sequence was likely to belong to G3 and G3f (Okonechnikov *et al.*, 2012).

Results

HEV

Of the 65 samples selected for HEV sequencing, eleven samples yielded HEV sequencing data using the G3 sequencing assay. The G1 PCR assay yielded no HEV sequence data. Of these eleven samples, ten were influent wastewater samples and one was a shellfish sample. Of the 55 samples which failed to yield HEV sequence data, 38 gave no nested PCR products and therefore were not included in the library preparation, and the remaining 16 yielded no sequence data but yielded amplicons with the expected size. No wastewater effluent or cetacean samples gave any HEV sequencing data. Figure 6.2 shows a box and whisker plot of the distribution of C_T values for samples which failed or succeeded to provide HEV sequences. The median C_T value from samples which failed to sequence was not significantly higher than that from samples which were successfully sequenced (one-sided Wilcoxon test, p= 0.559). The data showing the sequencing success can be seen in Table 6.2.

Table 6.2 Sample sequencing success and failure results

Sample	Sample type	CT	Sequencing result
09-465	Shellfish	39.81998	Fail
09-475	Shellfish	38.96326	Fail
10-113	Shellfish	38.81057	Success
18-361	Shellfish	38.31731	Fail
18-362	Shellfish	37.33453	Fail
C19/16	Cetacean	31.4999	Fail
C24/17	Cetacean	32.1837	Fail
C25/17	Cetacean	30.62367	Fail
C26/17	Cetacean	31.61662	Fail
C27/17	Cetacean	31.46813	Fail
C28/17	Cetacean	30.96494	Fail
C1/18	Cetacean	30.53666	Fail
C2/18	Cetacean	31.01212	Fail
C3/18	Cetacean	30.82375	Fail
C5/18	Cetacean	31.27773	Fail
C13/18	Cetacean	31.88618	Fail
C25/18	Cetacean	32.79505	Fail
C27/18	Cetacean	31.03182	Fail
C28/18	Cetacean	32.60235	Fail
C29/18	Cetacean	31.3209	Fail
C2/19	Cetacean	30.65009	Fail
C6/19	Cetacean	31.87744	Fail
C8/19	Cetacean	32.01376	Fail
SW04	Influent	39.51233	Success
SW06	Influent	39.33455	Fail
SW11	Effluent	39.47243	Fail
SW13	Effluent	41.16046	Fail
SW17	Effluent	39.38813	Fail
SW18	Influent	39.50611	Fail

SW20	Influent	33.57486	Success
SW22	Influent	36.46371	Success
SW24	Influent	37.24512	Success
SW25	Effluent	38.74826	Fail
SW26	Influent	38.27507	Success
SW32	Influent	37.37944	Success
SW34	Influent	39.76252	Fail
SW36	Influent	39.86499	Fail
SW38	Influent	39.58624	Fail
SW44	Influent	37.49087	Fail
SW46	Influent	37.53729	Fail
SW48	Influent	38.74213	Fail
SW50	Influent	38.37618	Fail
SW52	Influent	38.66098	Fail
SW60	Influent	40.48499	Fail
SW63	Effluent	40.44064	Fail
SW72	Influent	39.19169	Fail
SW78	Influent	40.27344	Success
SW66	Influent	40.5822	Success
SW80	Influent	41.34575	Fail
SW92	Influent	39.18997	Fail
SW96	Influent	36.18138	Success
SW101	Effluent	39.72198	Fail
SW103	Effluent	39.92864	Fail
SW106	Influent	38.79374	Success
SW107	Effluent	39.99926	Fail
SW108	Influent	39.98698	Fail
SW114	Influent	38.58137	Fail
SW116	Influent	40.4021	Fail
SW121	Effluent	42.81889	Fail
SW122	Influent	40.36576	Fail
SW123	Effluent	39.50692	Fail
SW127	Effluent	38.97312	Fail

SW130	Influent	38.97312	Fail
SW134	Influent	36.56873	Fail
SW140	Influent	43.71178	Fail

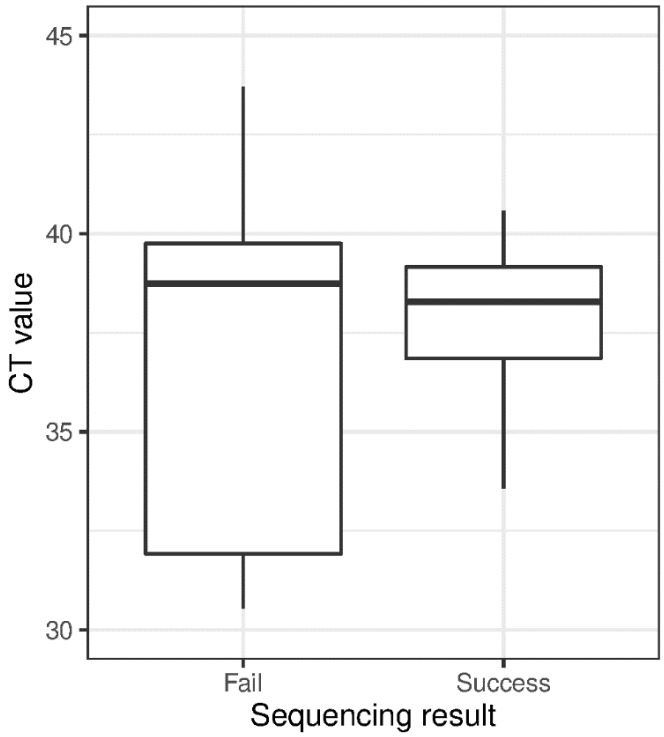


Figure 6.2 A box and whisker plot of the distribution of HEV C_T values

The distribution of C_T values which either succeeded or failed to yield sequencing data. The C_T values originate from qRT-PCR testing of HEV for samples which were included in this sequencing study.

The number of supporting reads per HEV amplicon sequence ranged from 2,115 to 401,595 after sequences of incorrect length were removed. The percentage coverage for all sequences was 100%. Samples which sequenced successfully yielded mostly just one sequence, but in a single case two different HEV sequences were obtained. The sequence read and coverage data can be seen in Table 6.3. All sequences were obtained in a single MinION run.

Table 6.3 Supporting reads and level of coverage for each HEV amplicon sequence obtained

Sample ID	Sequence ID	C_T	Supporting reads	Mean depth
SW20	Sewage_seq1	33.6	105610	99384
SW04	Sewage_seq2	39.5	2115	2049
SW22	Sewage_seq3	36.5	6375	6083
SW24	Sewage_seq4	37.2	82094	77388
SW26	Sewage_seq5	38.3	217785	211269
SW32	Sewage_seq6	37.4	256142	243621
SW78	Sewage_seq7	40.3	309055	300480
SW66	Sewage_seq8	40.6	384195	372624
SW96	Sewage_seq9	36.2	70643	67775
SW96	Sewage_seq10	36.2	8450	8114
SW106	Sewage_seq11	38.8	401595	389150
OY3	Shellfish_seq1	38.8	18834	17593

No correlation between the number of supporting reads from each sequence and the C_T values was observed (Spearman's rank correlation, Rho= 0.364 *p*= 0.206, 95% confidence intervals -0.510 and 0.843).

HEV typing using the RIVM Hepatitis E Virus Genotyping Tool showed that the small size of the amplicon created limitations for genotyping and subtyping (https://www.rivm.nl/mpf/typingtool/hev/). Except for one sequence, all samples were successfully genotyped as G3. Some sequences were subtyped as G3c. Although with a weak phylogenetic support, other sequences were genotyped as G3e and G3m. Table 6.4 shows a summary of the typing tool results. A distance matrix identified that Sewage_seq1 and Sewage_seq6 had identical HEV nucleotide sequences.

Table 6.4 Typing results for the eleven HEV sequences from the sewage and shellfish studies from RIVM

Sequence	BLAST result	BLAST score	Genotype result	Genotype support	Subtype result	Subtype support
Sewage_seq1	Hepeviridae Orthohepevirus A	90.2	HEV3	98.0	HEV3c	76.0
Sewage_seq2	Hepeviridae Orthohepevirus A	86.5	HEV3	91.0	Could not assign	NA
Sewage_seq3	Hepeviridae Orthohepevirus A	91.6	HEV3	99.0	Could not assign	NA
Sewage_seq4	Hepeviridae Orthohepevirus A	90.2	HEV3	98.0	HEV3c	78.0
Sewage_seq5	Hepeviridae Orthohepevirus A	89.7	HEV3	98.0	HEV3c	74.0
Sewage_seq6	Hepeviridae Orthohepevirus A	90.2	HEV3	98.0	HEV3c	76.0
Sewage_seq7	Hepeviridae Orthohepevirus A	85.1	HEV3	97.0	Could not assign	NA
Sewage_seq8	Hepeviridae Orthohepevirus A	90.7	HEV3	96.0	Could not assign	NA
Sewage_seq9	Hepeviridae Orthohepevirus A	88.4	HEV3	95.0	Could not assign	NA
Sewage_seq10	Hepeviridae Orthohepevirus A	92.1	HEV3	94.0	Could not assign	NA
Sewage_seq11	Hepeviridae Orthohepevirus A	85.6	HEV3	95.0	Could not assign	NA
Shellfish_seq1	Hepeviridae Orthohepevirus A	86.0	Could not assign	NA	NA	NA

A nucleotide BLAST of the sequences showed the majority clustered most closely with GenBank HEV sequences derived from humans (Altschul *et al.*, 1990). The results with the highest percentage identity and lowest E value per sequence can be seen in table 6.5. Some of the sequences were equally related to several published sequences; possibly because these database sequences were identical to each other in the region sequenced. Longer amplicon length may have helped to identify which GenBank sequences were more highly related to the HEV strains within the samples. Unsurprisingly, five of the twelve sequences were most closely related to other HEV strains from human infections in the UK, however some sequences were also closely related to human strains in France, and a G3 strain found in a wild boar in Italy.

Table 6.5 Nucleotide BLAST results for the amplicon sequences

Sequence	BLAST result	Percentage identity	E value	Score	BLAST result host	BLAST result Location
Sewage_seq1	MH504137.1	97.7	$2.75x10^{-98}$	370	Human	Poole, UK
Sewage_seq2	MH504146.1	98.1	$5.92x10^{-100}$	375	Human	Chichester, UK
Sewage_seq3	MH504128.1	97.2	$4.61x10^{-96}$	363	Human	Brighton, UK
Sewage_seq4	MH504137.1	97.7	$2.75x10^{-98}$	370	Human	Poole, UK
Sewage_seq5	MW355362.1	97.7	$2.75x10^{-98}$	370	Human	France
Sewage_seq6	MH504137.1	97.7	$2.75x10^{-98}$	370	Human	Poole, UK
Sewage_seq7	MH504146.1	95.8	$1.29x10^{-91}$	348	Human	Chichester, UK
	MT840367.1	95.8	$1.29x10^{-91}$	348	Wild boar	Italy
Sewage_seq8	MT362711.1	97.2	$4.61x10^{-96}$	363	Human	The Netherlands
	MW355220.1	97.2	$4.61x10^{-96}$	363	Human	France
Sewage_seq9	MF444141.1	98.1	$5.92x10^{-100}$	375	Human	France
	MF444109.1	98.1	$5.92x10^{-100}$	375	Human	France
	MW355403.1	98.1	$5.92x10^{-100}$	375	Human	France
	MT840367.1	98.1	$5.92x10^{-100}$	375	Wild boar	Italy
Sewage_seq10	MF444030.1	98.6	$1.27x10^{-101}$	381	Human	France
Sewage_seq11	MH504146.1	95.8	$6.00x10^{-90}$	342	Human	Chichester, UK
	MT840367.1	95.8	$6.00x10^{-90}$	342	Wild boar	Italy
Shellfish_seq1	MF444105.1	87.3	$6.26x10^{-60}$	243	Human	France

As the RIVM typing tool was unable to subtype several of the sequences from this study, BLAST sequences with the highest homology were typed to identify the possible subtypes for the sequences. The results of this can be seen in Table 6.6. It

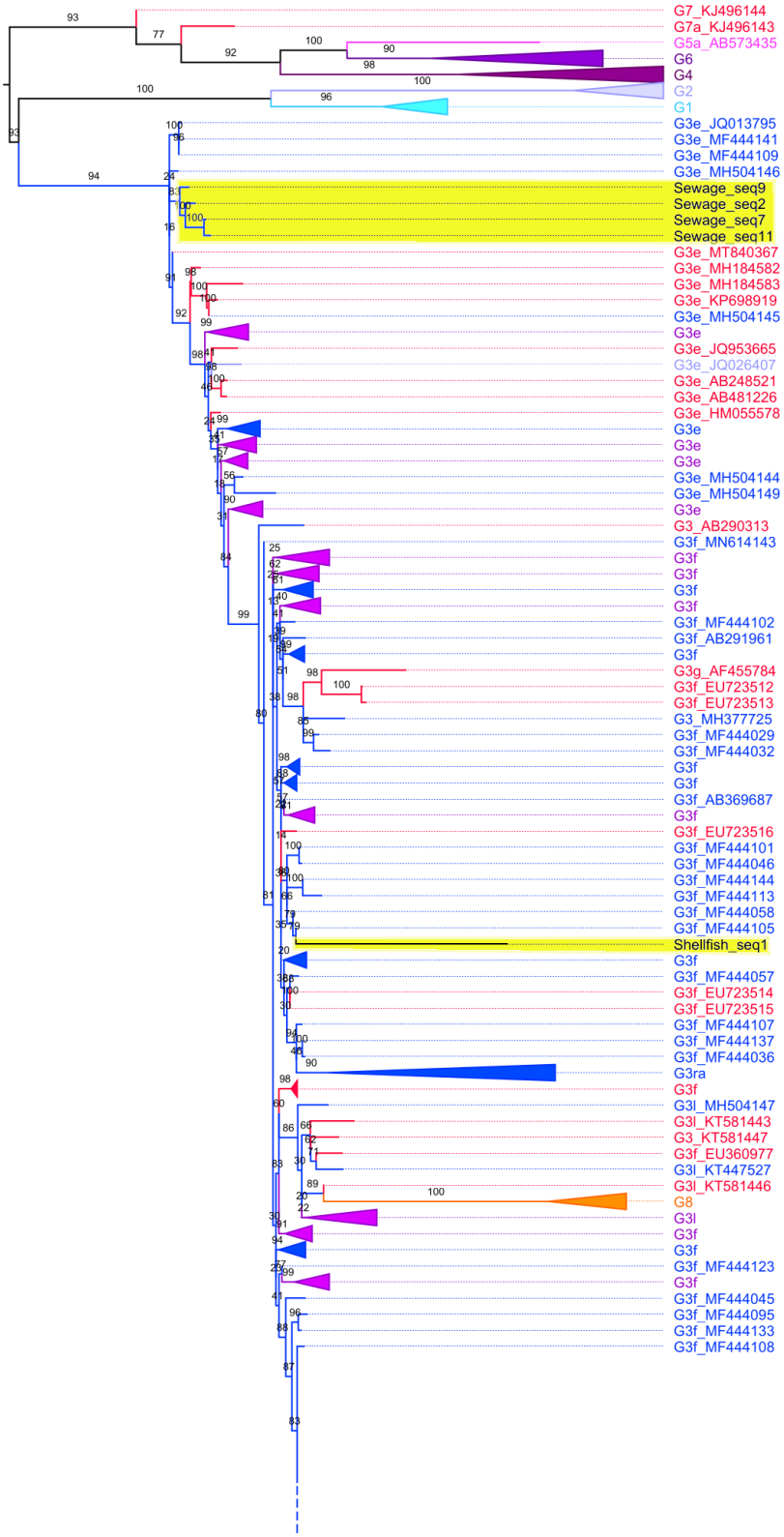
should be noted that the nearest BLAST result for the shellfish sequence is very dissimilar (87.3%) and therefore the subtype assigned to the most similar BLAST result may not accurately reflect the subtype of the sequence within the sample.

Table 6.6 Typing results for the BLAST results with highest nucleotide similarity to the HEV amplicon sequences

Sequence	RIVM typing tool subtype	BLAST result	Percentage identity	BLAST result subtype
Sewage_seq1	HEV3c	MH504137.1	97.7	G3c
Sewage_seq2	Could not assign	MH504146.1	98.1	G3e
Sewage_seq3	Could not assign	MH504128.1	97.2	G3c
Sewage_seq4	HEV3c	MH504137.1	97.7	G3c
Sewage_seq5	HEV3c	MW355362.1	97.7	G3c
Sewage_seq6	HEV3c	MH504137.1	97.7	G3c
Sewage_seq7	Could not assign	MH504146.1	95.8	G3e
		MT840367.1	95.8	G3e
Sewage_seq8	Could not assign	MT362711.1	97.2	G3c
		MW355220.1	97.2	G3c
Sewage_seq9	Could not assign	MF444141.1	98.1	G3e
		MF444109.1	98.1	G3e
		MW355403.1	98.1	G3e
		MT840367.1	98.1	G3e
Sewage_seq10	Could not assign	MF444030.1	98.6	G3m
Sewage_seq11	Could not assign	MH504146.1	95.8	G3e
		MT840367.1	95.8	G3e
Shellfish_seq1	Could not assign	MF444105.1	87.3	G3f

Phylogenetic analysis of the HEV sequences showed that the BLAST result subtypes accurately predicted where the HEV amplicon sequences cluster on the cladogram, except for Sewage_seq10 which clusters closely with sequences from subtypes G3c and G3m. The phylogenetic tree of the HEV amplicon sequences from this study alongside previously published sequences can be seen in Figure 6.3. This tree has been coloured by the host organism the HEV sequences was obtained from. Based on this phylogenetic analysis, it appears that the sequence obtained from the shellfish sample (Shellfish_seq1) may not fall into any existing genotypes or subtypes and may

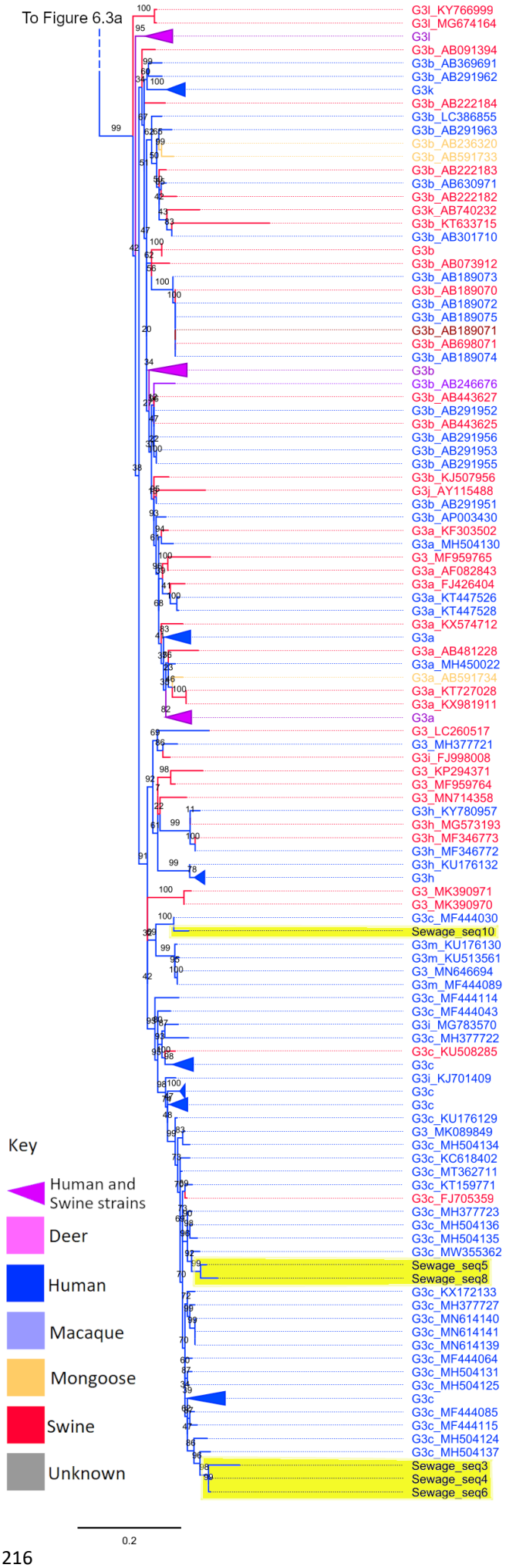
possibly be a new genotype or subtype. The tree showed that the amplicon sequences obtained in the sewage study cluster closely with human sequences, but also sequences from swine. The sequence from the shellfish sample is quite distantly related to sequences identified in humans.



To Figure 6.3b

Figure 6.3 Phylogenetic tree of HEV sequences

Phylogenetic tree of a 215bp fragment from 514 HEV sequences, including ten novel sequences detected in our samples. Only genotypes 3 and 8 are shown. All genotype branches had bootstrap support >70%. Some branches within G3 were collapsed due to low phylogenetic support for the branch or within the branch. Where the diagram has been split the branch between G3f and G3l subtypes has been extended. The full, complete tree can be seen in supplementary data 6.7 (not coloured by host, compatible for colour blindness conditions protanopia, tritanopia and tritanomaly). Sewage_seq1 was not included in the tree as it was identical to Sewage_seq6. The sequences from this study are shown with black labels, with highilghting. The other sequences are coloured by host, which can be found in the key. The scale bar shows the length of branch that represents an amount of genetic change of 0.2.



The phylogenetic tree shows that sequences 9, 2, 7 and 11 cluster together within subtype 3e, close to both human and swine sequences. Sequence 10 clusters within G3c and G3m, close to human and swine sequences. Sequences 5 and 8 cluster together within G3c, most closely with other human sequences. Sequences 3, 4 and 6 also clustered within G3c, but most closely to human sequences. The Hamming dissimilarity matrices (not shown) which were calculated for comparison of the shellfish sequence to existing G3 and G3f strains revealed that the G3 reference sequences were 76 – 96% similar to one another, and that the shellfish sequence was 79 – 86% similar to the G3 reference sequences. This suggests that the shellfish sequence belongs to genotype 3. However, of the available G3f sequences from GenBank which were fully or partially complete, these sequences were all 87 – 99% similar to one another. The shellfish sequence on the other hand was only 81 – 86% similar to the G3f strains. Therefore, this sequence might be best considered a representative of a novel G3 subtype. Figure 6.4 shows a zoomed in view of the shellfish sequence from Figure 6.3 for clarity.

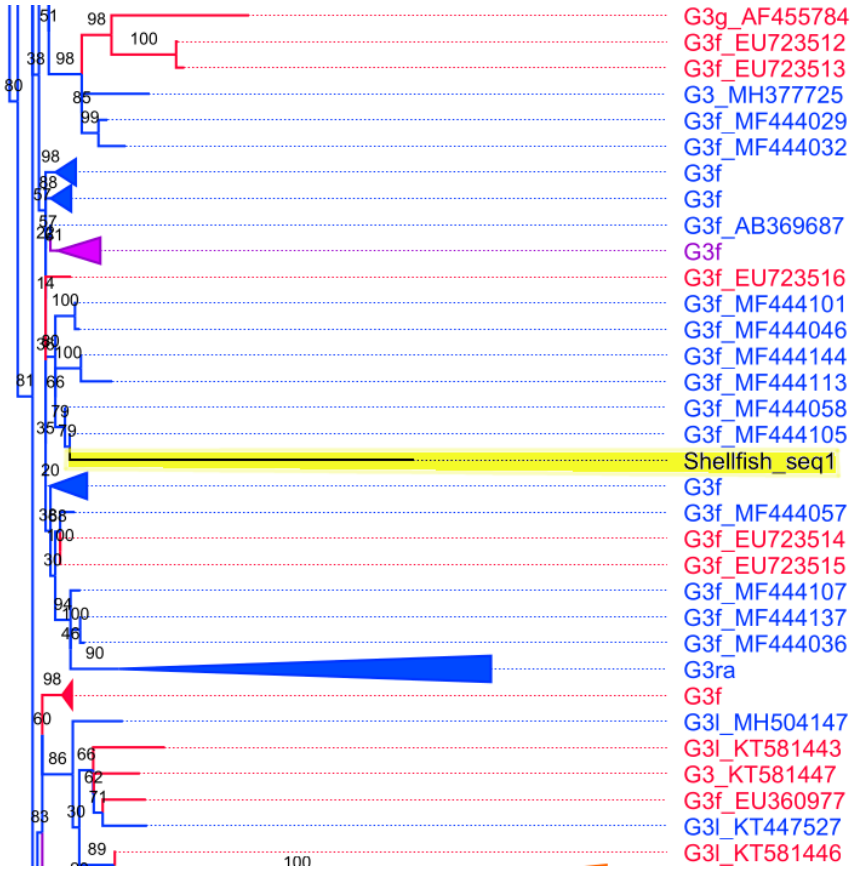


Figure 6.4 The shellfish sequence and surrounding G3 HEV sequences

A zoomed in version of the shellfish sequence from Figure 6.3.

Norovirus

Of the 42 influent and effluent wastewater samples which were selected for norovirus GII sequencing, 23 provided valid sequencing data (sequences with over 1,000 reads). Of these, 12 were influent and 11 were effluent samples. Fifteen of the samples which failed to sequence did not amplify using the nested PCR assay and therefore were not included in the library preparation. One sample provided <1,000 reads and so was not analysed further. The remaining three failed samples did not provide norovirus sequencing data (despite obtaining a nested PCR band during electrophoresis and being included in the library preparation). Figure 6.5 shows a box and whisker plot of the distribution of C_T values for samples which failed and succeeded.

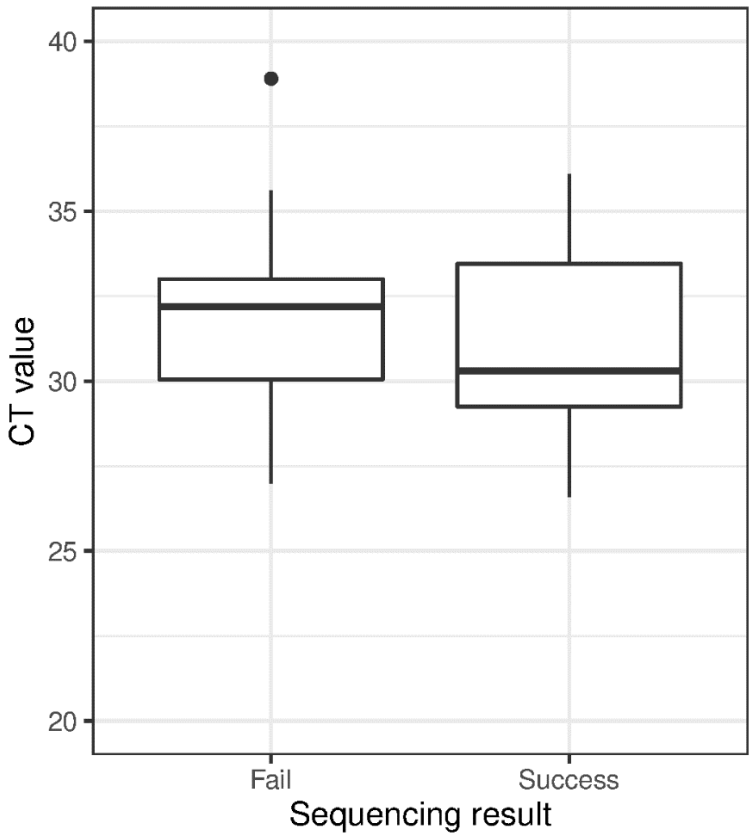


Figure 6.5 A box and whisker plot of the distribution of GII norovirus C_T values

The distribution of C_T values from qRT-PCR testing of GII norovirus for samples which were included in this sequencing study. The C_T values ranged from 26 – 39.

Whilst the median C_T for samples which sequenced successfully was lower than the median C_T for samples which failed to sequence, this difference was not significant (one-sided (greater) Wilcoxon test, p= 0.165). The number of reads aligning to each GII norovirus type varied between 1,329 and 93,423. The negative control produced

no norovirus reads. The percentage coverage for the sequences was 100%. Between one and ten different GII norovirus consensus sequences were observed per sample. The sequence read data can be seen in Figure 6.6. Depth of coverage can be seen for each sample in supplementary data 6.8. There was no correlation between C_T values and the number of supporting reads for each sequence (Spearman rank correlation, Rho= 0.078, *p*= 0.453, 95% confidence intervals -0.131 and 0.283); nor between C_T values and total supporting reads for each sample (Rho= -0.142, *p*= 0.517, 95% confidence intervals -0.457 and 0.253).

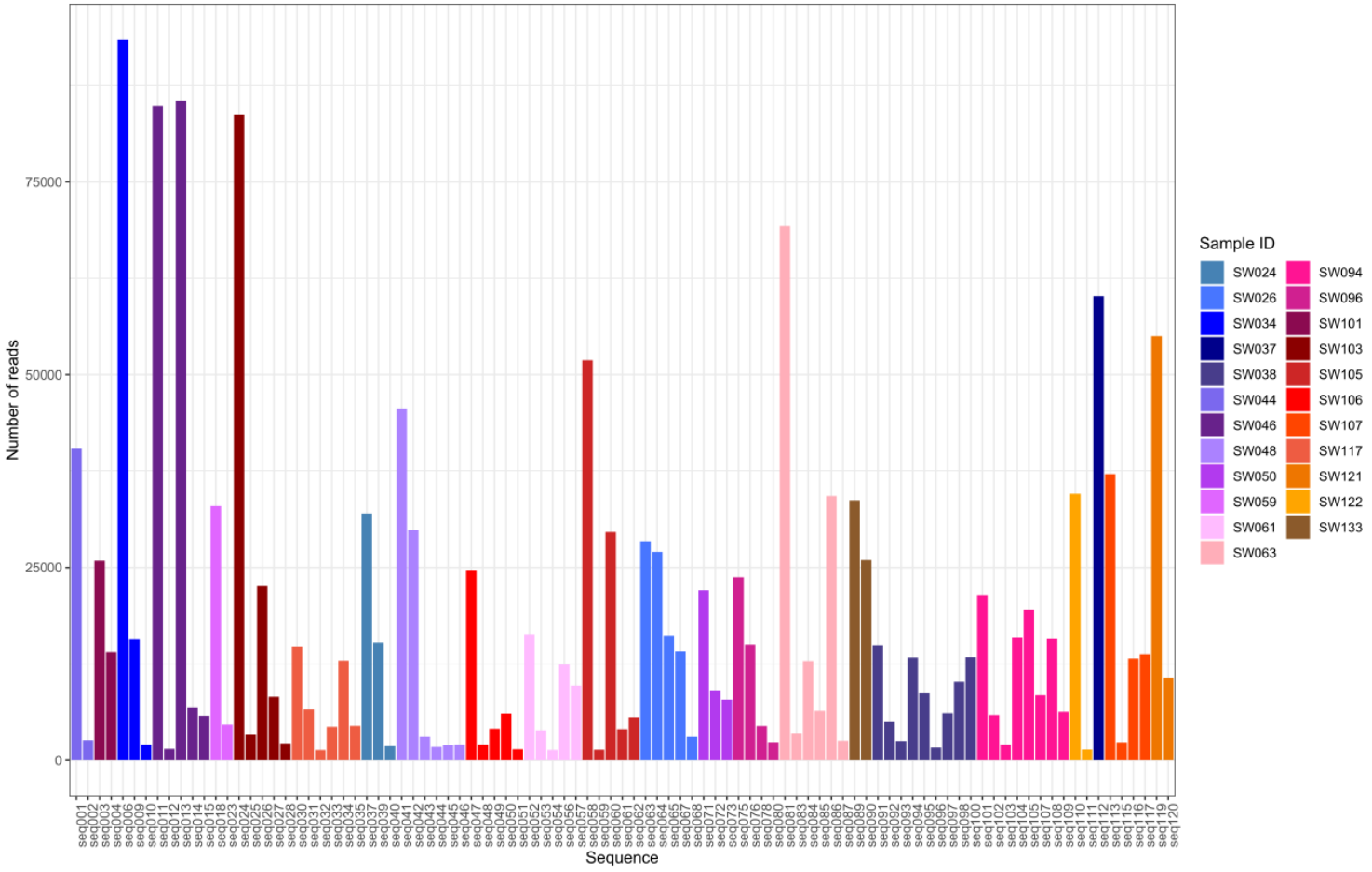


Figure 6.6 Supporting reads for each GII norovirus amplicon sequence obtained

Graph to show the number of supporting reads for each GII norovirus amplicon sequence, coloured by the sample ID.

The RIVM Norovirus Typing Tool was used to determine what genotypes the consensus sequences belonged to (Kroneman *et al.*, 2011). The sequences fells into eight different genotypes: GII.2, GII.3, GII.4, GII.6, GII.7, GII.9, GII.13 and GII.17. Two of the sewage samples contained only one genotype of GII norovirus, but the remaining samples contained between two and six genotypes. These results are presented in Figure 6.7.

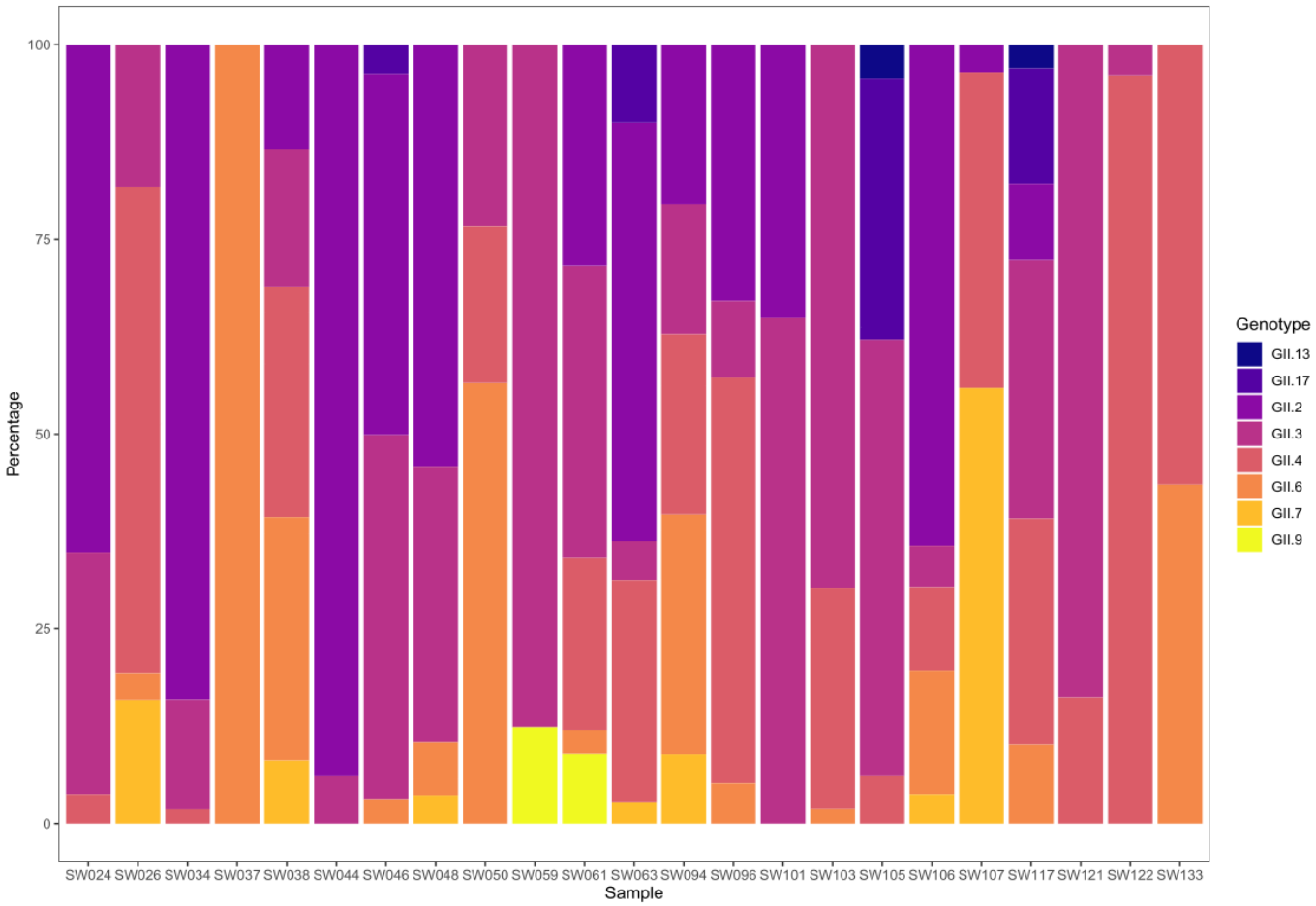


Figure 6.7 Plot of the percentage of reads which were attributed to a certain genotype of GII norovirus

A plot showing the percentage of the total number of reads per sewage sample which were attributed to specific GII norovirus genotypes. An alternative version suitable for the condition tritanopia can be seen in supplementary data 6.9.

The genotype which was detected most frequently (within the most samples) was GII.4, which generated 25 different sequences. Interestingly, the genotype with the most reads attributed to it was GII.3. The 23 influent samples yielded 55 sequences within genotypes GII.2, GII.3, GII.4, GII.6, GII.7 and GII.17. The 11 effluent samples yielded 40 sequences within the same genotypes as the influent samples, in addition to genotypes GII.9 and GII.13. The most common genotype found in influent samples was GII.6 (14 sequences), closely followed by GII.4 and GII.3 (13 sequences). The most common genotype within effluent was GII.4 (12 sequences). The genotypes with the highest proportion of reads within the influent samples was GII.2 (median of 31,999 reads), and for effluent samples it was GII.3 (median of 29,419 reads). Sequences from GII.2 and GII.3 were detected in the influent and effluent of each

WWTP which yielded sequencing results (five of seven). The number of sequences attributed to each genotype can be seen in Table 6.7.

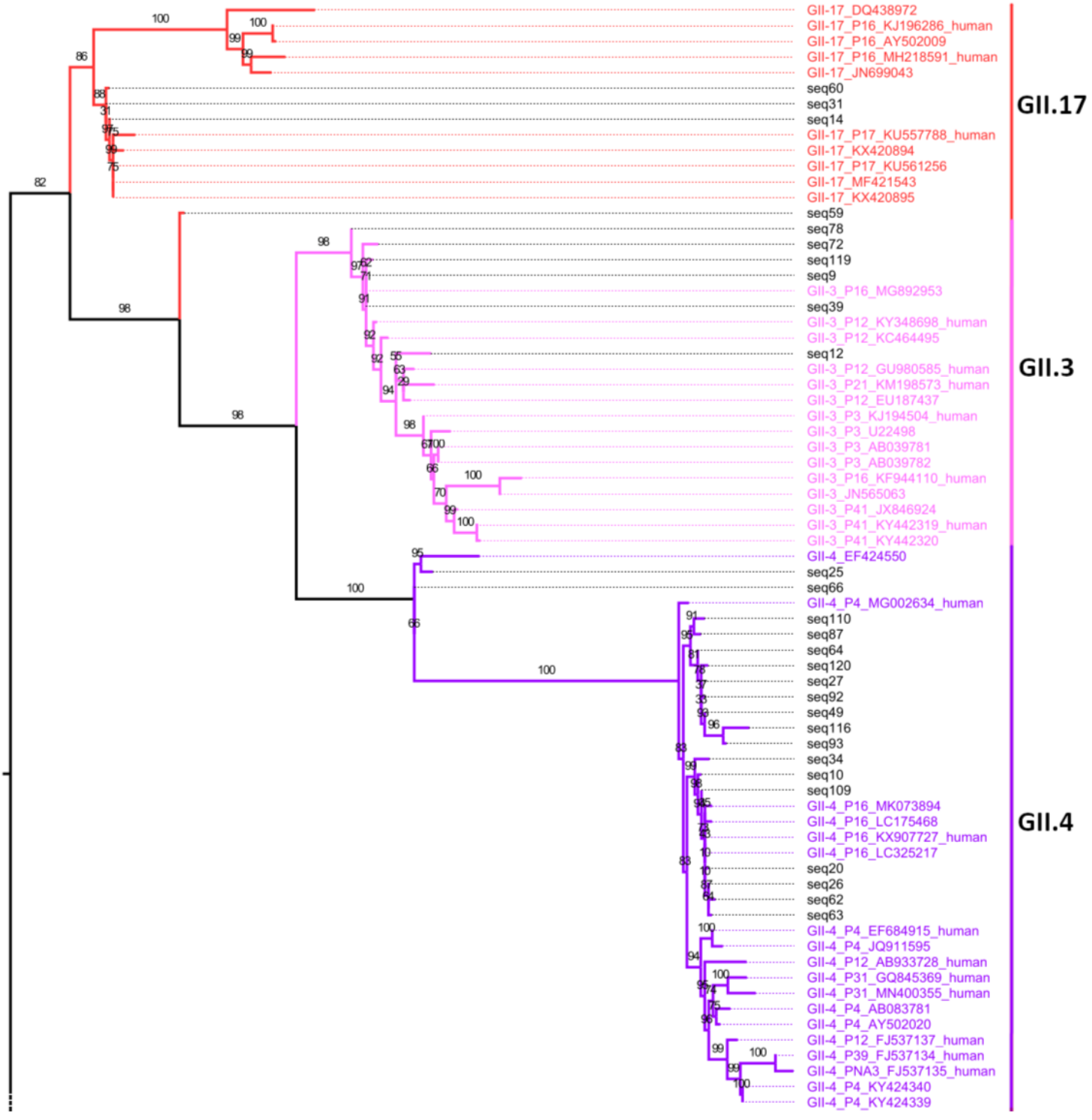
Table 6.7 Sequences attributed to each norovirus GII genotype

Genotype	Influent			Effluent			Total number of sequences	Total reads
	Number of sequences	Number of reads	Median number of reads	Number of sequences	Number of reads	Median number of reads		
GII.2	9	366,374	31,999	5	102,357	12,405	14	468,731
GII.3	13	212,129	13,378	8	286,904	29,419	21	499,033
GII.4	13	173,969	7,869	12	170,424	11,775	25	344,393
GII.6	14	98,011	4,421	5	94,163	4,484	19	192,174
GII.7	5	33,156	6,128	2	40,566	20,283	7	73,722
GII.9	0	0	0	2	8,553	4,277	2	8,553
GII.13	0	0	0	2	5,381	2,691	2	5,381
GII.17	1	6,798	6,798	4	50,444	9,748	5	57,242

Nucleotide BLAST results with the highest E value and percentage identity for each sequence showed the nucleotide sequences were 91 – 100% similar to existing GII norovirus sequences available in GenBank. Phylogenetic analysis of the capsid region within the amplicon sequences shows the clustering within the genotypes (Figure 6.8). Some sequences within different samples were shown to be identical (Hamming dissimilarity matrix) and were not included in Figure 6.8. These samples were above the 1,000 read minimum threshold and therefore suggest possible dominant strains circulating widely within the community.

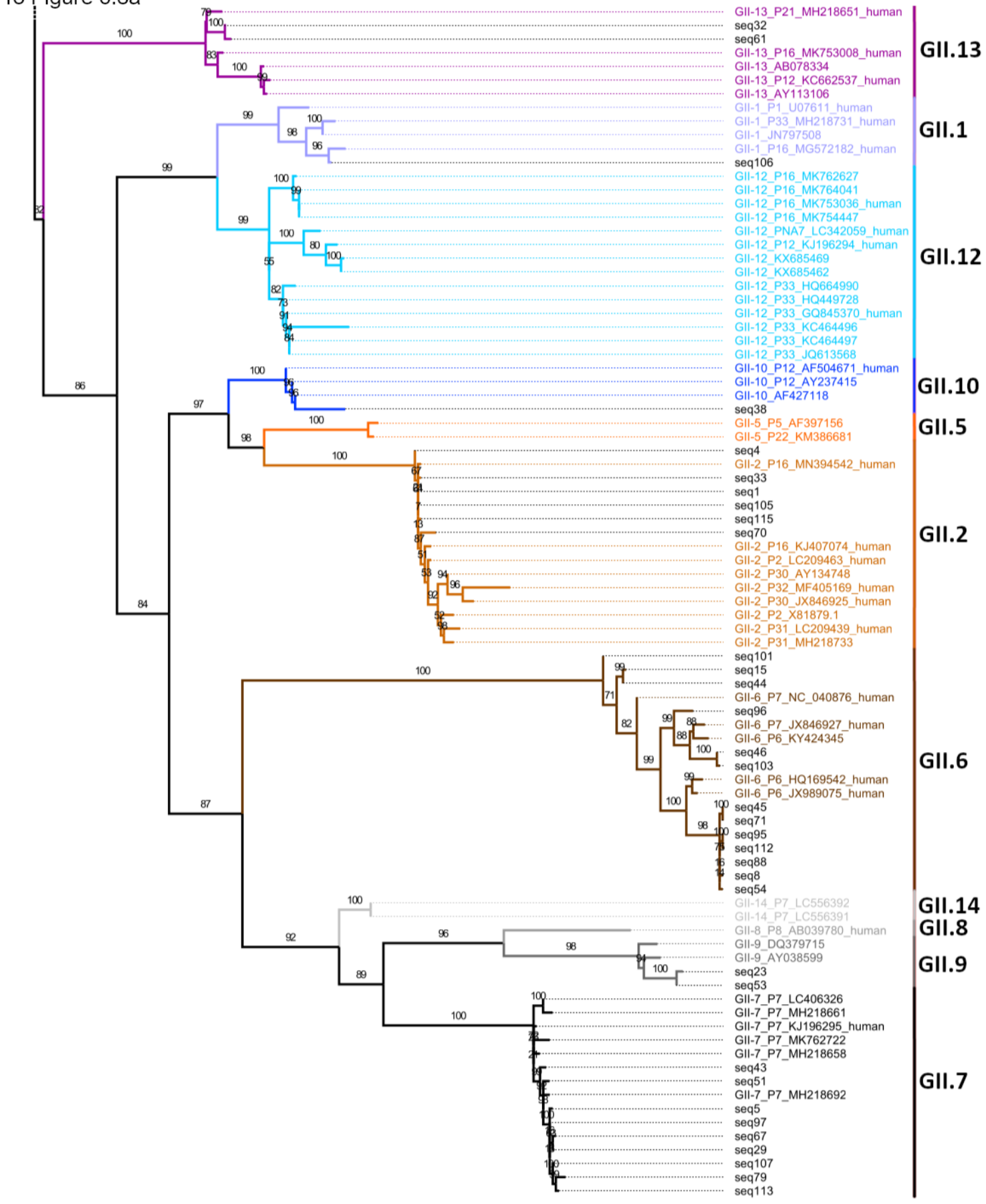
Figure 6.8 Phylogenetic tree of GII norovirus sequences

Phylogenetic tree constructed using a 282bp fragment of GII norovirus capsid gene. Amplicon sequences are labelled in black text (seq#). Bootstrap support was >70% for all major genotype clades. The phylogenetic tree can be viewed online in supplementary data 6.10. Genotypes not featured in the analysis had poor bootstrap support (<70%). Versions suitable for the colour blindness conditions protanopia, deuteranopia and tritanopia can be seen in supplementary data 6.11. The scale bar shows the length of branch that represents an amount of genetic change of 0.08.



To Figure 6.8b

To Figure 6.8a



Discussion

This study investigated GII norovirus and hepatitis E virus sequences within the samples obtained in the previous chapters. HEV presence within sewage and shellfish samples from Southern England was confirmed, and eight different genotypes of GII norovirus were identified within sewage samples. Most of the HEV sequences are likely to have originated from humans or swine, and GII norovirus sequences other than GII.4 were prominent within the sewage samples. This is the first detection of HEV in sewage and shellfish in England and the first known use of ONT metabarcoding of HEV amplicons for sequencing of samples with a very low viral concentration.

HEV sequences in sewage and shellfish

Of 65 samples of cetacean liver, sewage influent and effluent and shellfish (all of which reported high C_T values during qRT-PCR for HEV), eleven provided HEV sequencing data through this PCR metabarcoding approach. The C_T values of these samples ranged from 34 – 41 (1 – 113 copies/ml), and the range of reads was 2,115-401,595 within one sequencing run, showing that even samples with low levels of virus can be sequenced using this technique. The lack of sequencing data which could be obtained from the cetacean samples means that the qRT-PCR results from chapter 4 are inconclusive. There was no difference in the median C_T values of samples which failed or succeeded to sequence and no correlation between C_T values and the number of reads per sequence or sample. This could be due to sample integrity or presence of RNases or inhibitors. As discussed previously in chapter 4, it was not possible to test the sewage or archived shellfish samples for a sample integrity control due to an inability to create integrity controls for the sewage, and the unlikelihood that the shellfish samples would provide positive results (due to the speed of mRNA decay and age of the samples (Friedel *et al.*, 2009)). However, as studied within Weesendorp et al., (2010), viral RNA can persist for days within sample matrices which are decaying, and therefore these samples could still provide valuable insights into the viruses within this study. Indeed, this study has shown that even samples which may have been affected by factors which can degrade viral RNA can provide meaningful sequencing data.

It was shown that all of the HEV sequences within the samples were 87.3-98.6% similar to existing HEV sequences. Given that the amplicons were of a small size

(215bp), this may have led to multiple strains with similarities in this particular region being most closely related to the amplicon sequences. However, the database sequences may also have been identical. The sequences from the sewage samples were 95.8-98.6% similar to published HEV sequences on GenBank (NCBI) and the sequence with the highest dissimilarity (87.3%) belonged to the archived shellfish sample, which was distantly related to subtype G3f. This subtype is known to be highly diverse (Smith *et al.*, 2020b), however the RIVM HEV Typing Tool and phylogenetic analysis show that the sequence from this sample clearly has an unclear genotype and subtype. The analysis using the dissimilarity matrices for G3 and G3f suggest that the shellfish sequence could be considered a new subtype of genotype 3, though more research would be required to confirm this. All other HEV sequences belonged to genotype 3, and were shown to cluster within subtypes G3c, G3e, and G3f; one sewage sample was inconclusive. It is interesting that all of the sewage samples were so distantly related to the sequence within the shellfish sample (G3c and G3e); however, the sewage samples were collected ten years later, so dominant G3 subtypes within the community are likely to have changed, and sample sizes are also too small and geographically limited to conclude that no sequences with this unknown type are still present within English sewage, and that no other subtypes are present in English shellfish. It should be considered that the shellfish may have been contaminated through alternative routes, such as animal waste. This could originate from run off from pig or deer farms, or from other farms housing animals which are infected with HEV (Grierson *et al.*, 2015), or could perhaps originate from wild animals (Anheyer-Behmenburg *et al.*, 2017) or even sewer-dwelling animals such as rats (Kanai *et al.*, 2012).

The sequences from this study matched closely to HEV sequences from humans in most cases but were also closely related to swine HEV sequences for others, suggesting that these species were the major sources of the viruses within the samples. Sewage sequences 9, 2, 7 and 11 cluster together within G3e, close to both human and swine sequences, suggesting they could be of either human or swine origin, or perhaps may have been identified in both. Sewage sequence 10 had an undetermined subtype within G3, but clustered closely with human and swine sequences from G3c and G3m. Sewage sequences 5 and 8 clustered closely together within G3c, close to other human sequences, as did sewage sequences 3, 4 and 6. It is possible that if there were more animal sequences publicly accessible

that these results may have been less biased towards a link with human sequences, however this can only be assessed when more HEV sequences from animals and food matrices become available. However, with the consideration that most of the WWTPs were fed primarily by human sewage, it seems most likely that most of the HEV sequences originated from humans rather than animal sources. Due to the dissimilarity of the shellfish sequence compared to other published sequences, it is difficult to identify the origin of this sequence, though it clusters most closely (though distantly) with sequences from humans. Some subtypes have become more dominant in the UK in the past two decades; Ijaz *et al.*, (2013) and Grierson *et al.*, (2015) showed the emergence of new phylotypes of G3 emerging within the UK, showing that HEV subtypes seemed to form two major clades, one of which had been dominant between 2003 and 2010 and one of which became more dominant from 2011 (Ijaz *et al.*, 2013). Clade 1 includes subtypes 3e, 3f, 3g and clade 2 includes subtypes 3a, 3b, 3c, 3d, 3h, 3i, 3j (Ijaz *et al.*, 2013; Smith *et al.*, 2021). Interestingly, in the UK in 2013, pigs were shown to generally be infected with viruses from clade 1, whilst humans were generally infected with viruses from clade 2 (Grierson *et al.*, 2015); meanwhile pig sequences from Europe in the same time frame appeared to cluster with clade 2. A study on a limited number of UK infections in blood donors from 2018 – 2019 also showed most infections to belong to clade 2 (Smith *et al.*, 2021). It appears that the virus subtypes identified within this study fall evenly into both groups, and as swine are thought to be the main reservoir of HEV, this may mean that both UK and European swine strains circulate within the UK, however it is possible the swine data from the previous studies was too limited, and a phylogenetic comparison of the sewage sequences to swine strains from UK pigs was not possible (due to lack of UK swine HEV sequences). Unfortunately, due to the low number of samples which provided sequencing data, it was not possible to draw conclusions on whether these two major groups may be circulating more or less than previously reported, and there is no current data to suggest which subtypes were more dominant in the population in the UK in 2019.

It is apparent that HEV presence in both sewage, shellfish and the community is likely to be rare in the UK, due to the low prevalence identified in this and previous studies; such as the identification of HEV in 3% of Scottish shellfish samples sold in a supermarket (O'Hara *et al.*, 2018), and annual reports of clinically diagnosed HEV cases (Public Health England, 2019). However, despite low prevalence in these

areas, HEV does contaminate the aquatic environment in the UK, most likely due to release of untreated human (and possibly animal) sewage into water courses through combined sewer overflows (CSOs), which are allowed to spill into water courses due to storm weather conditions. Considering that CSOs across the UK spilt into water courses for over 3 million hours in 2020 (Environment Agency, 2020a), it is likely that HEV contaminates the aquatic environment from human faecal sources. It seems less likely that HEV within treated effluent would be a large source of contamination for the aquatic environment as no effluent samples from this study provided any HEV sequencing data, perhaps due to low copy number or RNA degradation. However, some effluent samples were qRT-PCR positive, so it cannot be ruled out.

GII Norovirus sequences in sewage

Of the 42 sewage samples with C_T values 26 – 39 (1 – 8,984 copies/ml), 23 samples were successfully sequenced. There was no association between C_T value and sequencing success, and no correlation between C_T values and the number of supporting reads for the sequence. This could perhaps be due to sample degradation or mismatches in the annealing region of the primers of the semi-nested PCR assay. During sequencing, 1,329 – 93,423 reads were obtained per sequence, which is a lower range than what was obtained for the HEV samples, however the GII norovirus run may not have been run for the exact same length of time.

The 23 samples contained eight different GII genotypes, with individual samples containing as many as six different genotypes. The GII norovirus sequences identified in this study were shown to have a nucleotide sequence similarity between 91 and 100% with published sequences on Genbank using nucleotide BLAST, however many of the sequences were equally similar to multiple published sequences, possibly due to repeated sequences on GenBank. Some sequences were also detected (all with >1,000 reads) in multiple samples, suggesting possible widely circulating strains, though this was not possible to confirm due to the small length of the amplicon. The effluent samples contained all eight genotypes, whilst the influent samples contained only six. This may be a result of influent and effluent samples being collected simultaneously, and therefore the treated effluent was not representative of the same raw influent. It also demonstrates how variable the pathogens in sewage are even over short spans of time. Unsurprisingly, GII.4

sequences were detected most commonly within the samples; this genotype has been the most frequently detected in the world since 2014 (Public Health England, 2020). However, GII.3 sequences had the highest median number of reads, suggesting that viruses from this genotype may have been very abundant within the samples. GII.4 sequences were most common within effluent samples, but GII.6 was most common within influent samples. However, GII.3 and GII.2 gave the highest median number of reads within effluent and influent respectively. GII.2 and GII.3 were the only genotypes which were detected at all of the WWTPs which were able to yield sequencing data from the 42 chosen samples (5/7 WWTPs).

Despite the abundance of different genotypes within the sewage samples, clinical data from (Public Health England, 2020) at the time of the study shows that though GII.4 and GII.6 made up a large proportion of cases, other detected genotypes such as GII.9 and GII.13 were not detected in cases from the public. This is likely to be explained by under-reporting of clinical cases by the public, or by asymptomatic illness, as norovirus cases are estimated to reach 3 million annually (Gherman *et al.*, 2020) and UK Health Security Agency reported only 6,172 symptomatic infections between 2018 and 2019 (Public Health England, 2021a). This finding brings further testament to the uses of sewage in the surveillance of viruses which cause outbreaks, as techniques such as these can lead to the detection of potential pandemic strains before they become more widely distributed.

Limitations

As so few of the HEV samples were capable of being sequenced, whether the remaining samples were true HEV positives at the qRT-PCR stage, or whether the semi-nested PCRs were perhaps too specific to amplify the samples is questionable. This is especially true for the cetacean samples, of which eighteen samples with the highest C_T values yielded no sequencing data. This could perhaps be due to a different genotype of HEV infecting the cetaceans, or perhaps a HEV-related virus within the cetaceans which was not detected using the G1 and G3 nested PCRs for sequencing. However, this hypothesis requires testing. In addition to this limitation, the G1 HEV PCR yielded no qRT-PCR or sequencing data. Whether this is due to genuine low abundance of G1 HEV within the samples or is perhaps due to a faulty nRT-PCR method cannot be known as there was no available positive control material for this assay. However, as G1 HEV is only caused by travel associated

infections which are rarely reported within the UK, this was a less important investigation regarding indigenous HEV transmission compared with G3. The sequencing results have been determined to be most closely related to other human and swine sequences, however there are not enough other animal sequences publicly available to say whether there may be alternative sources of HEV – such as cows, sheep, goats, deer, rabbits, or other animals. Another limitation is that the RIVM Hepatitis E Virus Genotyping tool (originally based on the classification proposed by Smith *et al.*, (2016) has not yet been updated with the new classification system (proposed by Smith *et al.*, (2020b). This study endeavoured to use the updated classification system as much as possible.

A significant limitation of NGS is that the threshold for deciding whether a sequencing run has cross contamination or incorrectly assigned barcodes is generally set at 1,000, but this is an arbitrary number with no real scientific basis behind it. This threshold has been applied to this data; however, if a threshold of 500 sequences has been used, this would have meant one other sample had sequencing data, and that two more genotypes of GII norovirus were present. Further investigations of NGS data analysis need to be performed to assign a threshold which does not compromise the data.

Another limitation of the study is that small amplicons were sequenced, which has prevented possible detection of new HEV and GII norovirus variants as there is not enough phylogenetic resolution. Sequencing of longer amplicons or whole genomes was unlikely to be successful due to the high CT values for most of the samples, and it has been observed elsewhere that longer amplicons than those of the qRT-PCR for detection can lead to lower sensitivity (Aprea *et al.*, 2016; Grierson *et al.*, 2015). However, the use of small amplicons has allowed sequencing data to be obtained from more samples for GII norovirus compared to long amplicons (data not shown), and small amplicons have been used to successfully genotype HEV and norovirus sequences. The size of the amplicon is enough to be able to genotype the strain in most cases, however longer amplicons would provide the benefit of identifying significantly different changes in sequences, and possibly identifying new variants. In addition, despite error correction during the bioinformatics pipeline, it is known that ONT sequencing can generate erroneous reads due to the method of basecalling relying on changes in potential difference across a membrane (Stefan *et al.*, 2021),

rather than a more exact method such as fluorescent base tagging. ONT sequencing has been quoted to have an individual read error rate of 5-20% previously (Kono and Arakawa, 2019), however the modal accuracy rate for the chemistry and high accuracy basecalling method used in this study is estimated to be 97.8% (Oxford Nanopore Technologies, 2021). Moreover, consensus accuracies close to 100% have been reported in studies using similar sequencing and bioinformatic approaches (McNaughton *et al.*, 2019; Bull *et al.*, 2020), although this was not determined in the present study.

Conclusion

In this study a metabarcoding approach to genotype HEV and GII norovirus was successfully developed and applied This could have several advantages for the sequencing of samples with low viral concentration in future studies, and the use of the ONT platform for this method could enable greater portability of HEV and GII norovirus sequencing, as well as improving affordability over other sequencing platforms (such as Illumina). The study has shown that HEV is present in sewage and shellfish in southern England and therefore that contamination of the aquatic environment with HEV does occur in the UK, though rarely. It has also shown that many different genotypes of GII norovirus are present in sewage, and that national surveillance of clinical norovirus cases may not be fully representative of all of the circulating genotypes within the population. Considering the high levels of faecal pollution of surface waters and shellfish harvesting areas throughout the UK due to CSO use, it must be considered that pathogens within human sewage, such as HEV, contaminate UK surface waters, and indeed there is existing proof that norovirus does this in the UK, and that HEV does this in other countries. An assessment of the levels of contamination of surface waters must be conducted to identify the risk to people who undergo recreational water activities and consume shellfish. In conclusion, viruses such as norovirus and HEV within sewage can contaminate the aquatic environment, which may lead to the contamination of shellfish harvesting areas and bathing waters with these viruses. This in turn can lead to public health risks. More must be done to limit or stop faecal pollution of bathing waters to prevent illnesses from recreational water activities and the consumption of shellfish.

References

Agrawal, S., Orschler, L. and Lackner, S. (2021) 'Long-term monitoring of SARS-CoV-2 RNA in wastewater of the Frankfurt metropolitan area in Southern Germany', *Scientific reports,* 11(1), pp. 5372-5372.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) 'Basic local alignment search tool', *J Mol Biol,* 215(3), pp. 403-10.

Anheyer-Behmenburg, H. E., Szabo, K., Schotte, U., Binder, A., Klein, G. and Johne, R. (2017) 'Hepatitis E virus in wild boars and spillover infection in red and roe deer, Germany, 2013–2015', *Emerging infectious diseases,* 23(1), pp. 130.

Aprea, G., Amoroso, M. G., Di Bartolo, I., D'Alessio, N., Di Sabatino, D., Boni, A., Cioffi, B., D'Angelantonio, D., Scattolini, S., De Sabato, L., Cotturone, G., Pomilio, F., Migliorati, G., Galiero, G. and Fusco, G. (2018) 'Molecular detection and phylogenetic analysis of hepatitis E virus strains circulating in wild boars in south-central Italy', *Transboundary and Emerging Diseases,* 65(1), pp. e25-e31.

Batista, F. M., Stapleton, T., Lowther, J. A., Fonseca, V. G., Shaw, R., Pond, C., Walker, D. I., van Aerle, R. and Martinez-Urtaza, J. (2020) 'Whole Genome Sequencing of Hepatitis A Virus Using a PCR-Free Single-Molecule Nanopore Sequencing Approach', *Front Microbiol,* 11, pp. 874.

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. and Sayers, E. W. (2013) 'GenBank', *Nucleic Acids Res,* 41(Database issue), pp. D36-42.

Bull, R. A., Adikari, T. N., Ferguson, J. M., Hammond, J. M., Stevanovski, I., Beukers, A. G., Naing, Z., Yeang, M., Verich, A., Gamaarachchi, H., Kim, K. W., Luciani, F., Stelzer-Braid, S., Eden, J.-S., Rawlinson, W. D., van Hal, S. J. and Deveson, I. W. (2020) 'Analytical validity of nanopore sequencing for rapid SARS- CoV-2 genome analysis', *Nature Communications,* 11(1), pp. 6272.

Desdouits, M., Wacrenier, C., Ollivier, J., Schaeffer, J. and Le Guyader, F. S. (2020) 'A Targeted Metagenomics Approach to Study the Diversity of Norovirus GII in Shellfish Implicated in Outbreaks', *Viruses,* 12(9), pp. 978.

Eden, J.-S., Hewitt, J., Lim, K. L., Boni, M. F., Merif, J., Greening, G., Ratcliff, R. M., Holmes, E. C., Tanaka, M. M. and Rawlinson, W. D. (2014) 'The emergence and evolution of the novel epidemic norovirus GII. 4 variant Sydney 2012', *Virology,* 450, pp. 106-113.

Environment Agency. (2020) 'Event Duration Monitoring - Storm Overflows - 2020' [Database of CSO use throughout UK in 2020]. Available at: https://environment.data.gov.uk/dataset/21e15f12-0df8-4bfc-b763-45226c16a8ac (Accessed.

Friedel, C. C., Dölken, L., Ruzsics, Z., Koszinowski, U. H. and Zimmer, R. (2009) 'Conserved principles of mammalian transcriptional regulation revealed by RNA half- life', *Nucleic acids research,* 37(17), pp. e115-e115.

Gao, J., Wu, H., Shi, X., Huo, Z., Zhang, J. and Liang, Z. (2016) 'Comparison of Next-Generation Sequencing, Quantitative PCR, and Sanger Sequencing for Mutation Profiling of EGFR, KRAS, PIK3CA and BRAF in Clinical Lung Tumors', *Clin Lab,* 62(4), pp. 689-96.

Gherman, I., Holland, D., Clifford, R., Mahmoudzadeh, N., Uy, F., Adkin, A., Kainth, B., Cook, P., Day, A. and Wilson, A. (2020) 'Technical Report: Review of Quantitative Risk Assessment of foodborne norovirus transmission', *Science,* 1, pp. 1.

Grierson, S., Heaney, J., Cheney, T., Morgan, D., Wyllie, S., Powell, L., Smith, D., Ijaz, S., Steinbach, F. and Choudhury, B. (2015) 'Prevalence of hepatitis E virus infection in pigs at the time of slaughter, United Kingdom, 2013', *Emerging infectious diseases,* 21(8), pp. 1396.

Iizuka, S., Oka, T., Tabara, K., Omura, T., Katayama, K., Takeda, N. and Noda, M. (2010) 'Detection of sapoviruses and noroviruses in an outbreak of gastroenteritis linked genetically to shellfish', *Journal of medical virology,* 82(7), pp. 1247-1254.

Ijaz, S., Said, B., Boxall, E., Smit, E., Morgan, D. and Tedder, R. S. (2013) 'Indigenous hepatitis E in England and Wales from 2003 to 2012: evidence of an emerging novel phylotype of viruses', *The Journal of infectious diseases,* 209(8), pp. 1212-1218.

Johne, R., Reetz, J., Ulrich, R., Machnowska, P., Sachsenröder, J., Nickel, P. and Hofmann, J. (2014) 'An ORF 1-rearranged hepatitis E virus derived from a chronically infected patient efficiently replicates in cell culture', *Journal of viral hepatitis,* 21(6), pp. 447-456.

Kanai, Y., Miyasaka, S., Uyama, S., Kawami, S., Kato-Mori, Y., Tsujikawa, M., Yunoki, M., Nishiyama, S., Ikuta, K. and Hagiwara, K. (2012) 'Hepatitis E virus in Norway rats (Rattus norvegicus) captured around a pig farm', *BMC Res Notes,* 5, pp. 4.

Kenney, S. P. (2019) 'The current host range of hepatitis E viruses', *Viruses,* 11(5), pp. 452.

Kojima, S., Kageyama, T., Fukushi, S., Hoshino, F. B., Shinohara, M., Uchida, K., Natori, K., Takeda, N. and Katayama, K. (2002) 'Genogroup-specific PCR primers for detection of Norwalk-like viruses', *J Virol Methods,* 100(1-2), pp. 107-14.

Kokkinos, P., Kozyra, I., Lazic, S., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A. M., Kaupke, A. and Legaki, E. (2012) 'Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries', Food and environmental virology, 4(4), pp. 179-191.

Kono, N. and Arakawa, K. (2019) 'Nanopore sequencing: review of potential applications in functional genomics', *Development, growth & differentiation,* 61(5), pp. 316-326.

Kroneman, A., Vennema, H., Deforche, K., v d Avoort, H., Peñaranda, S., Oberste,M. S., Vinjé, J. and Koopmans, M. (2011) 'An automated genotyping tool for enteroviruses and noroviruses', *J Clin Virol,* 51(2), pp. 121-5.

Ledesma, J., Williams, D., Stanford, F. A., Hewitt, P. E., Zuckerman, M., Bansal, S., Dhawan, A., Mbisa, J. L., Tedder, R. and Ijaz, S. (2019) 'Resolution by deep sequencing of a dual hepatitis E virus infection transmitted via blood components', *Journal of General Virology,* 100(11), pp. 1491-1500.

Loisy, F., Atmar, R., Guillon, P., Le Cann, P., Pommepuy, M. and Le Guyader, F. (2005) 'Real-time RT-PCR for norovirus screening in shellfish', *Journal of*

virological methods, 123(1), pp. 1-7.

Lu, J., Fang, L., Zheng, H., Lao, J., Yang, F., Sun, L., Xiao, J., Lin, J., Song, T., Ni, T., Raghwani, J., Ke, C., Faria, N. R., Bowden, T. A., Pybus, O. G. and Li, H. (2016) 'The Evolution and Transmission of Epidemic GII.17 Noroviruses', *The Journal of Infectious Diseases,* 214(4), pp. 556-564.

Malmberg, M. M., Spangenberg, G. C., Daetwyler, H. D. and Cogan, N. O. I. (2019) 'Assessment of low-coverage nanopore long read sequencing for SNP genotyping in doubled haploid canola (Brassica napus L.)', *Sci Rep,* 9(1), pp. 8688.

Mancini, P., Bonanno Ferraro, G., Iaconelli, M., Suffredini, E., Valdazo-González, B., Della Libera, S., Divizia, M. and La Rosa, G. (2019) 'Molecular characterization of human Sapovirus in untreated sewage in Italy by amplicon-based Sanger and next- generation sequencing', *Journal of applied microbiology,* 126(1), pp. 324-331.

Martin, J., Klapsa, D., Wilton, T., Zambon, M., Bentley, E., Bujaki, E., Fritzsche, M., Mate, R. and Majumdar, M. (2020) 'Tracking SARS-CoV-2 in Sewage: Evidence of Changes in Virus Variant Predominance during COVID-19 Pandemic', *Viruses,* 12(10), pp. 1144.

McNaughton, A. L., Roberts, H. E., Bonsall, D., de Cesare, M., Mokaya, J., Lumley, S. F., Golubchik, T., Piazza, P., Martin, J. B., de Lara, C., Brown, A., Ansari, M. A., Bowden, R., Barnes, E. and Matthews, P. C. (2019) 'Illumina and Nanopore methods for whole genome sequencing of hepatitis B virus (HBV)', *Sci Rep,* 9(1), pp. 7081.

Meng, X.-J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., Purcell, R. H. and Emerson, S. U. (1998) 'Genetic and experimental evidence for cross-species infection by swine hepatitis E virus', *Journal of virology,* 72(12), pp. 9714-9721.

Meng, X.-J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. and Emerson, S. U. (1997) 'A novel virus in swine is closely related to the human hepatitis E virus', *Proceedings of the National Academy of Sciences,* 94(18), pp. 9860-9865.

Mulder, A. C., Kroneman, A., Franz, E., Vennema, H., Tulen, A. D., Takkinen, J., Hofhuis, A. and Adlhoch, C. (2019) 'HEVnet: a One Health, collaborative, interdisciplinary network and sequence data repository for enhanced hepatitis E virus molecular typing, characterisation and epidemiological investigations', *Euro Surveill,* 24(10).

Nguyen, L. T., Schmidt, H. A., von Haeseler, A. and Minh, B. Q. (2015) 'IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies', *Mol Biol Evol,* 32(1), pp. 268-74.

Oeser, C., Vaughan, A., Said, B., Ijaz, S., Tedder, R., Haywood, B., Warburton, F., Charlett, A., Elson, R. and Morgan, D. (2019) 'Epidemiology of Hepatitis E in England and Wales: A 10-Year Retrospective Surveillance Study, 2008–2017', *The Journal of Infectious Diseases,* 220(5), pp. 802-810.

Okonechnikov, K., Golosova, O., Fursov, M. and team, t. U. (2012) 'Unipro UGENE: a unified bioinformatics toolkit', *Bioinformatics,* 28(8), pp. 1166-1167.

O'Hara, Z., Crossan, C., Craft, J. and Scobie, L. (2018) 'First Report of the Presence of Hepatitis E Virus in Scottish-Harvested Shellfish Purchased at Retail Level', *Food and environmental virology*, pp. 1-5.

Oxford Nanopore Technologies. (2021) *Guppy v5.0.7 release note*. Oxford Nanopore Technologies Community Posts. Available at: https://community.nanoporetech.com/posts/guppy-v5-0-7-release-note (Accessed: 02 September 2021 2021).

Pallerla, S. R., Schembecker, S., Meyer, C. G., Linh, L. T. K., Johne, R., Wedemeyer, H., Bock, C. T., Kremsner, P. G. and Velavan, T. P. (2021) 'Hepatitis E virus genome detection in commercial pork livers and pork meat products in Germany', *Journal of Viral Hepatitis,* 28(1), pp. 196-204.

Pellerin, M., Hirchaud, E., Blanchard, Y., Pavio, N. and Doceul, V. (2021) 'Characterization of a Cell Culture System of Persistent Hepatitis E Virus Infection in the Human HepaRG Hepatic Cell Line', Viruses, 13(3).

Public Health England . (2019) *Hepatitis E: symptoms, transmission, treatment and prevention*. gov.uk: Public Health England. Available at: https://www.gov.uk/government/publications/hepatitis-e-symptoms-transmission-prevention-treatment/hepatitis-e-symptoms-transmission-treatment-and-prevention (Accessed: 27 August 2019).

Public Health England. (2020) *PHE National norovirus and rotavirus Report*, GOV.UK: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/871753/Norovirus_update_2020_weeks_08_to_09.pdf.

Public Health England. (2021) *2018 to 2019 Season Norovirus Report National norovirus laboratory and outbreak data in England*, gov.uk: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/966775/2021-01-29-Norovirus_annual_report_2018_to_2019.pdf.

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T. F. and Beutler, N. A. (2017) 'Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples', *nature protocols,* 12(6), pp. 1261.

R Core Team (2018) *R: A Language and Environment for Statistical Computing* (Version 3.4.3). Available at: https://www.R-project.org/.

Rivadulla, E., Varela, M. F., Mesquita, J. R., Nascimento, M. S. and Romalde, J. L. (2019) 'Detection of hepatitis E virus in shellfish harvesting areas from Galicia (Northwestern Spain)', *Viruses,* 11(7), pp. 618.

Robilotti, E., Deresinski, S. and Pinsky, B. A. (2015) 'Norovirus', *Clin Microbiol Rev,* 28(1), pp. 134-64.

Sakon, N., Sadamasu, K., Shinkai, T., Hamajima, Y., Yoshitomi, H., Matsushima, Y., Takada, R., Terasoma, F., Nakamura, A., Komano, J., Nagasawa, K., Shimizu, H., Katayama, K. and Kimura, H. (2018) 'Foodborne Outbreaks Caused by Human Norovirus GII.P17-GII.17–Contaminated Nori, Japan, 2017', *Emerging Infectious Disease journal,* 24(5), pp. 920.

Silva, A. J., Yang, Z., Wolfe, J., Hirneisen, K. A., Ruelle, S. B., Torres, A., Williams-Hill, D., Kulka, M. and Hellberg, R. S. (2021) 'Application of whole-genome sequencing for norovirus outbreak tracking and surveillance efforts in Orange County, CA', Food Microbiology, 98, pp. 103796.

Slot, E., Zaaijer, H. L., Molier, M., Van den Hurk, K., Prinsze, F. and Hogema, B.

M. (2017) 'Meat consumption is a major risk factor for hepatitis E virus infection', *PloS one,* 12(4), pp. e0176414.

Smith, C., Halse, T. A., Shea, J., Modestil, H., Fowler, R. C., Musser, K. A., Escuyer, V. and Lapierre, P. (2020a) 'Assessing Nanopore Sequencing for Clinical Diagnostics: a Comparison of Next-Generation Sequencing (NGS) Methods for Mycobacterium tuberculosis', *J Clin Microbiol,* 59(1).

Smith, D. B., Izopet, J., Nicot, F., Simmonds, P., Jameel, S., Meng, X.-J., Norder, H., Okamoto, H., van der Poel, W. H. and Reuter, G. (2020b) 'Update: proposed reference sequences for subtypes of hepatitis E virus (species Orthohepevirus A)', *Journal of General Virology*, pp. jgv001435.

Smith, D. B., Simmonds, P., Izopet, J., Oliveira-Filho, E. F., Ulrich, R. G., Johne, R., Koenig, M., Jameel, S., Harrison, T. J. and Meng, X.-J. (2016) 'Proposed reference sequences for hepatitis E virus subtypes', *Journal of General Virology,* 97(3), pp. 537-542.

Smith, I., Said, B., Vaughan, A., Haywood, B., Ijaz, S., Reynolds, C., Brailsford, S., Russell, K. and Morgan, D. (2021) 'Case–Control Study of Risk Factors for Acquired Hepatitis E Virus Infections in Blood Donors, United Kingdom, 2018–2019', *Emerging Infectious Diseases,* 27(6), pp. 1654-1661.

Stefan, C. P., Hall, A. T., Graham, A. S. and Minogue, T. D. (2022) 'Comparison of Illumina and Oxford Nanopore Sequencing Technologies for Pathogen Detection from Clinical Matrices Using Molecular Inversion Probes', The Journal of Molecular Diagnostics, 24(4), pp. 395-405.

Tamada, Y., Yano, K., Yatsuhashi, H., Inoue, O., Mawatari, F. and Ishibashi, H. (2004) 'Consumption of wild boar linked to cases of hepatitis E', *Journal of Hepatology,* 40(5), pp. 869-870.

Todt, D., Gisa, A., Radonic, A., Nitsche, A., Behrendt, P., Suneetha, P. V., Pischke, S., Bremer, B., Brown, R. J. and Manns, M. P. (2016) 'In vivo evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome', *Gut,* 65(10), pp. 1733-1743.

Treagus, S., Wright, C., Baker-Austin, C., Longdon, B. and Lowther, J. (2021) 'The Foodborne Transmission of Hepatitis E Virus to Humans', *Food and Environmental Virology*, pp. 1-19.

Verhoef, L., Kouyos, R. D., Vennema, H., Kroneman, A., Siebenga, J., van Pelt, W. and Koopmans, M. (2011) 'An integrated approach to identifying international foodborne norovirus outbreaks', *Emerg Infect Dis,* 17(3), pp. 412-8.

Ward, P., Müller, P., Letellier, A., Quessy, S., Simard, C., Trottier, Y. L., Houde, A. and Brassard, J. (2008) 'Molecular characterization of hepatitis E virus detected in swine farms in the province of Quebec', *Can J Vet Res,* 72(1), pp. 27-31.

Weesendorp, E., Willems, E. M. and Loeffen, W. L. A. (2010) 'The effect of tissue degradation on detection of infectious virus and viral RNA to diagnose classical swine fever virus', *Veterinary Microbiology,* 141(3), pp. 275-281.

Chapter 7

Quantitative Microbial Risk Assessment of HEV and Norovirus Presence in the UK Aquatic Environment

Abstract

Norovirus and hepatitis E virus are enteric pathogens which are shed in the faeces and urine of infected individuals. Both viruses are transmitted by the faecal-oral route but have different epidemiological patterns. Norovirus is well known to cause outbreaks through person-to-person transmission, the consumption of shellfish and other foods such as fruit and vegetables, and through exposure to faecally polluted water. HEV is endemic in pigs, leading to food borne transmission through consumption of contaminated pork products, and has been detected in sewage and shellfish in many countries, though no outbreaks from shellfish consumption or recreational water activities within polluted waters have been conclusively confirmed. This study endeavoured to assess the risk of illness from exposure to sewage containing norovirus and HEV, and from consumption of shellfish containing norovirus and HEV, using concentrations of the viruses obtained from chapters 5 and 6. It was estimated that there is a high risk of norovirus infection in people undergoing recreational water activities, even in areas where sewage is highly diluted by surface waters, and a high risk of norovirus from shellfish consumption. We also identified that the risk of HEV from these sources was low.
However, we must consider that model limitations and viability assumptions may affect these estimates.

Introduction

Norovirus is a gastrointestinal virus which is highly infectious and causes millions of cases worldwide annually. Hepatitis E virus (HEV) is also classed as a gastrointestinal virus, but it causes comparatively fewer cases than norovirus annually and spreads much less readily than norovirus. In the UK, 6,719 laboratory-confirmed cases of norovirus were reported in the 2017 – 2018 norovirus season (Public Health England, 2018b), but 3 million cases are estimated to occur in the UK yearly (Gherman *et al.*, 2020). This discrepancy is likely due to under-reporting by

the public, or under-testing by clinicians, and asymptomatic cases. In 2018, 1,002 HEV cases were reported in England and Wales (Public Health England, 2019), and this may also be an underestimate due to asymptomatic cases (Guillois *et al.*, 2015; Yin *et al.*, 2019), though the severity of symptomatic cases makes under-reporting unlikely in those affected. Though HEV and norovirus are clinically very different, they share common features in that they are both transmitted through a faecal-oral route, often through contaminated food and water (Treagus *et al.*, 2021; Alfano-Sobsey *et al.*, 2012; Di Bartolo *et al.*, 2015; Guillios *et al.*, 2015; Tripathy *et al.*, 2019).

Food and water become contaminated with human pathogens, such as norovirus and HEV, through exposure to blood, faeces and urine of infected individuals and animal hosts (Bouwknegt *et al*., 2009; Halbur *et al.*, 2001; Grierson *et al.*, 2015; Ledesma *et al.*, 2019). This may be due to cross-contamination during preparation of food by infected food handlers, the use of polluted irrigation water for crops, or other activities. Norovirus is estimated to have caused 383,182 cases from foodborne disease in the UK alone in 2018 (Holland and Mahmoudzadeh, 2020), and many cases occur in other countries also. For example, the largest known foodborne outbreak of norovirus occurred in Germany in 2012, where nearly 11,000 cases of norovirus originated from frozen strawberries imported from China (Bernard *et al.*, 2014). In addition, norovirus outbreaks from contaminated water are a common occurrence, often due to sewage contamination of drinking water sources. For example, in Denmark in 2012, at least 339 people were affected by norovirus infections attributed to intrusion of wastewater into the mains water supply (van Alphen *et al.*, 2014). Norovirus has also been identified as a common contaminant of surface waters, due to human faecal pollution (Di Bartolo *et al.*, 2015; Lodder *et al.*, 2005). This can be from treated sewage effluent or raw sewage from combined sewer overflows (CSOs). Norovirus has been detected in the surface waters of many countries, such as the Netherlands where norovirus was detected at high levels in the rivers Maas and Waal (maximum $4.9x10^6$ copies/ml) (Lodder and de Roda Husman, 2005). Additionally, surface water contamination has caused outbreaks during recreational water activities, such as beach and lake swimming or bathing (Kauppinen *et al.*, 2017; Di Bartolo *et al.*, 2015; Schets *et al.*, 2018; Wade *et al.*, 2018; Graciaa *et al.*, 2018). Contamination of surface waters can also lead to outbreaks of norovirus from the consumption of

bivalve shellfish, such as oysters and mussels, as they filter feed from their surrounding waters. For example, a shellfish-borne outbreak from a restaurant in the UK affected 240 people (Smith *et al.*, 2012). Though there is less available data for HEV, probably due to a lower prevalence worldwide, HEV is hypothesised to be transmitted in similar ways to norovirus. Most HEV outbreaks have currently been linked to the consumption of undercooked meat from pigs and deer. In France, an outbreak of HEV occurred after the consumption of pork from a spit-roasted piglet at a wedding (Guillois *et al.*, 2015). Additionally, a HEV outbreak within a household in Japan was shown to have been caused by the consumption of raw deer meat (Tei *et al.*, 2003). Whilst pigs and deer are known reservoirs for HEV, it has been speculated that HEV could also be transmitted through the consumption of shellfish in a similar way to norovirus. Studies around the world have detected HEV in surface waters and shellfish. In Italy, a study of the Tiber River in Rome showed the presence of many faecally-derived pathogens, including HEV and noroviruses (Marcheggiani *et al.*, 2015), and in the Philippines, genotype 3 HEV was shown to be present in the waters of two rivers in Manila city (Li *et al.*, 2014). HEV has also been detected in shellfish from China, England, Italy, Japan, Scotland, Spain (see chapters 2 and 5) (Gao *et al.*, 2015; La Rosa *et al.*, 2018; Li et al., 2007; Crossan *et al.*, 2012; O'Hara *et al.*, 2018; Mesquita *et al.*, 2016; Rivadulla *et al.*, 2019). No studies have yet identified an epidemiological link between HEV outbreaks and shellfish consumption. However, a retrospective study aiming to identify the cause of a HEV outbreak on a cruise ship concluded that shellfish were the probable source (Said *et al.*, 2009).

Previous studies have endeavoured to assess the risk of becoming ill from recreational use of surface waters, though most do not focus on specific pathogens such as norovirus or HEV, but illness generally (Leonard *et al.*, 2018). Prüss, (1998) showed, through a meta-analysis, that 19 studies (of 22 reviewed) identified a dose-response relationship between faecal indicator or pathogenic bacteria and the rate of gastrointestinal illness. Wade *et al.*, 2003 also used a systematic review and meta-analysis of 27 studies to identify that there was an increased relative risk of illness when there were increases in levels of faecal indicator bacteria, such as *E. coli* and *enterococci*. The study showed that the relative risk of illness from swimming in polluted waters was higher, where polluted waters were determined by the volumes of faecal indicator or pathogenic bacteria

present in the water. A later study by Leonard *et al.*, (2020)also identified that there was a higher risk of any illness, including ear, respiratory and gastrointestinal illness in bathers compared to non-bathers. Van Dijk et al., (1996) also identified that surfing/diving was associated with an increase in self-reporting of illness. Additionally, an outbreak of gastrointestinal illness from a swimming event in the river Thames was investigated, and ingestion of water during swimming was identified as a significant risk factor for illness (Hall *et al.*, 2017). Wade et al., (2018) also identified that people who had swam at a tropical beach were more likely to have had a norovirus exposure than non-swimmers and non-swimmers and waders. Many surface water risk assessment studies focus on viable faecal indicator or pathogenic bacteria to quantify viral risk as this is what is used to monitor bathing water quality. However, some studies have identified no association between the risk of illness and the presence of faecal indicator bacteria (Van Dijk *et al.*, 1996; Corbett *et al.*, 1993; Arnold *et al.*, 2013), and suggest that the risks of illness from viruses in surface waters have been underestimated (Corbett *et al.*, 1993; Arnold *et al.*, 2013). In addition, faecal indicator bacteria have been shown to be inadequate indicators for the presence of faecally derived viruses, such as norovirus, within both sewage and surface water (Kitajima *et al.*, 2014; Gibson *et al.*, 2011). However, some studies have focussed on the risks which viruses in the aquatic environment may pose using Quantitative Microbial Risk Assessment (QMRA). QMRA usually involves four steps, hazard identification, exposure assessment, dose response analysis and risk characterization (He and Huang, 2020). Hazard identification requires identifying the micro-organism which may cause a hazard to human health. Exposure assessment then requires identification of the exposure doses, the times of exposure and the frequency. The dose response analysis then utilizes mathematical models to predict the dose response, whether this leads to infection with the micro-organism or not. Finally, risk characterization will look to identify the annual risk from the exposure to that hazard (He and Huang, 2020).

QMRA tends to vary specifically by the micro-organism identified as the hazard. Dose-response models which have been calibrated to the specific dose-response studies for that organism can also vary. The most used dose-response models are the beta-Poisson model and the exponential model (He and Huang, 2020). Most virus models have utilized either a beta Poisson or fractional Poisson model (He

and Huang, 2020; *REF*). However, newer models such as the fractional-Poisson model have also been proposed and used (Messner *et al.*, 2014; Vergara *et al.*, 2016), and some more complex QMRA studies have incorporated multiple mathematical models into the calculations to improve modelling assumptions (Williams and O'Brien, 2019). A weakness of QMRA tends to be the assumptions made around factors such as micro-organism viability, the state of aggregation of a micro-organism, and the vulnerable population, taking into account susceptibility and acquired immunity (van Abel *et al.*, 2017). That being said, QMRA has been used to assess the risks of microbes in food, wastewater and other matrices, including microbes within recreational waters and shellfish. For instance, Vergara et al., (2016) used a Markov Chain Monte Carlo simulation alongside a fractional-Poisson dose response model (developed by Messner et al., (2014)) to determine the probability of norovirus illness from primary contact recreation (prolonged water exposure where water may be accidentally swallowed or ingested) to be approximately 0.61% for adults, and 0.89% for children in Singapore. A later study by Bortagaray *et al.*, (2020) determined the daily risk of rotavirus infection and illness from direct exposure during recreational activities in two rivers in Uruguay to be 6.41% and 3.2% respectively, using a beta-Poisson model from Haas et al., (2014).

QMRA has also been used to assess the rate of illness from shellfish consumption. Williams and O'Brien, (2009) authored a large, multi-centred study which used multiple mathematical models to predict individual factors which might contribute to becoming ill with norovirus after consuming an oyster meal. The study found that the risk of infection per meal was 6.78/1000 meals, or approximately one infection per 150 meals in the UK (Williams and O'Brien, 2009). A study in Thailand also investigated the risk of HEV and hepatitis A virus from raw oyster consumption, using a beta-Poisson model to calculate that 604 cases of HEV were estimated to result from farmed oyster consumption annually, and that there was relatively low risk in comparison to that from hepatitis A (Ruchusatsawat *et al.*, 2021).

However, no studies have yet assessed the risk of HEV infection from shellfish in the UK. Many countries, including the UK, have a shellfish monitoring system in place to reduce the risk of illness from pathogenic bacteria and viruses in shellfish. However, these systems commonly monitor faecal indicator bacteria

concentrations, such as *E. coli*, and do not monitor virus presence. This can lead to an underestimation of virus presence due to a lack of correlation between faecal indicator bacteria and norovirus concentrations (Lowther *et al.*, 2012; Sharp *et al.*, 2021), which is aggravated by the fact that shellfish purification techniques such as depuration and relaying can often reduce *E. coli* concentrations but have little effect on norovirus concentrations (Ueki *et al.*, 2007; Choi and Kingsley, 2016).

To our knowledge, no UK studies have yet determined the risk of HEV and norovirus illness from recreational water activities using quantitative data from sewage. A study by Leonard *et al.*, (2015) collated the volumes of water ingested during recreational water activities through a review of available literature; the data was then used to calculate the amount of antibiotic-resistant *E. coli* ingested during recreational water activities in the UK. In the present study the number of virus particles which may be ingested during a water activity, or during the consumption of shellfish was calculated. These calculations were based on the concentration of norovirus and HEV detected in sewage and shellfish samples previously and were used as the dose of norovirus or HEV. These calculations were made using the ingested water volumes per water activity from Leonard *et al.*, (2015). The probability of infection and illness was calculated from the virus dose using previously published dose-response models. This enabled an estimate of the risk of illness from norovirus or HEV, if people were to be exposed to the viral concentrations detected in sewage and shellfish samples in the previous studies (chapter 4 and 5). A calculation of the number of water activity sessions likely to cause illness, and the number of people who may become ill with norovirus from oyster consumption was also carried out.

Materials and Methods

Determining number of virus particles ingested during water activities

The paper by Leonard *et al.*, (2015) reviewed and described the volumes of water ingested during recreational water activities, and that information is summarised in Table 7.1.

Table 7.1 Estimated volumes of water ingested during recreational water activities from Leonard *et al.*, (2015)

Activity	Volume of water ingested (ml)	Study
Boating	3.7 ml per session	Dorevitch *et al.*, (2011)
Canoeing	3.9 ml per session	Dorevitch *et al.*, (2011)
Diving	9.9 ml per session	Schijven and de Roda Husman (2006)
Fishing	3.6 ml per session	Dorevitch *et al.*, (2011)
Kayaking	3.8 ml per session	Dorevitch *et al.*, (2011)
Rowing	3.5 ml per session	Dorevitch *et al.*, (2011)
Surfing	170.6 ml per session	Stone *et al.*, (2008)
Swimming	16-37 ml per 45-minute swimming session*	Dufour *et al.*, (2006)
Wading/splashing	3.7 ml per session	Dorevitch *et al.*, (2011)

*Lower estimate provided was for people over the age of 18; higher estimate for people under the age of 18

Using calculations from Leonard *et al.*, (2015), estimates were made for how many norovirus and HEV virus particles are ingested during each water activity, the dose. This was calculated using the formula:

$$C \times Vi \times D \times V = Virus\ dose$$

Where C is the median or maximum copies/ml of virus in influent or effluent. Vi is a viability constant, $Vi = 0.5$, which was estimated for norovirus infectivity in a faecal inoculum previously by Teunis *et al.*, (2008a), and also applied to HEV (as it is also a non-enveloped single stranded RNA virus), though there is no information on viability estimates for HEV. D is the theoretical dilution factor, and V is the mean volume of water ingested during a specified water activity.

We used a range of dilutions to simulate different dilution factors, to account for distances from sewage outfall pipes to areas where recreational water activities commonly take place. These dilution factors are: 0.1, 0.01, 0.001, 0.0001, 0.00001. For norovirus we used the median and maximum concentrations in sewage influent and effluent obtained in chapter 4 to calculate the number of virus particles which may be ingested during each different water related activity, under the assumption of releasing the influent as raw sewage through CSOs into surface waters, as a

worst- case scenario. For HEV, only the maximum concentration was used for these calculations due to very low levels of HEV in effluent samples, and a median value of 0 copies/ml in influent due to generally very low HEV concentrations. This was done to present a worst-case scenario, where influent containing the highest HEV levels is released into the environment, e.g., through the use of CSOs. These calculations gave estimates of the dosage of virus ingested at each dilution during different water activities. These were then used to determine the probability of infection and illness. All calculations and graphs were made using R (R Core Team, 2018).

Determining number of virus particles ingested from consumption of raw oysters

For shellfish consumption, calculation of the dose required determining the virus copies in an average oyster. As quantification of the viruses was performed only on the digestive tissues of oysters, as other oyster tissues can cause PCR inhibition issues, a calculation was required to convert the virus copies/g of digestive tissues to the virus copies in an average oyster. This required multiple factors, including the virus copies/g of digestive tissues, the average weight of digestive tissues in a single oyster, a constant for estimated viability for the viruses, and the proportion of virus which is contained within the oyster digestive tissues. The dose was calculated using the median and maximum virus copies/g of digestive tissues (C). The average weight of digestive tissues in a single oyster (G) was obtained from a previous oyster study (Lowther, J. Personal Communication, 2022). G was determined to be 1.2 g. The digestive tissues contain approximately 98% of bioaccumulated norovirus within oysters (Mcleod *et al.*, 2009; Maalouf *et al.*, 2010). Therefore, we assumed that 98% of norovirus and HEV within our shellfish samples was contained within the oyster digestive tissues. We used this for both HEV and norovirus, assuming the same bioaccumulation pattern occurs for HEV as it does for norovirus within oysters, though there are no studies proving this to date. The viability constant (Vi) was determined using the infectivity ratios described in Lowther *et al.*, (2019) and provided by personal communication (Lowther, J. Personal Communication, 2022). The constant was determined by identifying the average ratio of PCR detectable GII F-specific bacteriophage to cultured GII phage from oyster digestive tissues, using the bacteriophage as a norovirus surrogate. Vi was determined to be 0.07 using this data (Lowther, J. Personal Communication,

2022). We again applied the same viability constant to HEV though there is no information on HEV viability within oysters.

$$\frac{C \times Vi \times G}{0.98} = Virus\ copies\ within\ an\ average\ oyster$$

We then identified common oyster meal sizes (number of oysters consumed in one sitting), according to Degner and Petrone, (1994) and Pouillot *et al.*, (2021), and calculated the median and maximum dosage of norovirus and HEV which might be expected from different oyster meal sizes. These meal sizes were 1, 6, 12, 18, and 24.

$$Virus\ copies\ within\ an\ average\ oyster \times Meal\ size = Virus\ dose\ in\ an\ oyster\ meal$$

These calculations gave estimates of the dosage of virus ingested during oyster consumption for different meal sizes. These were then used to determine the probability of infection and illness.

Determining the risk of illness

We used calculations from dose response studies published by Messner et al., (2014) and Vergara et al., (2016) to estimate the probability of norovirus infection and illness from the calculated norovirus doses. For HEV, we used the dose response models from Ruchusatsawat *et al.*, (2021) to estimate the probability of hepatitis. We did this for virus doses calculated from water activities and shellfish consumption.

The calculation of the probability of infection of norovirus was derived from a fractional-Poisson model previously published by Messner et al., (2014), with modifications based on the publications by Van Abel *et al.*, (2017) and Vergara et al.,
(2016). The calculation utilises the parameter P which is derived from the fractional-Poisson dose response model and represents the proportion of secretor positive fully susceptible individuals (those that secrete histo-blood group antigen structures into the gut, which norovirus utilises during infection). This parameter limits the maximum probability of infection to 72%, as it was calculated by Messner et al., (2014) that this was the percentage of individuals which were susceptible to norovirus within the

dose-response populations. The parameter μ is the calculated mean virus aggregate
size. Many risk assessment studies assume that viruses in recreational waters are disaggregated, however the best practice for predicting norovirus risk may be to provide calculations based on both aggregated and disaggregated doses (Van Abel *et al.*, 2017). For disaggregated doses, $\mu = 1$ and for aggregated doses, the conservative estimate of $\mu = 399$ was used (Vergara et al., 2016; Van Abel *et al.*, 2017). Aggregate size is estimated using a Poisson distribution, and an aggregate size of 399 was used as this was the lower 95% confidence interval for mean aggregate size from Messner et al., (2014). Messner et al., (2014) predicted that virus aggregates could have a mean size of 1106 virions, however Van Abel *et al.*, (2017) suggested that aggregates are not this large. The calculation for the probability of norovirus infection can be seen below, where P = 0.72, D is the dose of virus, and μ = 1 or 399, as defined by Vergara et al., (2016) and Messner et al., (2014).

$$P_{inf} = P\left(1 - e^{-\frac{D}{\mu}}\right)$$

To calculate the probability of norovirus illness (P_{ill}), we used a probability of illness given norovirus infection (P_{igi}) of 60%, which has been adopted elsewhere (Vergara et al., 2016; McBride *et al.*, 2013; Soller *et al.*, 2010; Viau, Lee and Boehm, 2011; Teunis *et al.*, 2008a). As the probability of infection is limited to 72%, this in turn, limits the probability of illness in an exposed individual to a maximum of 43.2%. The calculation can be seen below.

$$P_{ill} = P_{inf} \times P_{igi}$$

The calculation of the probability of developing symptomatic hepatitis from HEV was derived from a beta-Poisson dose-response model previously published by Ruchusatsawat *et al.*, (2021). The calculation utilises two parameters which define the model, α and N_{50} which is the dose at which 50% of an exposed population are expected to be infected. The calculation for the probability of hepatitis from HEV can be seen below, where D is the dose of virus, $N_{50} = 3.03 \times 10^7$, and $\alpha = 216.89$, as defined by Ruchusatsawat *et al.*, (2021). There is no known data on HEV aggregation yet.

$$P_{HEV} = 1 - \left(1 - \frac{D}{N_{50}}(2^{-\alpha} - 1)\right)^{-\alpha}$$

Calculating exposure events

The number of exposure events per year for each water activity were calculated using different possible scenarios. Where exposure events were calculated using effluent norovirus doses, this assumes a scenario where only effluent was being released into surface waters and that no influent was also released. Where exposure events were calculated using influent, this assumes a scenario where influent from combined sewer overflows was released into the environment, and that no effluent contributed to this release. Additionally, the norovirus doses were calculated separately, assuming they were either disaggregated or aggregated. The number of exposure events caused by norovirus in influent and in effluent, for aggregated or disaggregated doses, was calculated.

To calculate the number of water activity sessions which could cause illness annually, the number of water activity sessions per person in England and Wales was taken from EFTEC, (2002), as used previously by Leonard *et al.*, (2015). We utilised similar calculations to those of Leonard *et al.*, (2015) to identify the number of water activity sessions per year, and therefore the number of sessions resulting in illness, utilising population data for England and Wales from 2019 (Office for National Statistics, 2020). The risk data obtained from the 1,000-fold dilution of norovirus in sewage was used to calculate the number of water activity sessions likely to cause illness annually as this was the median dilution factor. The median dose illness risk data from the 1,000-fold dilutions was used because it was the mid-point of the dataset. The risk of illness for each water activity was multiplied by the number of sessions of each activity per year. The workflow of the calculations can be seen in Figure 7.1.

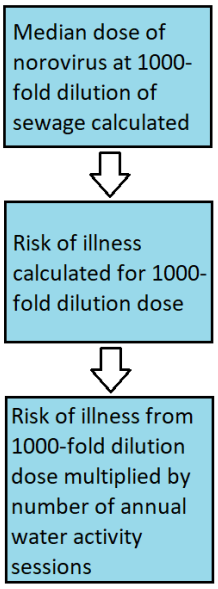


Figure 7.1 The process for calculating the number of water activities leading to illness

As HEV was calculated using the maximum concentration identified in sewage, and the median concentration was 0 copies/ml, population exposure estimates could not be made for HEV as they would potentially overestimate the risk.

To calculate the number of people who are at risk of norovirus illness from shellfish consumption in the UK annually, an estimate of the number of oyster meals per year was taken from (Williams and O'Brien, 2019), and this was multiplied by the population of the UK in 2019 (Office for National Statistics, 2020). Calculations were made treating the dose as consisting of either 100% aggregated or 100% disaggregated virus particles. The median dose data was used to calculate the number of people who may become ill from oyster meals per year as using the maximum dose data would lead to overestimates in risk (as not all shellfish contain the maximum norovirus levels). We used the risk of illness from an oyster meal size of 12 as this was the most common meal size in (Degner and Petrone, 1994; Pouillot *et al.*, 2021). We multiplied the risk of illness from 12 oysters by the number of oyster meals consumed per year to calculate the number of people who may become ill from oyster consumption per year. A workflow for the calculations can be seen in Figure 7.2.

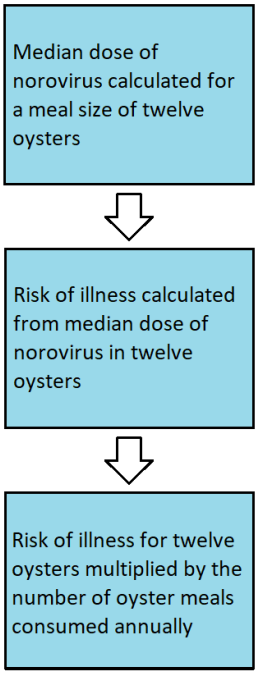


Figure 7.2 Workflow for calculating the number of oyster meals which may cause illness per year

Calculations for the number of oyster meals which may cause HEV illness per year were not made as the maximum doses were used to calculate the risk of illness from oyster meals, while the median concentration was 0 copies/g. Therefore, population exposure estimates could not be made for HEV as they may overestimate the population exposure to HEV. Additionally, the HEV shellfish data was from a study on archived shellfish samples, and so the quantification of HEV within the shellfish may not be reliable.

Results

Calculation of viral dose during recreational water activities

The number of ingested HEV and norovirus particles were calculated for each dilution, and for median and maximum HEV and norovirus concentrations in the sewage influent and effluent samples. The median and maximum norovirus and HEV concentrations are displayed in Table 7.2. As the median HEV was 0, this was not used for any further calculations.

Table 7.2 Median and maximum HEV and norovirus levels in influent and effluent sewage samples

Virus	Sewage type	Median copies/ml	Maximum copies/ml
Norovirus	Influent	250	9,034
	Effluent	18	2,634
HEV	Influent	0	113
	Effluent	0	5

All dose calculation results can be seen in supplementary data 7.1. The norovirus doses for the median copies/ml of norovirus in influent and effluent can be seen in Figure 7.3, whilst the doses for the maximum copies/ml of norovirus in influent and effluent can be seen in Figure 7.4. These graphs show the number of virus particles which are estimated to have been ingested during each recreational water activity, based on the dilution factors, which are in logarithmic form.

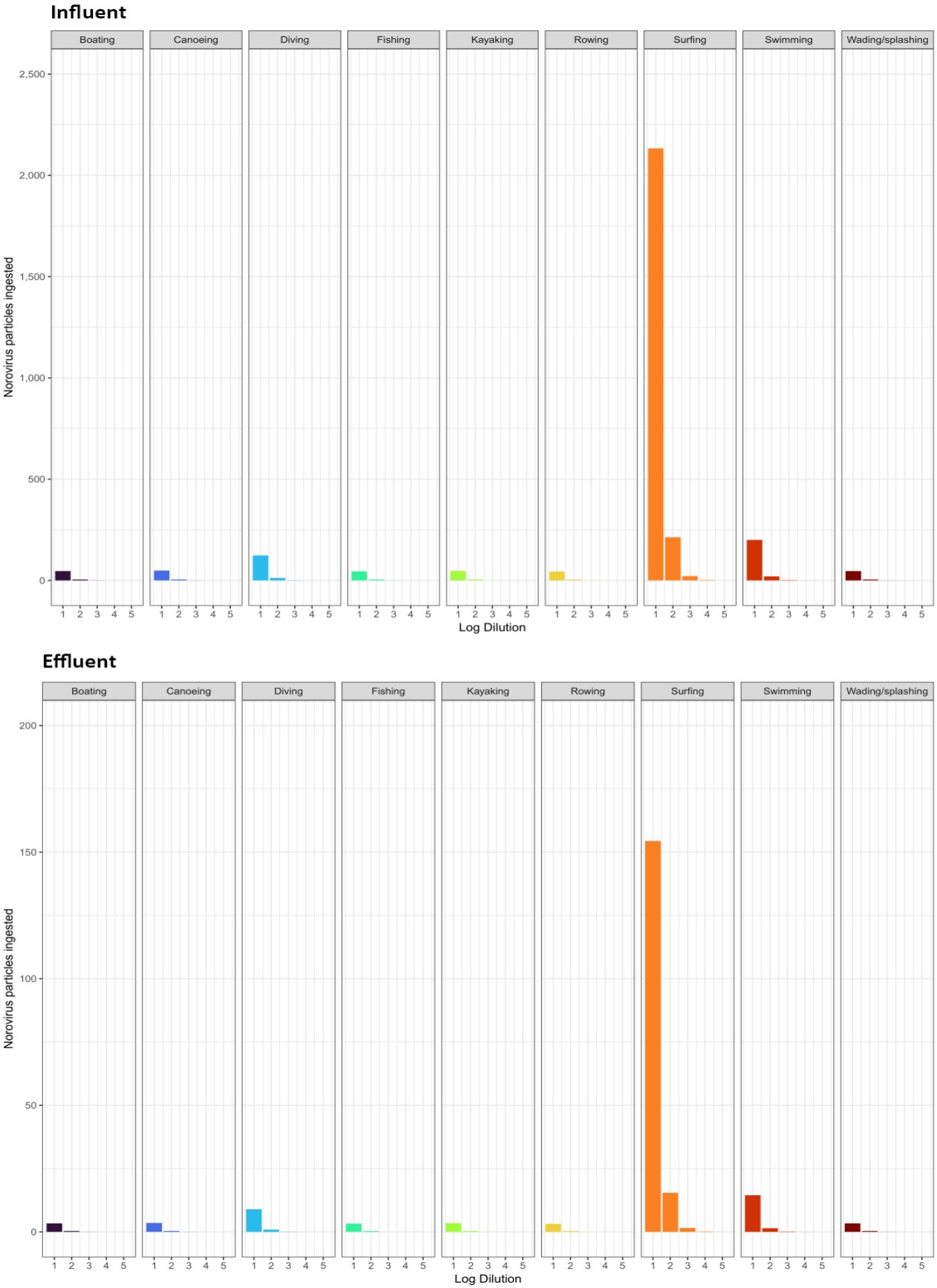


Figure 7.3 Norovirus particles ingested during each recreational water activity based on median levels

The median norovirus particles ingested during different recreational water activities, depending on the dilution of the influent or effluent. Calculations based on the median concentrations of norovirus observed within influent and effluent samples within chapter 4. The dilutions are from 1-5 which represents the log dilution factors: 0.1, 0.01, 0.001, 0.0001, 0.00001. For swimming, the ingestion estimate for adults was used. The axes differ from influent to effluent due to lower norovirus levels in effluent.

The highest doses of norovirus were observed for the 10-fold dilution of influent, where exposed individuals may have ingested between 1,581 and 77,059 norovirus particles depending on the water activity undertaken. At a 100-fold dilution, the range of particles ingested falls to between 158 and 7,706, with the highest levels seen for surfers. Exposure to a 1,000-fold dilution may have caused surfers to ingest 771 norovirus particles, and individuals conducting other water activities may have ingested between 16 and 72 particles. At a 10,000-fold dilution, the number of particles ingested ranged from 2 to 77, and at a 100,000-fold dilution swimming had 1 ingested particle whilst surfing had 8, all other water activities had negligible amounts. For individuals exposed to effluent, between 461 and 22,471 particles were ingested for all water activities, with the highest seen in surfers. For the 100-fold dilution, the number of particles ingested by exposed individuals ranged between 46 and 2,247. At the 1,000-fold dilution, the number of particles ingested reduces to between 4 and 21 for all activities except surfing, with 225 ingested particles. For a 10,000-fold dilution the range of ingested particles falls to between 1 and 22. At a 100,000-fold dilution, the number of particles ingested becomes negligible for all activities bar surfing, with 2 ingested particles.

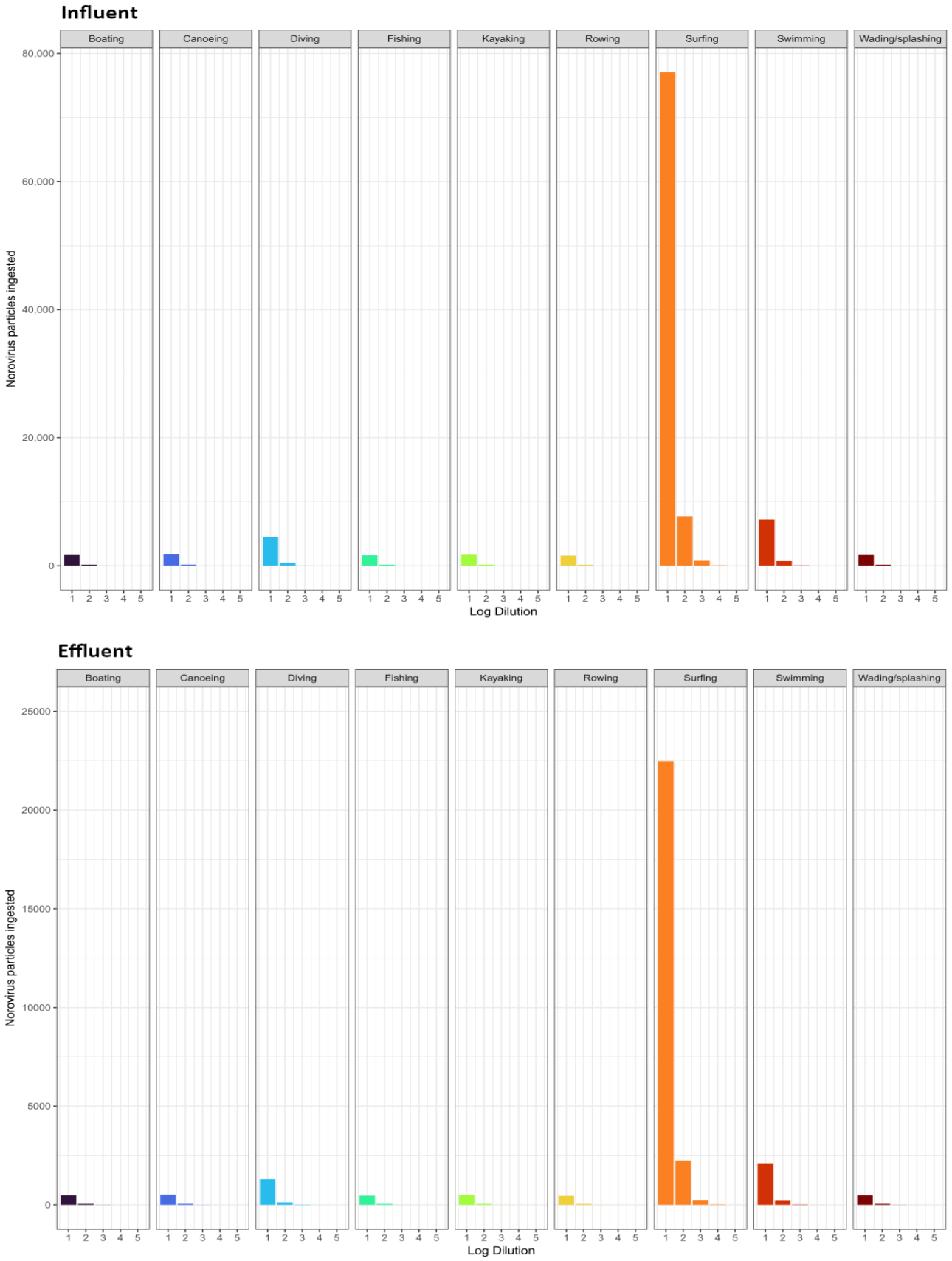


Figure 7.4 Norovirus particles ingested during each recreational water activity based on maximum levels

The maximum norovirus particles ingested during different recreational water activities, depending on the amount of dilution of the influent or effluent. Calculations based on the maximum concentrations of norovirus observed within influent and effluent samples within chapter 4. The log dilutions are from 1-5 which represents the dilution factors: 0.1, 0.01, 0.001, 0.0001, 0.00001. For swimming, the ingestion estimate for adults was used. The axes differ from influent to effluent due to lower norovirus concentrations in effluent.

The maximum HEV copies/ml identified in the influent samples was used to calculate the dosage of HEV from each water activity. Figure 7.5 shows the number of virus particles which are estimated to have been ingested during each water activity, for each dilution factor.

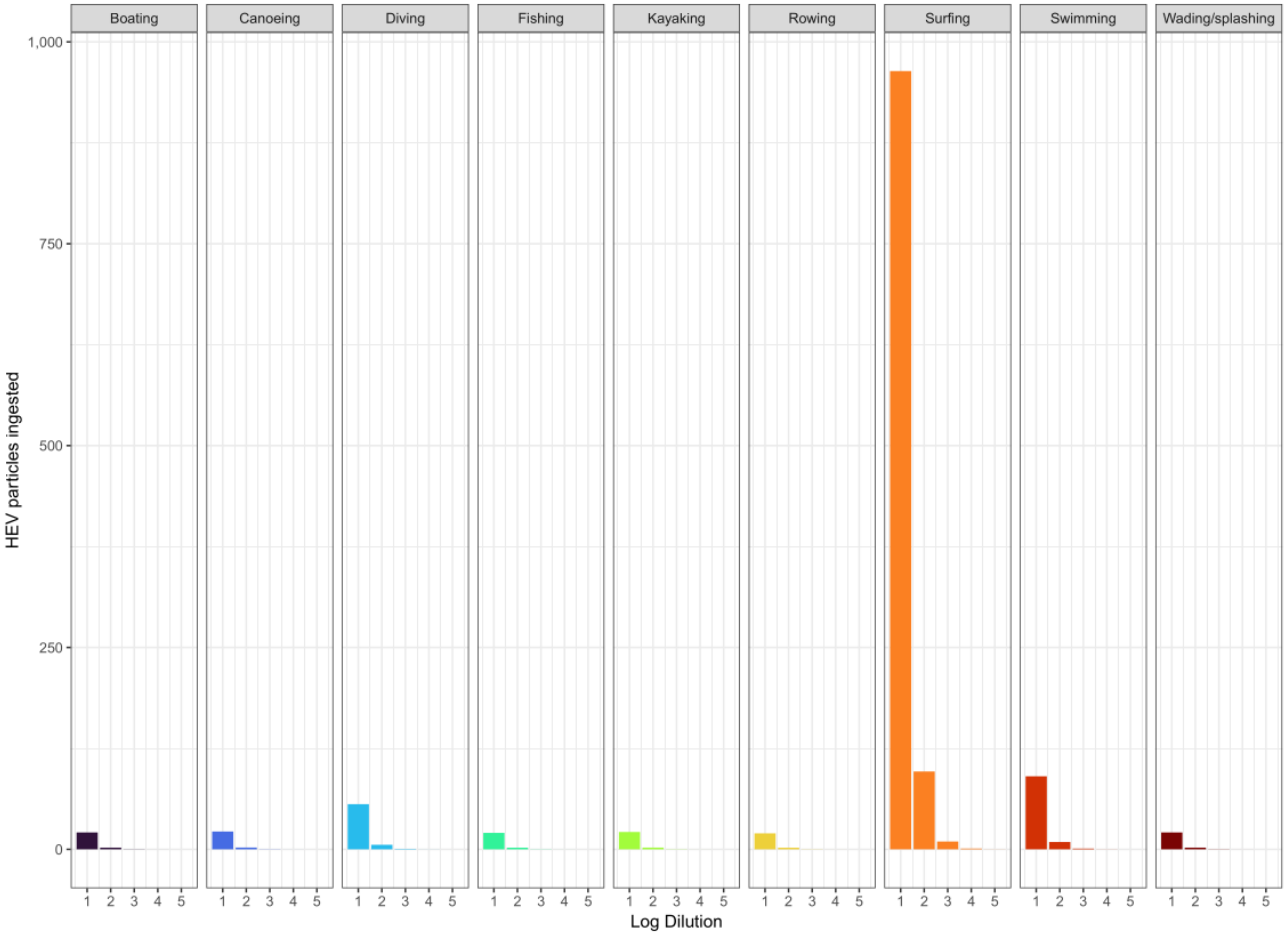


Figure 7.5 HEV particles ingested during each recreational water activity based on maximum levels

The maximum HEV particles which are ingested during each recreational water activity, for each dilution, based on the maximum HEV copies/ml of influent observed within chapter 4. Calculated using the above calculation. The log dilutions are from 1-5 which represents the dilution factors: 0.1, 0.01, 0.001, 0.0001, 0.00001. For swimming, the ingestion estimate for adults was used.

For a 10-fold dilution, the highest doses of HEV during exposure were observed, at a maximum of 964 ingested particles for surfing, and a minimum of 20 particles for rowing. At a 100-fold dilution, the dose ranged from 2 – 96 particles for all activities, and at a 1,000-fold dilution the number of particles ingested ranged from 0 – 10. At a 10,000-fold dilution the particles ingested were negligible for all activities except surfing, with 1 ingested particle.

Probability of norovirus illness

Utilising the calculations for the probability of infection from an aggregated or disaggregated dose of norovirus (seen in supplementary data 7.2), we calculated the probability of illness to exposed individuals (calculation results in supplementary data 7.3). Normally, norovirus within water is considered disaggregated, however whether norovirus is disaggregated in sewage is unknown. Therefore, we present the calculations for both the disaggregated and aggregated dose equations. The probability of illness from sewage containing the median disaggregated doses can be seen in Figure 7.6. The data shows that assuming exposure to the median norovirus doses from influent with a 10-fold dilution, there is a probability of illness of 43.2% for all activities (this is the maximum probability of illness for an exposed individual - see Methods). A 100-fold dilution of sewage fails to reduce this risk for surfing and reduces it only slightly for swimming and other water activities, with the range of illness risk between 42.7 and 43.2%. At a 1,000-fold dilution, the risk of illness stays the same for surfing, at 43.2%, but for the other water activities, the risk declines to between 15.3 and 37.3%. At a 10,000-fold dilution, the risk is between 1.9 and 7.8% for all water activities bar surfing, which still maintains a high risk of illness of 38.1%. At the 100,000-fold dilution, the risk reduces to between 0.19 and 8.3% for all water activities. For the median norovirus doses from effluent, a 10-fold dilution gives a risk range of 41.4 – 43.2% for all water activities. For a 100-fold dilution, the risk of illness does not reduce for surfing, staying at the maximum ceiling of 43.2%, but for other water activities the risk range is 11.7 – 33.0%. At the 1,000-fold dilution, the risk for surfing is at 34.0%, but the other water activities are between 1.3 and 5.8%. At the 10,000-fold dilution, the risk of illness is between 0.14 and 6.2% for all water activities, and for the 100,000-fold dilution the risk of illness is negligible for all activities except surfing, with a risk of 1.1%.

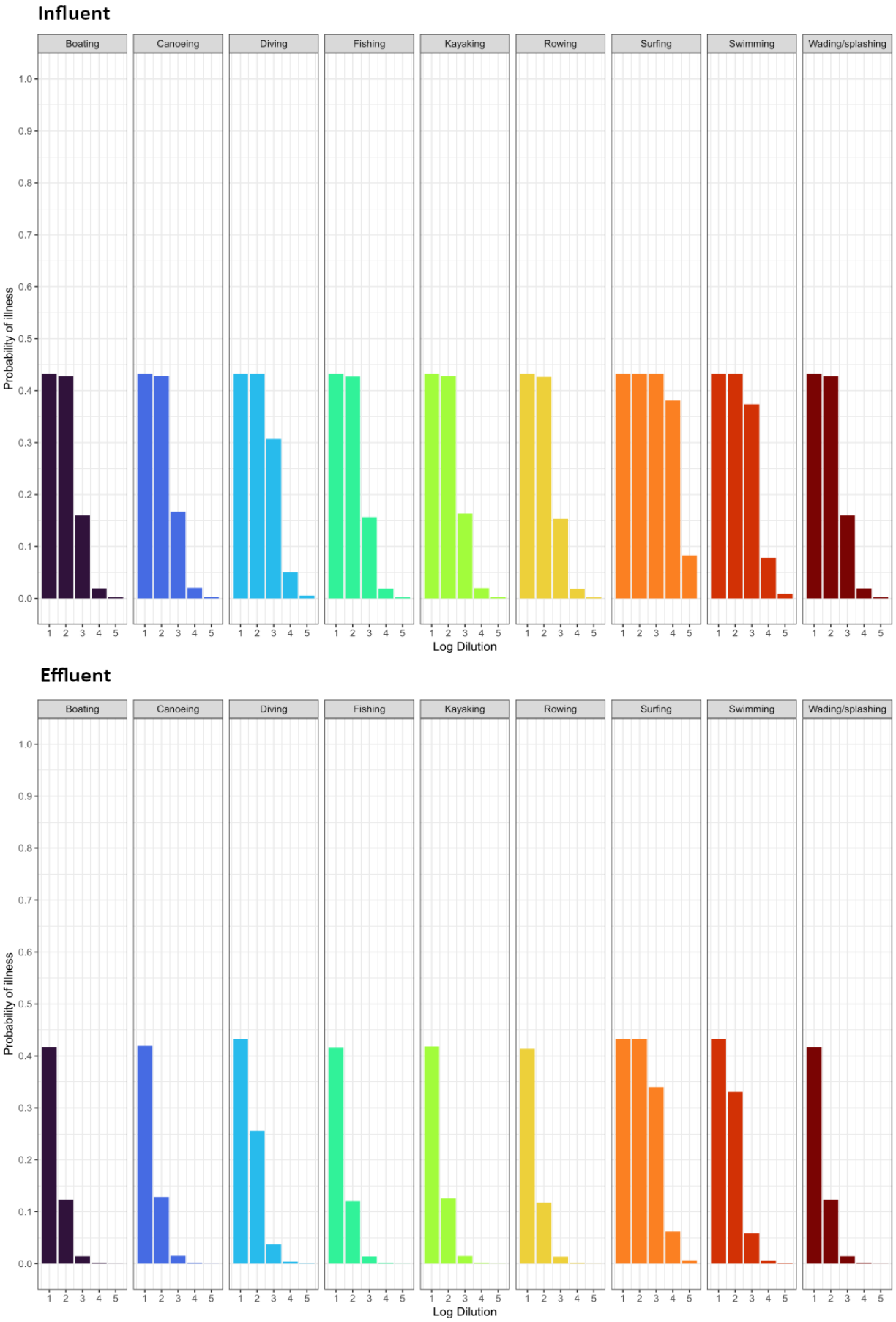


Figure 7.6 The probability of norovirus illness from median disaggregated doses

The probability of norovirus illness for an exposed individual from consumption of a median dose of disaggregated norovirus, dependent on the dilution of sewage in recreational water. The log dilutions are from 1-5 which represents the dilution factors: 0.1, 0.01, 0.001, 0.0001, 0.00001. For swimming, the ingestion estimate for adults was used.

The probability of illness from sewage containing the maximum disaggregated (Figure 7.7) doses of norovirus show that for influent doses, all activities for dilution factors 0.1, 0.01 and 0.001 have the highest risk of illness (at the maximum ceiling) of 43.2%. At the 10,000-fold dilution of influent, the risk ranges from 34.3 – 43.2% for all water activities, with the highest risk for surfing. At the 100,000-fold dilution, the risk still ranges from 6.3 – 43.1%, with the highest risk for surfing. For the maximum disaggregated doses of norovirus from effluent doses, the risk is again at the maximum ceiling of 43.2% for all water activities at the dilution factors 0.1 and 0.01. At a 1,000-fold dilution, the risk ranges from 42.8 – 43.2%, and at a 10,000-fold dilution, the risk ranges from 16.0 – 43.2%, with the highest risk for surfing. At a 100,000-fold dilution, excluding surfing, the risk ranges from 1.9 – 8.2%, but the risk for surfing is still 38.6%.

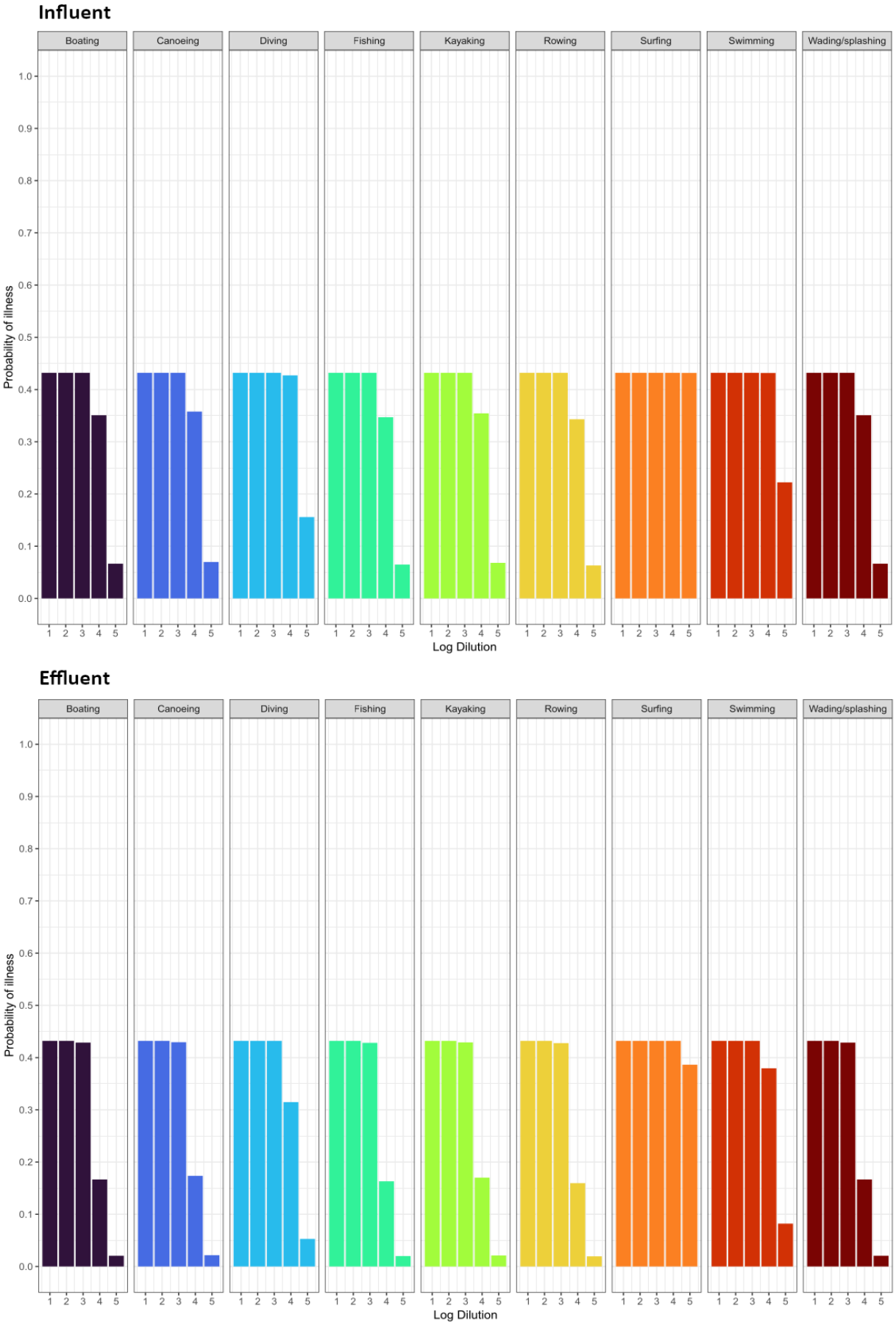


Figure 7.7 The probability of norovirus illness from maximum disaggregated doses

The probability of norovirus illness for an exposed individual from consumption of a maximum dose of disaggregated norovirus, dependent on the dilution of sewage in recreational water. The log dilutions are from 1-5 which represents the dilution factors: 0.1, 0.01, 0.001, 0.0001, 0.00001. For swimming, the ingestion estimate for adults was used.

Considering sewage/recreational water to give an aggregated dose of norovirus reduces the probability of infection during water activities. The probability of illness from sewage containing the median aggregated doses can be seen in Figure 7.8. For median doses of aggregated norovirus in influent, there is still a high risk of illness for surfers, swimmers, and divers (11.5 – 43.0%) within waters where the sewage is diluted 10-fold. At a 100-fold dilution, the risk ranges from 0.48 – 17.9%, with the highest risk in surfers. At a 1,000-fold dilution, the risk ranges from 0.05 – 2.2% for all activities. At the 10,000-fold dilution, the risk for all water activities is negligible, except for surfing which is 0.23%. All risks at the 100,000-fold dilution are negligible. For median doses of aggregated norovirus in effluent, assuming 10-fold dilution of the sewage, the risk for all water activities is between 0.34 and 13.9%. At a 100-fold dilution, the range of illness risk for all activities is 0.03 – 1.6%, and for the 1,000-fold dilution, all risks are negligible except for surfing, with a risk of 0.17%.

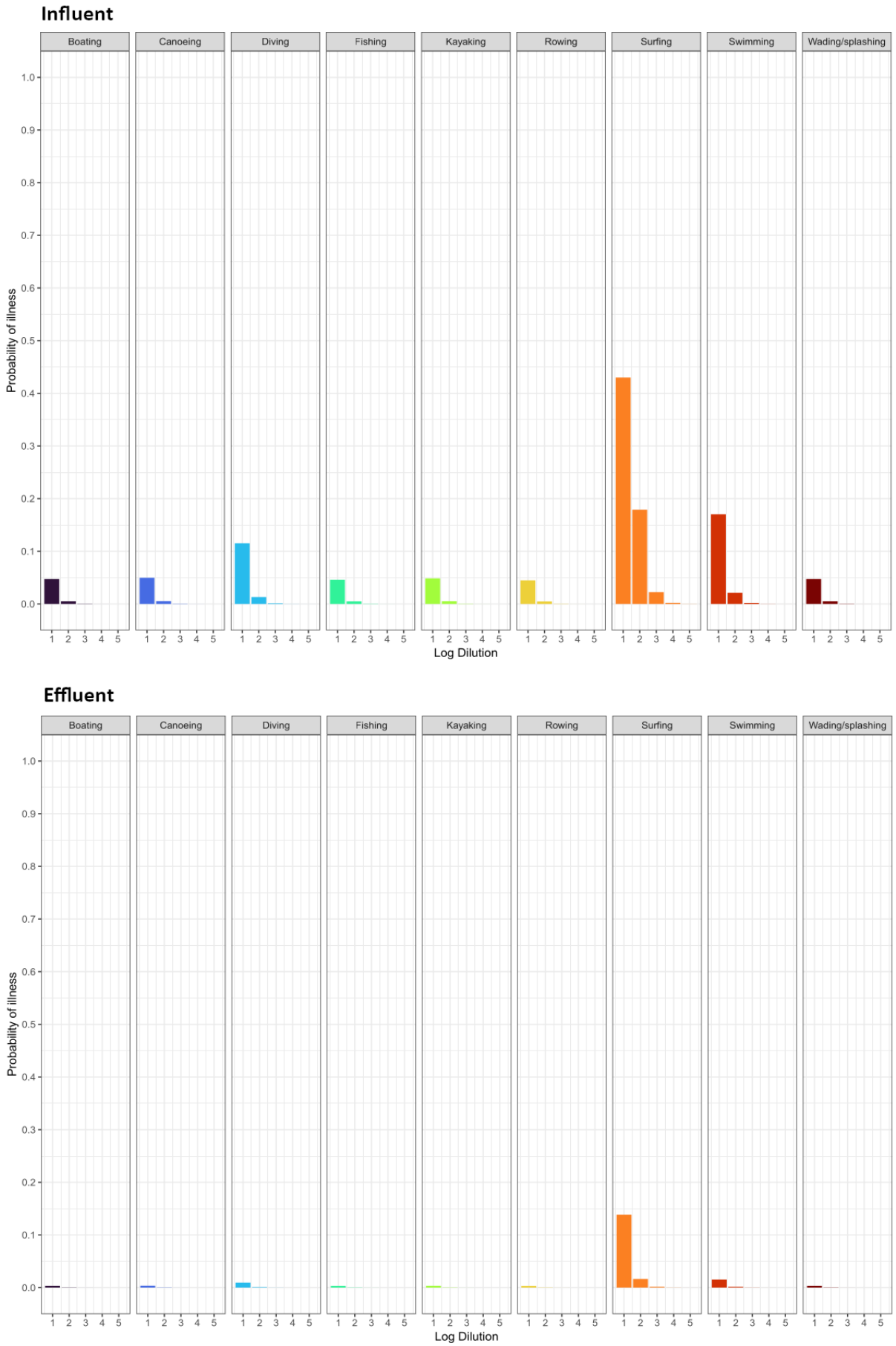


Figure 7.8 The probability of norovirus illness from median aggregated doses

The probability of norovirus illness for an exposed individual from median aggregated doses of norovirus from sewage, dependent on the dilution factor of the sewage in recreational waters. The log dilutions are from 1-5 which represents the dilution factors: 0.1, 0.01, 0.001, 0.0001, 0.00001. For swimming, the ingestion estimate for adults was used.

The probability of illness from sewage containing the maximum aggregated doses can be seen in Figure 7.9. For the maximum aggregated norovirus dose in influent, the risk of illness is at 42.5 – 43.2% (43.2% being the maximum ceiling) 10-fold diluted sewage for all water activities. The risk for 100-fold dilutions is still above 14.4% for all water activities, with the risk for surfing still at the maximum ceiling of 43.2%. At 1,000-fold dilutions the risk is above 1.7% for all, with the risk for surfing at 36.9%. At a 10,000-fold dilution, the risks decline to 0.17 – 7.6%. The risks at the 100,000-fold dilution are negligible. For effluent, the 10-fold dilution gives a risk of illness of 29.6 – 43.2% for all water activities, with the highest risk for surfing. At the 100-fold dilution, excluding surfing, the risk is between 4.7 and 17.7%, with the risk for surfing at 43.0%. At a 1,000-fold dilution, the risk ranges from 0.5 – 18.6%, depending on the water activity. The risk at a 10,000-fold dilution ranges from 0.05 – 2.4%. The risks at the 100,000-fold dilution are negligible.

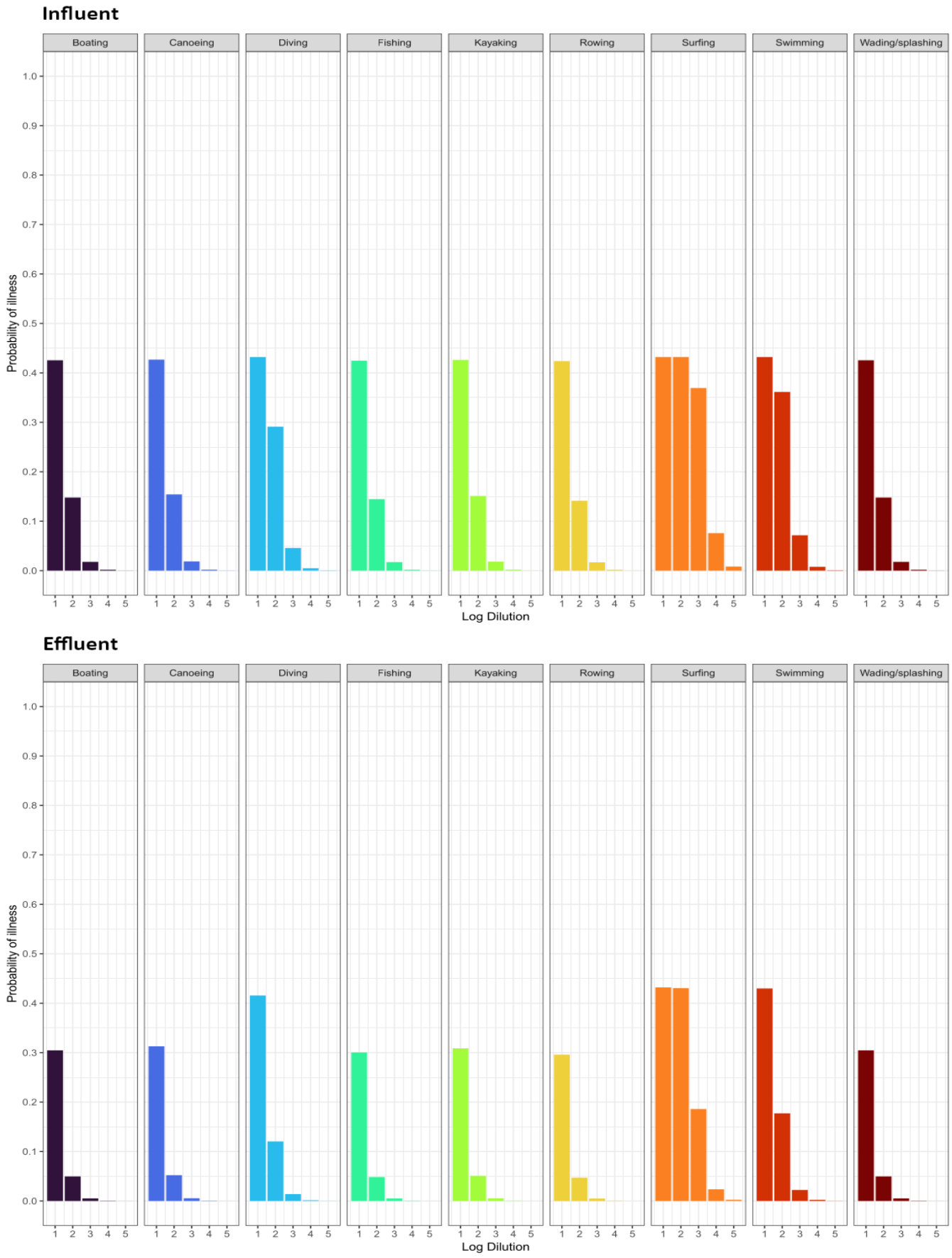


Figure 7.9 The probability of norovirus illness from maximum aggregated doses

The probability of norovirus illness for an exposed individual from maximum aggregated doses of norovirus from sewage, dependent on the dilution factor of the sewage in recreational waters. The log dilutions are from 1-5 which represents the dilution factors: 0.1, 0.01, 0.001, 0.0001, 0.00001. For swimming, the ingestion estimate for adults was used.

Probability of Hepatitis from HEV

The calculated probability of hepatitis from HEV from the maximum calculated HEV dosage in influent can be seen in Figure 7.10. The highest risk (to surfers with 1:10 dilution of sewage) is relatively low, compared with equivalent figures for norovirus, at 0.69%. The risk appears to be negligible for the other water activities and for higher dilution factors for surfing also.

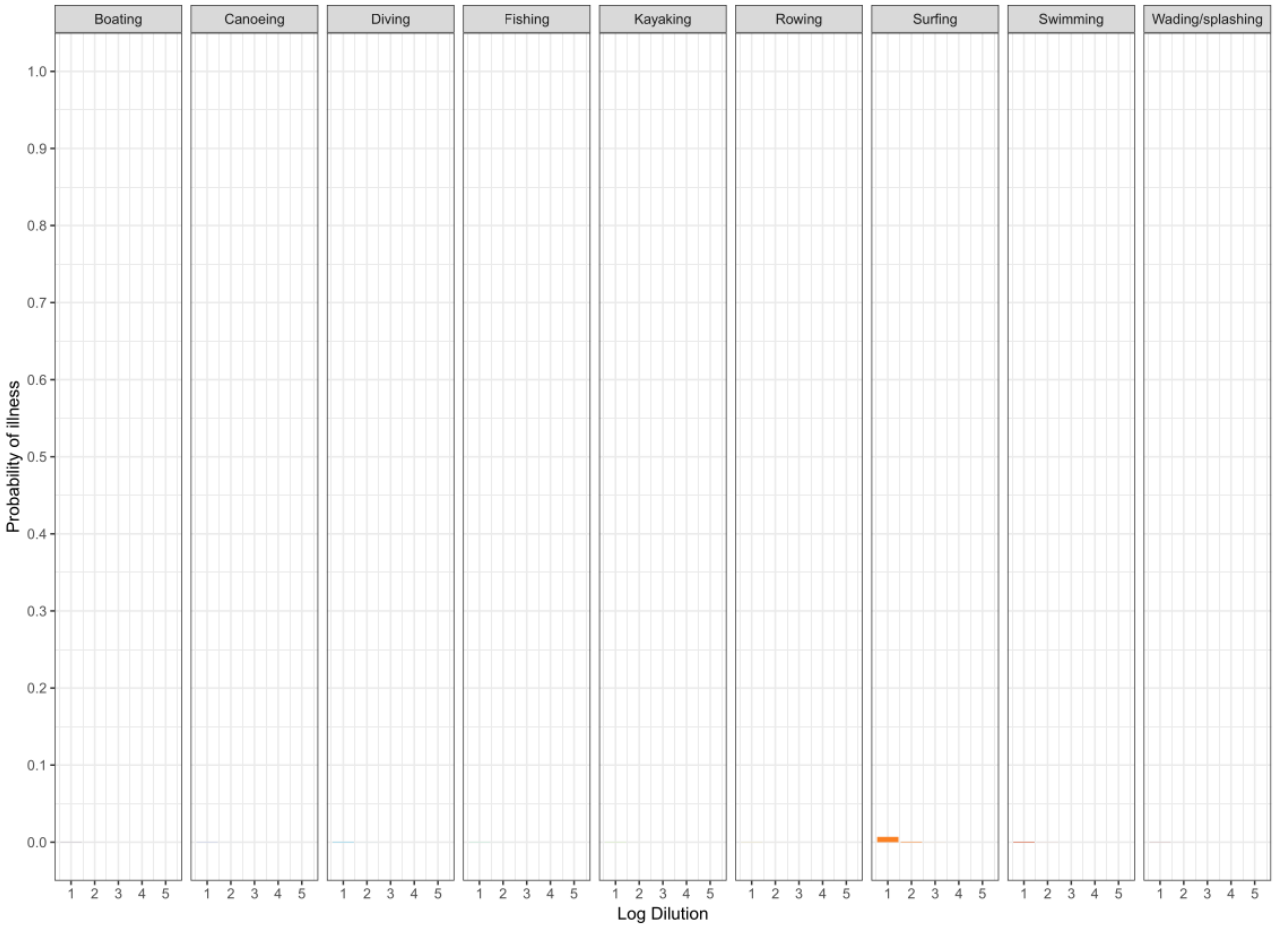


Figure 7.10 The calculated probability of hepatitis from HEV during different water activities based on maximum levels

The probability of hepatitis from HEV for an exposed individual based on the maximum HEV dosage received during different recreational water activities, depending on the dilution of the influent. The log dilutions are from 1-5 which represents the dilution factors: 0.1, 0.01, 0.001, 0.0001, 0.00001. For swimming, the ingestion estimate for adults was used.

Norovirus and HEV doses from oysters

The median concentration of norovirus in oyster digestive tissues was 106 copies/g and the maximum was 1,526 copies/g. These results were obtained from shellfish purchased from retailers in 2019. For HEV, five shellfish samples

from harvesting areas from the years 2009-2018 were determined to be positive. The median quantified HEV, considering all samples, was 0 copies/g of oyster digestive tissues and therefore was not used in further calculations, and the maximum was 121 copies/g of oyster digestive gland.

The doses of norovirus and HEV from oysters were calculated from the median and maximum virus copies/g of oyster digestive tissues and the number of oysters consumed (oyster meal size). This can be seen in Figure 7.11.

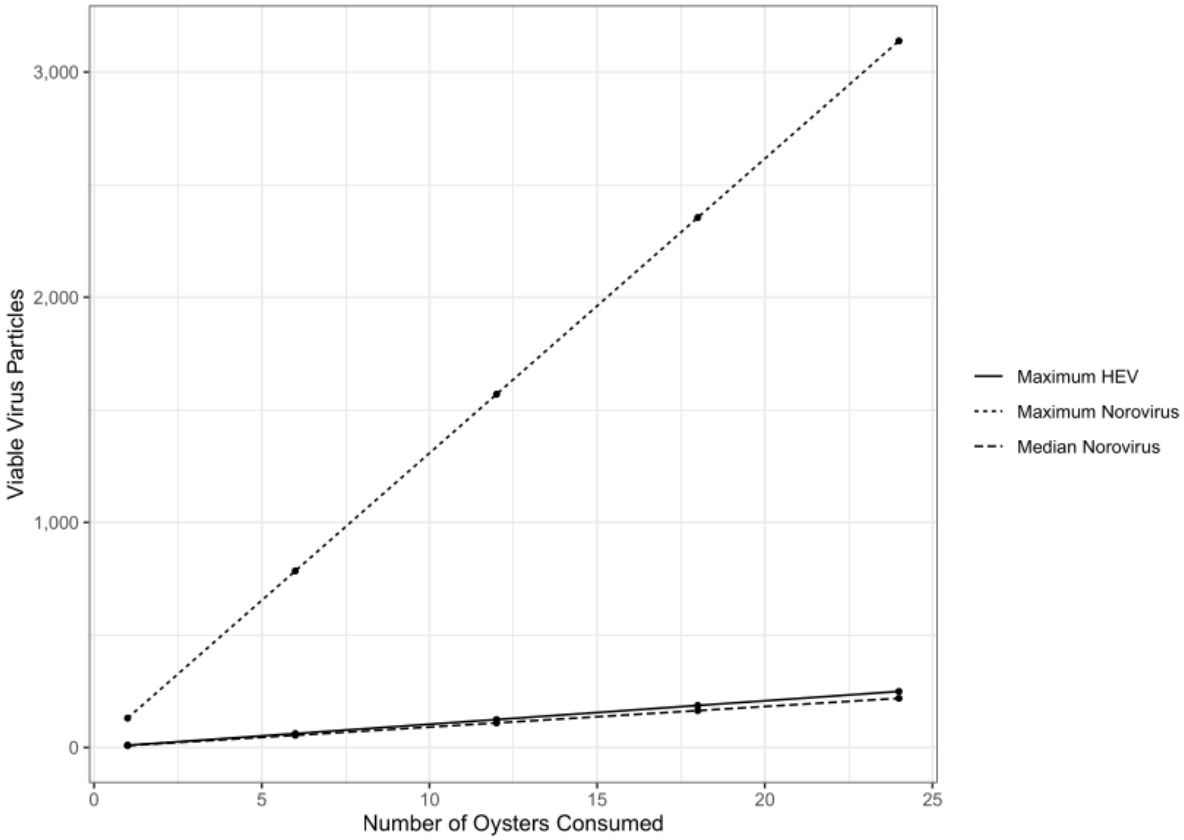


Figure 7.11 The norovirus and HEV doses from the number of oysters consumed (meal size)

The doses of norovirus and HEV calculated from the median or maximum copies/g of shellfish tissues and the number of oysters consumed during an oyster meal. Virus dose is in viable virus particles.

Probability of norovirus illness from oyster consumption

The probability of norovirus illness was calculated using the median and maximum norovirus doses for different meal sizes for shellfish sold at retail in

2019. We used the formulae for both a disaggregated dose of norovirus and an aggregated dose. For the median and maximum disaggregated doses, the risk of illness for all meal sizes was 43.2%.

For the aggregated median and maximum doses, the risk of illness was much lower than the disaggregated doses, which can be seen in Figure 7.12 For a meal size of one oyster, the maximum aggregated norovirus dose gave a probability of illness of 12.1%, whilst a meal size of 12 gave a risk of 42.4%. For the median aggregated norovirus dose, the risk from one oyster was 0.98%. For a meal size of 12, the risk was 10.4%. The probability of infection calculation results can be found in supplementary data 7.2).

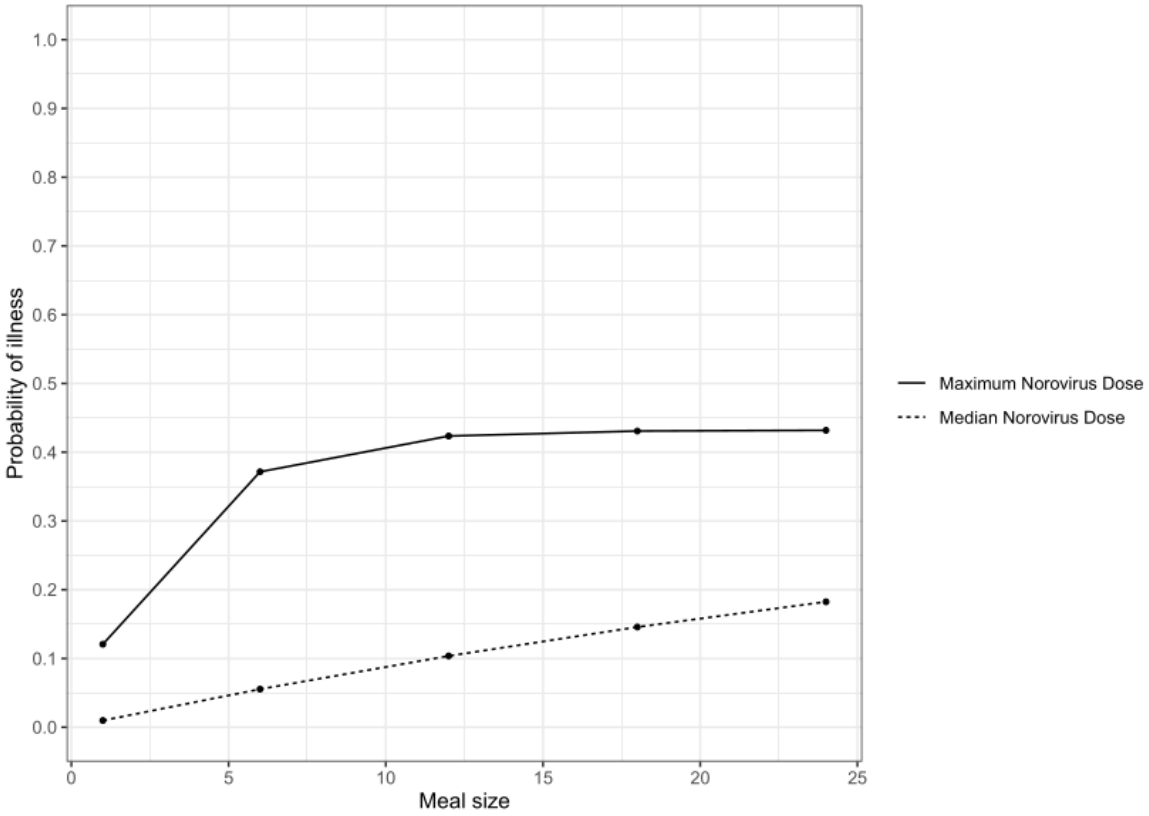


Figure 7.12 Probability of norovirus illness from infection with an aggregated dose through oyster consumption

The probability of norovirus illness from consuming oysters of the median and maximum aggregated doses, based on the number of oysters consumed during a meal.

Probability of illness from HEV in shellfish

The probability of illness from HEV during oyster meals of various sizes can be seen in Table 7.3. The risk of HEV was 7.44×10^{-3}% from consumption of a

single oyster and 0.089% for twelve oysters.

Table 7.3 Probability of illness from HEV infection with a maximum dose from oyster consumption

Meal size	HEV probability of illness (%)
1	7.44×10^{-3}
6	4.46×10^{-2}
12	8.92×10^{-2}
18	1.34×10^{-2}
24	1.78×10^{-3}

Calculation of exposure events for recreational water activities

The median aggregated and disaggregated dose data was used for calculation of exposure events in England and Wales for Boating, Canoeing, Diving, Surfing and Swimming. Exposure events could only be calculated for water activities which had data on the number of sessions per person per year. The risk of illness from the 1,000-fold dilution of sewage (using the median dose) was multiplied by the number of water activity sessions annually to calculate the number of water activities which cause norovirus illness annually.

Effluent release

Where dose data has been taken from effluent samples, a scenario where only effluent is released into surface waters is assumed, and no input of norovirus from combined sewage overflows contributes to the norovirus dose from surface waters. Table 7.4 shows the estimates for the number of water activities annually which could cause illness from a disaggregated or aggregated dose of norovirus from effluent. In total we calculated that 13,272,718 water activity sessions in England and Wales could lead to norovirus illness yearly when a median disaggregated dose of norovirus from effluent was involved (8.9% of annual water activities). We also found that 53,533 water activity sessions in England and Wales could lead to norovirus illness from a median aggregated dose of norovirus in effluent (0.036% of annual water activities).

Table 7.4 The number of water activity sessions which may result in norovirus illness annually, calculated using a median dose of norovirus from effluent

Activity	Sessions per person per year (EFTEC, 2002)	Sessions per year	Risk of illness from disaggregated effluent	Risk of illness from aggregated effluent	Sessions resulting in NoV illness from disaggregated effluent	Sessions resulting in NoV illness from aggregated effluent
Boating	0.5	29,719,920	$1.42x10^{-2}$	$3.60x10^{-5}$	422,796	1,077
Canoeing	0.2	11,887,968	$1.50x10^{-2}$	$3.80x10^{-5}$	178,100	454
Diving	0.2	11,887,968	$3.71x10^{-2}$	$9.70x10^{-5}$	440,114	1,153
Surfing	0.4	23,775,936	0.34	$1.67x10^{-3}$	8,077,889	39,668
Swimming	1.2	71,327,808	$5.82x10^{-2}$	$1.57x10^{-4}$	4,153,819	11,180
TOTAL	NA	148,599,600	NA	NA	13,272,718	53,533

Influent release

Where dose data has been taken from influent samples, a scenario where only influent is released into surface waters is assumed, using combined sewer overflows which release untreated sewage into surface waters. This scenario assumes that there is no input of norovirus from effluent contributing to the norovirus dose from surface waters. Table 7.5 shows the estimates for the number of water activities annually which could cause illness from an aggregated or disaggregated dose from influent. We found that 47,501,918 activities may have caused illness from median disaggregated influent doses (31.9% of annual water activities). Additionally, we calculated that a median aggregated dose of norovirus in influent could lead to 725,944 water activity sessions which cause illness annually (0.48% of annual water activities).

Table 7.5 The number of water activity sessions which may result in norovirus illness annually, calculated using a median dose of norovirus from influent

Activity	Sessions per person per year (EFTEC, 2002)	Sessions per year	Risk of illness from disaggregated influent	Risk of illness from aggregated influent	Sessions resulting in NoV illness from disaggregated influent	Sessions resulting in NoV illness from aggregated influent
Boating	0.5	29,719,920	0.17	$5.01x10^{-4}$	4,955,341	14,880
Canoeing	0.2	11,887,968	0.17	$5.28x10^{-4}$	1,982,136	6,273
Diving	0.2	11,887,968	0.31	$134x10^{-3}$	3,646,457	15,910
Surfing	0.4	23,775,936	0.43	$2.25x10^{-2}$	10,271,204	534,752
Swimming	1.2	71,327,808	0.37	$2.16x10^{-3}$	26,646,779	154,129
TOTAL	NA	148,599,600	NA	NA	47,501,918	725,944

Calculation of exposure events for shellfish consumption

To calculate the number of people who may become ill with norovirus from shellfish consumption annually, the median risk of norovirus illness was determined using the median oyster meal size of 12. The median risk of illness was multiplied by the calculated number of people who eat shellfish annually to give the number of shellfish meals which may cause illness from norovirus annually. The annual number of oyster meals consumed within the UK population in 2019 was calculated as 0.137 by Williams and O'Brien, (2019), so with a population of 66,796,807 in the UK in 2019, the number of annual shellfish meals was estimated to be 9,142,813. The risk of illness from shellfish consumption is based on two scenarios, a scenario where the norovirus dose is fully aggregated and a scenario where the norovirus dose is fully disaggregated. These affect the dose response calculations. In a scenario where the median norovirus dose was 100% disaggregated in all shellfish consumed, the number of shellfish meals which may cause illness from norovirus annually was calculated to be 3,949,695. For a scenario where the median norovirus dose was 100% aggregated in all shellfish consumed, the number of shellfish meals which may cause illness from norovirus annually was calculated to be 947,754.

Discussion

This study utilised previously published data from reviews and dose response models to assess the risk of infection and illness from the detected norovirus and HEV doses in shellfish and sewage samples collected for chapters 4 and 5.

The Risk of Virus Illness from Water Activities

We identified, using the study by Leonard *et al.*, (2015), the possible doses of norovirus and HEV ingested during different water activities. We did this considering a range of dilution factors of sewage in recreational waters, to simulate the risk to water users based on the proximity to sewage outfalls and the effects of factors such as tidal flushing. As the water ingestion volumes from Leonard *et al.*, (2015) for surfing, swimming, and diving were the highest, this led to high virus doses and risks for these activities, in agreement with Van Dijk et al., (1996) and Prüss, (1998). Using a fractional-Poisson model from Messner et al., (2014) for norovirus dose-response calculations, and a beta-Poisson model for HEV dose response (Ruchusatsawat *et al.*, 2021), we calculated the probability of infection and the probability of illness from exposure during water activities from our quantitative data. This has not been done in the UK before.

Norovirus

We determined that, for norovirus, the risk of infection and illness was very dependent on the size of virus aggregates, and that determining whether virus aggregation occurs in matrices such as water and shellfish is a much-needed step in the risk assessment process to gain the most accurate estimates. Norovirus is expected to be within a disaggregated state within water because the pH of environmental water is normally higher than the isoelectric point of norovirus (Van Abel *et al.*, 2017), however, whether the same can be said for sewage and shellfish matrices is unknown. The data which has been presented for norovirus doses and risks have been presented in the case of two scenarios, where the norovirus dose was 100% aggregated (and therefore less infectious (Van Abel *et al.*, 2017)); or where the norovirus dose is 100% disaggregated.

Many dose-response models have typically considered aggregation or disaggregation to be important factors within the model due to the higher risk of infection which disaggregation can cause. It could be assumed that a dose of norovirus from within a matrix such as sewage or shellfish may contain a mixture of aggregated and disaggregated virus particles, and therefore the real risk may lie somewhere between the estimates for the 100% aggregated and 100% disaggregated scenarios. When considering the data generated for these scenarios, it is difficult to compare the results to other studies due to the dilution factors used in this study. For this reason, we chose to compare the results of the median dilution factor (1,000-fold dilution) with other studies, as well as using this dilution for population exposure estimates. Realistically, each recreational water site will have a different ratio of sewage to water, and this ratio will vary daily due to factors such as tide and weather, and so using average dilution factors from specific bathing sites would be more accurate for the purposes of risk assessment, but unfortunately this was not possible.

Assuming a scenario where the dose of norovirus is 100% disaggregated and 1,000-fold diluted, people exposed to a median dose of effluent would have a 1.3 – 34.0% chance of illness depending on the water activity. A maximum dose of effluent would have a 42.8 – 43.2% chance of illness to exposed individuals, close to or at the maximum ceiling of possible risk of illness. Where waters are impacted by influent the risks are higher due to higher doses. Assuming the same scenario, people exposed to a median dose of influent would have a 15.3 – 43.2% risk of illness depending on the water activity, and a maximum dose would have a 43.2% risk of illness to exposed individuals for all water activities. These calculated risks are very high compared to other papers, but this may be attributed to the disaggregated status of the dose or to the use of the 1,000-fold dilution factor. A UK study by Kay *et al.*, (1994) identified that the likelihood of gastroenteritis was 14.8% in bathers compared to non-bathers (9.7%), and whilst the cause of gastroenteritis in these cases was not identified to be norovirus, the almost ubiquitous nature of norovirus in sewage (see chapter 4 and Campos *et al.*, (2016)) suggests that it may have been a contributing factor to the reported gastroenteritis cases. The identified risk of illness from a

disaggregated dose of influent or effluent is dependent on the water activity, but regardless the levels of risk identified in these calculations are much higher than the study by Kay *et al.*, (1994). Additionally, Vergara et al., (2016) identified that the risk from water activities which involved submersing the head had a risk of infection of just 0.61%, though this study used a Monte Carlo analysis to estimate the exposure to the population. The discrepancy could be because of a low level of population exposure, or possibly because the norovirus doses were more aggregated (and therefore have a lower risk of infection), or perhaps could be due to modelling assumptions, or factors which have not been considered (such as acquired immunity from exposure, which is not factored into the susceptibility calculations). However, an outbreak linked to a swimming event in the River Thames identified a 54% gastrointestinal illness rate (338 cases), showing that a high illness rate is possible, though at least five of these cases had been determined to be caused by *Giardia* and *Cryptosporidium* species (Hall *et al.*, 2017).

In comparison, if a scenario is assumed where the dose of norovirus is 100% aggregated and 1,000-fold diluted, individuals exposed to a median dose of effluent would have a 0.003 – 0.166% chance of illness depending on the water activity, and a maximum dose of effluent would give a 0.50 – 18.6% chance of illness. Assuming the same scenario, individuals exposed to a median dose of influent would have a 0.047 – 2.25% risk of illness, and those exposed to a maximum dose of influent would have a 1.68 – 36.9% chance of illness (depending on the water activity). These estimates are closer to those made by Vergara et al., (2016) and Kay *et al.*, (1994), which suggests that perhaps the use of the aggregated dose response model may be more accurate. However, again we do have to consider that the 1,000-fold dilution factor will not be representative of all recreational waters and therefore may not be appropriate for comparison to other studies.

Another issue with comparison of risk results to other papers is that these calculated risks are from scenarios where waters are contaminated only with effluent or only with influent. If waters are being spilled into by CSOs, effluent is likely also still being discharged. Therefore, the risk of illness from exposure in

the scenario where influent is impacting recreational waters may be an under-estimate as doses may be higher than calculated. However, we also have not considered that the viability of norovirus in effluent may be lower than that in influent if the sewage treatment practices can reduce the proportion of viable virus within sewage. Additionally, this study has calculated the risk of illness based on the assumption of exposure to a dose of norovirus and has not considered the probability of not being exposed to the norovirus dose. Though norovirus is almost ubiquitous within sewage, it may not be ubiquitous within recreational waters, and therefore the probability of exposure to a norovirus dose during a water activity may vary between sites used for recreational water activities. It is therefore easier to compare the results of population exposure estimates between studies instead of directly comparing the risk of illness, which is why subsequent calculations were made for calculating population exposure.

HEV

Using the maximum doses of HEV within sewage influent, we determined that the risk of hepatitis was nearly negligible for all water activities and dilutions except for surfing. For surfing, when receiving a 10-fold dilution of the maximum HEV dose in influent, the risk of illness was just 0.69%. All other water activities and dilutions gave negligible risks. This means that HEV release from influent into surface waters is unlikely to constitute a significant risk to water users. This is likely to have resulted from the large viral dose needed to cause infection compared to the virus copies in the influent doses. The virus copies needed to cause infection in 50% of individuals was estimated at 3.03×10^{7} virus copies during a dose response study (McCaustland *et al.*, 2000), whereas the maximum dose of HEV from influent was calculated as 964 viral copies for surfers. There is currently no other published data to compare these results to.

The Risk of Virus Illness from Oyster Consumption

Norovirus

Similar to the risk of illness calculated for the water activities, the estimates of

risk for norovirus illness from oyster consumption assumed either a scenario of 100% aggregated norovirus within the oysters, or 100% disaggregated norovirus within the oysters. Considering a scenario of exposure to a median or maximum disaggregated dose, the risk for all oyster meal sizes is 43.2%. These risk calculations are high compared to results from previous studies. Lowther *et al.*, (2010) observed an illness reporting rate from oyster meals of 0.21%, which is significantly lower. Additionally, Williams and O'Brien, (2019) identified a risk of illness per oyster meal of 0.68%, though the size of the meal used in the risk calculation is not clear. However, the report by Williams and O'Brien, (2019) suggests that norovirus is likely to be in an aggregated state within shellfish and therefore the risk estimates for the aggregated dose scenarios may be more realistic.

In a scenario of exposure to a median aggregated dose within the oysters, a single oyster would have a risk of 0.98%, whereas an oyster meal of twelve would have a risk of 10.4%. A meal size of twelve is the most common oyster meal size according to a Florida survey (Degner and Petrone, 1994). Considering this is a common portion size available to purchase in the UK, the data is likely to also be relevant to the UK population, though there is no UK data publicly available to support this assumption. In a scenario leading to exposure with a maximum aggregated dose of norovirus in the oysters, the risk from one oyster is 12.1%, and the risk from a meal size of twelve is 42.4%. The risk estimates from the median dose in a single oyster are much closer to those of Lowther *et al.*, (2010) and Williams and O'Brien, (2019), suggesting that the shellfish doses may indeed be aggregated within shellfish. The results mean that the chance of becoming ill from a norovirus-contaminated oyster are low, if the aggregated dose data is to be considered a more reliable estimate than the disaggregated data, though the risk rises quite significantly the more oysters are consumed in a meal.

HEV

The risks of contracting hepatitis from HEV in shellfish was even lower than the risk posed by norovirus contamination in shellfish (even when comparing to norovirus scenarios using an aggregated norovirus dose). Though no

assumptions were made about the state of aggregation of HEV (due to a lack of data) the risk of illness from a scenario where an individual consumed a single oyster containing the maximum dose of HEV was low, at 0.0074%. For a meal size of twelve oysters, the risk increased to just 0.089%. It is important to note that the shellfish samples used for the HEV data were kept in storage at -20°C for up to ten years, and therefore the detected number of virus genome copies within the samples may be lower than the original contamination levels, which may lead to an underestimation of risk. However, these shellfish were also collected from shellfish harvesting areas and had therefore not undergone any post-harvest processing, such as depuration and relaying. It is unknown whether these processing techniques could enable a reduction in HEV within the shellfish as depuration studies on HEV have currently not been performed, to our knowledge. However, in the case that HEV concentrations can be reduced in shellfish by depuration, this may mean that the risk of illness from oysters available to the consumer is lower than estimated here. The risks of HEV from water activities and shellfish consumption are considerably lower than for norovirus, and this is likely due to the much lower dose required for norovirus infection than HEV infection, combined with lower HEV concentrations in sewage due to lower HEV prevalence in the community. This may possibly explain why outbreaks of norovirus have been linked to shellfish consumption and water activities, whereas HEV transmission has not been.

Exposure Estimates for Norovirus

Water activities

In addition to the individual risks of illness from exposure to norovirus and HEV, we calculated the number of water activity sessions a year which may lead to norovirus illness from data on the number of water activity sessions a year and the risk data obtained from a 1,000-fold dilution of a median dose of sewage.

The data is again presented in scenarios, where the dose can be 100% disaggregated or 100% aggregated, and calculations were made from the median dose of influent or effluent. For a scenario where the dose is 100%

disaggregated, and 100% of the water activity sessions in a year cause exposure to this norovirus dose, the number of sessions resulting in norovirus annually from a median effluent dose would be 13,272,718, and the number of sessions resulting from a median influent dose would be 47,501,918. These estimates are much higher than the number of estimated norovirus cases which occur in the UK each year (3 million (Gherman *et al.*, 2020) and therefore seem like a vast overestimate. This may be due to the assumption of complete disaggregation; however, it is acknowledged that the number of water activity sessions being exposed to the median norovirus dose annually is unlikely to be as high as 100%. In a scenario where the norovirus dose is 100% aggregated (again assuming 100% exposure), the number of sessions resulting in norovirus annually from a median effluent dose would be 53,533 and the number resulting from a median dose of influent would be 725,944. Again, it should be acknowledged that it is unlikely that 100% of water activity sessions would cause exposure to this norovirus dose, and therefore if a probability of exposure were to be factored in, the number of sessions which cause illness annually would likely be lower. Data from (Economics for the Environment Consultancy Ltd, 2002) estimates that the number of gastrointestinal illnesses from water activities in 2002 was around 2.84 million, which is higher than the aggregated dose estimates but lower than the disaggregated dose estimates. The discrepancy may be due to several reasons, the first being that gastrointestinal illnesses can be caused by several pathogens within water (e.g., *Giardia*, astrovirus). Additionally, it may also be due to the scenarios presented. As mentioned previously, it is unlikely that when a CSO discharges influent into a water body, discharge of effluent stops. In fact, they are more likely to both be discharged simultaneously. This would therefore mean that the number of sessions which cause norovirus annually is likely to be between the values calculated for the influent and effluent scenarios, as you would never have risk caused purely by influent or purely by effluent in a yearlong period. In general, the aggregated dose estimates seem to be more reasonable than those for the disaggregated dose calculations.

Shellfish

The scenarios for a disaggregated and aggregated dose of norovirus from oyster consumption were used to calculate the number of shellfish meals which may cause illness annually. The illness risk data for an oyster meal size of 12 was used based on the data from (Degner and Petrone, 1994), and both dose scenarios assume that all annual oyster meals cause exposure to the median norovirus dose. For a scenario involving a disaggregated median dose, 3,949,695 meals would be capable of causing norovirus illness annually. For a scenario involving a median aggregated dose, 947,754 meals would be capable of causing norovirus illness annually. Similar to the previous results, the disaggregated dose risk estimates are very high, and are unlikely to be truly representative of the real risk. The aggregated dose data is also still high, and this may be attributed to the assumption that 100% of oyster meals consumed will cause ingestion of the same norovirus dose. It is important to note that norovirus is not ubiquitous within oysters, with Lowther *et al.*, (2018) identifying a 68.7% prevalence of norovirus RNA in oysters available to the UK consumer, and chapter 5 identifying a prevalence of 78.8% in oysters from retailers, this suggests that exposure, though high, is unlikely to be 100%. Additionally, the number of virus particles within an oyster is highly variable, and can range from zero to thousands, and the state of viability of these particles can only be predicted. The estimates from an aggregated dose are high in comparison to an estimate that norovirus would cause 14,000 infections per year from oyster consumption (Williams and O'Brien, 2019); and another estimate from Hassard *et al.*, (2017) which suggested 11,800 cases of norovirus were caused by shellfish consumption in the UK annually. To get a better idea of the true probability of illness from shellfish consumption and recreational water activities, a cohort survey study investigating the occurrence of illness between consumers of shellfish and people who undergo water activities, and the rest of the population may be useful to identify the discrepancy between the modelled risk and the population risk. This could then be used to estimate the effect that water activities and shellfish consumption have in causing norovirus outbreaks within the population.

Limitations

One limitation of this study is the calculation of the ingestion volumes for different water activities. The volumes of water ingested during different recreational water activities are likely to vary from person to person and also between age groups, for example, the data quoted by Leonard *et al.*, (2015) for swimmers suggested different ingestion volumes for children than for adults. Additionally, though some of the volume data was collected through objective means (such as measuring levels of cyanuric acid from swimming pools in swimmer's urine), other water activity volumes were calculated using surveys from water users, which is much more subjective. Another limitation to the dose calculations is that the virus dose has been calculated using qRT-PCR of sewage and shellfish samples, however the genome copies detected within the samples may not represent 100% of the virus in the sample due to sample processing procedures (such as wastewater concentration techniques and RNA extraction). Virus losses are difficult to quantify accurately, but this may mean that the virus doses calculated from the samples are slightly lower than the true viral concentration in the samples.

The dose-response models employed in this study also have some limitations, regarding the assumptions made to calculate certain parameters. For the norovirus fractional-Poisson model, the probability of infection is capped at 72% and the probability of illness at 43%. This is because the value of P is 0.72. P is the proportion of the population which are fully susceptible to norovirus and are "secretor positive", meaning that they secrete histo-blood group antigen (HGBA) complexes from the gut mucosa. These proteins help norovirus to infect patients, and therefore secretor-negative people are more resistant to norovirus infection. However, though norovirus is observed much more rarely in secretor negative people, outbreaks of norovirus from certain strains have been reported within secretor negative populations (Nordgren *et al.*, 2010), and therefore excluding them from the calculation of P may artificially limit the risk of infection and illness. Another factor which may not be considered within this susceptibility value (P) is the possibility of immunity due to previous exposure and infection, and the possibility of reinfection after antibodies have waned. This may

artificially inflate the P value, over-estimating the susceptible population. Additionally, most dose response models for norovirus have been based on the dose response generated by norovirus GI.1. However most large-scale outbreaks and pandemics of norovirus in the past decades have been caused by GII norovirus strains (particularly GII.4), and dose response models would likely benefit from norovirus data from different genogroups and genotypes (Messner et al., 2014).

For the HEV beta-Poisson model, some limitations are discussed by Teunis and Havelaar, (2000) on the use of the beta-Poisson model, with particular regard to the possibility of overinflation of risk at low doses. However, Teunis and Havelaar, (2000) focussed on beta-Poisson modelling of three different organisms, *Campylobacter jejuni*, *Vibrio cholerae* and rotavirus, all of which gave different dose-response curves and levels of uncertainty at lower doses. The results for rotavirus indicated very minor over-inflation of the maximum risk at the lowest doses, (10^{-2} – 10^{-1}), whereas the risk of illness for *Campylobacter* at most doses (10^{-3} – 10^{5}) was much more overinflated in comparison to the maximum risk. It appears that the model may be more appropriate for some organisms than others, however the dose-response curve modelled by Ruchusatsawat *et al.*, (2021) appeared to show no overinflation of risk at lower doses for HEV, and indeed the probability of illness identified for the low doses of HEV in this study are rare. Another limitation of this model is that the dose- response data used by Ruchusatsawat *et al.*, (2021) is very limited. The only dose response data available for use was that of McCaustland *et al.*, (2000), where chimpanzee dose-response was measured using HEV dose challenge. Only seven chimpanzees could be included within the study, and as a result, low dose data was not included. Because of the high dose needed to cause illness, as identified by this limited study, the N_{50} parameter for the model is high, at 3.03×10^{7}. However, oral dosage data from pigs suggests the N_{50} may be lower, (Casas *et al.*, 2009) and it is unknown whether chimpanzees (or pigs) would have the same N_{50} as humans. In addition, low dose-response data for the fractional-Poisson model by Messner et al., (2014) is lacking for

disaggregated norovirus, which may lead to inflation of the risks of infection and illness for lower doses of disaggregated norovirus.

Another limitation of this data is that dilution factors for the sewage in surface waters are uncertain. Sites of sewage release across the UK are likely to have a variety of dilution rates, and indeed this is true for England, which had a median dilution of 8 times but an average of 150 times across 170 sites in the study by Comber et al., (2020). It was not possible to ascertain the specific dilution factors for each release site and each sample within chapter 5, which is why a range of dilution factors was used within this study. A range of dilution factors was also used to simulate the norovirus concentrations which may reach recreational waters. Realistically, the dilution of sewage in recreational surface waters will be dependent on the distance from sewage discharge points and environmental factors such as tidal flushing. Additionally, it is important to note that the sewage samples were taken during the winter period between 2018 and 2019. Norovirus shows a pattern of seasonality, where human cases and levels of the virus in sewage are higher during colder months of the year (Campos *et al.*, 2016; Public Health England, 2021a). Whether there is a similar pattern of seasonality for HEV in the UK is unknown, but this means that the risk results of this study can only be applied to winter months, as the levels of norovirus in sewage during summer months is much lower, and therefore the exposure doses and risk of illness would be lower. For more specific and accurate risk assessment, dilution factors for specific sewage release sites should be used alongside norovirus levels in sewage and surface waters, and risk assessments should be conducted on a month-by-month basis, due to the changes in norovirus levels seen throughout the year, to better inform policies which reduce the risk of illness.

Finally, the models which have been discussed in this paper have utilised different viability factors depending on the sample matrix. The estimate used for the viability in wastewater originated from Teunis *et al.*, (2008a), which estimated that a single norovirus particle from a diluted inoculum had a 50% chance of causing illness. This was used as an estimate of viability for the wastewater samples, but realistically the matrix within the dose-response study

was unlikely to be representative of a wastewater sample diluted within environmental waters, and subject to factors such as temperature, pH and UV which may have affected virus viability within the environment. Additionally, a viability factor of 0.07 (7%) was used for norovirus in shellfish, based on the ratio of PCR detected and cultured surrogate bacteriophage (F-specific bacteriophage GII) levels detected in oyster samples. Whilst this is likely to be a better measure of norovirus viability than a capsid-integrity assay which may overestimate virus viability Williams and O'Brien, (2019), it is unknown whether the bacteriophage has the same susceptibility to environmental factors which may reduce viability as norovirus. Also, the viability ratio has been taken from the average ratio of PCR detectable to cultured bacteriophage, but the median (1.7%) and geometric mean (1.5%) of the viability data were much lower, and it is unclear which viability factor would be the most ideal to use due to the lack of knowledge on norovirus viability within shellfish. Additionally, we apply the same viability factors to HEV despite no known data on the viability of HEV. Norovirus has been cultured relatively recently within 3D intestinal organoid structures (Ettayebi *et al.*, 2016), and HEV is also culturable, however these culturing processes require high viral titres and specific strains and are likely to be inhibited by matrices such as environmental waters and sewage. Additionally, culturing facilities, resources and higher containment laboratories were not available for this project. Therefore, a level of uncertainty surrounds the viability factors used within the dose calculations, this is an ongoing issue within the virus research community.

Conclusion

In conclusion, this study is the first of its kind to apply dose-response models to quantitative virus levels from sewage from the UK to assess the risk of infection from these samples during recreational water activities. We also used dose-response models to estimate the risk of HEV and norovirus illness in shellfish. We identified that the risk of illness from recreational water activities and shellfish consumption was highly dependent on the dose type (aggregated or disaggregated) and that the risk of HEV illness from water activities and shellfish consumption was almost negligible, but that the risk from norovirus

was much higher, likely due to the higher prevalence of norovirus within the community in comparison to HEV. When considering exposure to influent, the risks are much higher for all water users due to higher doses, and this is significant because when combined sewer overflows (CSOs) discharge, they release raw sewage into surface waters which can impact recreational waters. Considering that combined sewer overflows were used for over 3 million hours collectively in 2020 (Environment Agency, 2020a), it would be a fair assumption that surface waters in the UK are consistently becoming contaminated with faecal pollution, and this may cause significant risk to recreational water users, and it is known to lead to contamination of shellfish harvesting areas. However, with a lack of virus monitoring within surface waters, and bathing water quality being assessed using faecal indicator bacteria (Wyer *et al.*, 2018), which correlate poorly with virus concentration (Kitajima *et al.*, 2014; Gibson *et al.*, 2011), the extent of the risks to human health is yet unknown. Quantitative risk assessment of viruses within recreational waters and shellfish still requires work to identify the best practices, such as the best model type, the level of virus aggregation and viability. However, given more information on these variables, this could become a promising technique for identifying the risk of illness from norovirus for oyster consumers and recreational water users, and in turn could encourage stricter policies on the release of raw sewage into surface waters in the UK.

References

Alfano-Sobsey, E., Sweat, D., Hall, A., Breedlove, F., Rodriguez, R., Greene, S., Pierce, A., Sobsey, M., Davies, M. and Ledford, S. L. (2012) 'Norovirus outbreak associated with undercooked oysters and secondary household transmission', *Epidemiol Infect,* 140(2), pp. 276-82.

Arnold, B. F., Schiff, K. C., Griffith, J. F., Gruber, J. S., Yau, V., Wright, C. C., Wade, T. J., Burns, S., Hayes, J. M., McGee, C., Gold, M., Cao, Y., Weisberg, S. B. and Colford, J. M. J. (2013) 'Swimmer Illness Associated with Marine Water Exposure and Water Quality Indicators: Impact of Widely Used Assumptions', *Epidemiology,* 24(6), pp. 845-853.

Bernard, H., Faber, M., Wilking , H., Haller, S., Höhle, M., Schielke, A., Ducomble, T., Siffczyk, C., Merbecks, S. S., Fricke, G., Hamouda, O., Stark, K., Werber, D. and Team, o. b. o. t. O. I. (2014) 'Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012', *Eurosurveillance,* 19(8), pp. 20719.

Bortagaray, V., Girardi, V., Pou, S., Lizasoain, A., Tort, L. F. L., Spilki, F. R., Colina, R. and Victoria, M. (2020) 'Detection, Quantification, and Microbial Risk Assessment of Group A Rotavirus in Rivers from Uruguay', *Food Environ Virol,* 12(2), pp. 89-98.

Bouwknegt, M., Rutjes, S. A., Reusken, C. B., Stockhofe-Zurwieden, N., Frankena, K., de Jong, M. C., de Roda Husman, A. M. and van der Poel, W. H. (2009) 'The course of hepatitis E virus infection in pigs after contact-infection and intravenous inoculation', BMC veterinary research, 5(1), pp. 7.

Campos, C. J., Avant, J., Lowther, J., Till, D. and Lees, D. N. (2016) 'Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes', *Water research,* 103, pp. 224-232.

Casas, M., Pina, S., de Deus, N., Peralta, B., Martín, M. and Segalés, J. (2009) 'Pigs orally inoculated with swine hepatitis E virus are able to infect contact sentinels', *Vet Microbiol,* 138(1-2), pp. 78-84.

Choi, C. and Kingsley, D. H. (2016) 'Temperature-dependent persistence of human norovirus within oysters (Crassostrea virginica)', *Food and environmental virology,* 8(2), pp. 141-147.

Comber, S. D. W., Gardner, M. J. and Ellor, B. (2020) 'Seasonal variation of contaminant concentrations in wastewater treatment works effluents and river waters', *Environmental Technology,* 41(21), pp. 2716-2730.

Corbett, S. J., Rubin, G. L., Curry, G. K. and Kleinbaum, D. G. (1993) 'The health effects of swimming at Sydney beaches. The Sydney Beach Users Study Advisory Group', *Am J Public Health,* 83(12), pp. 1701-6.

Crossan, C., Baker, P. J., Craft, J., Takeuchi, Y., Dalton, H. R. and Scobie, L. (2012) 'Hepatitis E virus genotype 3 in shellfish, United Kingdom', *Emerging infectious diseases,* 18(12), pp. 2085.

Degner, R. L. and Petrone, C. (1994) 'Consumer and restaurant manager reaction to depurated oysters and clams', *FAMRC industry report/(USA)*.

Di Bartolo, I., Pavoni, E., Tofani, S., Consoli, M., Galuppini, E., Losio, M. N., Ruggeri, F. M. and Varisco, G. (2015) 'Waterborne norovirus outbreak during a summer excursion in Northern Italy', New Microbiol, 38(1), pp. 109-12.

Dorevitch, S., Panthi, S., Huang, Y., Li, H., Michalek, A. M., Pratap, P., Wroblewski, M., Liu, L., Scheff, P. A. and Li, A. (2011) 'Water ingestion during water recreation', Water research, 45(5), pp. 2020-2028.

Dufour, A. P., Evans, O., Behymer, T. D. and Cantu, R. (2006) 'Water ingestion during swimming activities in a pool: A pilot study', Journal of Water and Health, 4(4), pp. 425-430.

EFTEC (2002) 'Valuation of benefits to England and Wales of a revised bathing water quality directive and other beach characteristics using the choice experiment methodology', *Final Rep.*

Environment Agency. (2020) 'Event Duration Monitoring - Storm Overflows - 2020' [Database of CSO use throughout UK in 2020]. Available at: https://environment.data.gov.uk/dataset/21e15f12-0df8-4bfc-b763-45226c16a8ac (Accessed 23 Jun 2021).

Ettayebi, K., Crawford, S. E., Murakami, K., Broughman, J. R., Karandikar, U., Tenge, V. R., Neill, F. H., Blutt, S. E., Zeng, X. L., Qu, L., Kou, B., Opekun, A. R., Burrin, D., Graham, D. Y., Ramani, S., Atmar, R. L. and Estes, M. K. (2016) 'Replication of human noroviruses in stem cell-derived human enteroids', *Science,* 353(6306), pp. 1387-1393.

Gao, S., Li, D., Zha, E., Zhou, T., Wang, S. and Yue, X. (2015) 'Surveillance of hepatitis E virus contamination in shellfish in China', *International journal of environmental research and public health,* 12(2), pp. 2026-2036.

Gherman, I., Holland, D., Clifford, R., Mahmoudzadeh, N., Uy, F., Adkin, A., Kainth, B., Cook, P., Day, A. and Wilson, A. (2020) 'Technical Report: Review of Quantitative Risk Assessment of foodborne norovirus transmission', *Science,* 1, pp. 1.

Gibson, K. E., Opryszko, M. C., Schissler, J. T., Guo, Y. and Schwab, K. J. (2011) 'Evaluation of human enteric viruses in surface water and drinking water resources in southern Ghana', *The American journal of tropical medicine and hygiene,* 84(1), pp. 20-29.

Graciaa, D. S., Cope, J. R., Roberts, V. A., Cikesh, B. L., Kahler, A. M., Vigar, M., Hilborn, E. D., Wade, T. J., Backer, L. C., Montgomery, S. P., Secor, W. E., Hill, V. R., Beach, M. J., Fullerton, K. E., Yoder, J. S. and Hlavsa, M. C. (2018) 'Outbreaks Associated with Untreated Recreational Water - United States, 2000-2014', MMWR Morb Mortal Wkly Rep, 67(25), pp. 701-706.

Grierson, S., Heaney, J., Cheney, T., Morgan, D., Wyllie, S., Powell, L., Smith, D., Ijaz, S., Steinbach, F. and Choudhury, B. (2015) 'Prevalence of hepatitis E virus infection in pigs at the time of slaughter, United Kingdom, 2013', Emerging infectious diseases, 21(8), pp. 1396.

Guillois, Y., Abravanel, F., Miura, T., Pavio, N., Vaillant, V., Lhomme, S., Le Guyader, F. S., Rose, N., Le Saux, J.-C. and King, L. A. (2015) 'High proportion of asymptomatic infections in an outbreak of hepatitis E associated with a spit-

roasted piglet, France, 2013', *Clinical Infectious Diseases,* 62(3), pp. 351-357.

Haas, C. N., Rose, J. B. and Gerba, C. P. (2014) *Quantitative microbial risk assessment.* John Wiley & Sons.

Hall, V., Taye, A., Walsh, B., Maguire, H., Dave, J., Wright, A., Anderson, C. and Crook, P. (2017) 'A large outbreak of gastrointestinal illness at an open-water swimming event in the River Thames, London', *Epidemiology and Infection,* 145(6), pp. 1246-1255.

Halbur, P., Kasorndorkbua, C., Gilbert, C., Guenette, D., Potters, M., Purcell, R., Emerson, S., Toth, T. and Meng, X. (2001) 'Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human', Journal of clinical microbiology, 39(3), pp. 918-923.

Hassard, F., Sharp, J. H., Taft, H., LeVay, L., Harris, J. P., McDonald, J. E., Tuson, K., Wilson, J., Jones, D. L. and Malham, S. K. (2017) 'Critical review on the public health impact of norovirus contamination in shellfish and the environment: a UK perspective', *Food and environmental virology,* 9(2), pp. 123-141.

He, X. and Huang, K. (2020) 'Chapter 7 - Assessment technologies for hazards/risks of wastewater', in Ren, H. and Zhang, X. (eds.) *High-Risk Pollutants in Wastewater*: Elsevier, pp. 141-167.

Holland, D. and Mahmoudzadeh, N. (2020) *Foodborne Disease Estimates for the United Kingdom in 2018*.

Kauppinen, A., Al-Hello, H., Zacheus, O., Kilponen, J., Maunula, L., Huusko, S., Lappalainen, M., Miettinen, I., Blomqvist, S. and Rimhanen-Finne, R. (2017) 'Increase in outbreaks of gastroenteritis linked to bathing water in Finland in summer 2014', *Eurosurveillance,* 22(8), pp. 30470.

Kay, D., Jones, F., Wyer, M. D., Fleisher, J. M., Salmon, R. L., Godfree, A. F., Zelenauch-Jacquotte, A. and Shore, R. (1994) 'Predicting likelihood of gastroenteritis from sea bathing: results from randomised exposure', *The Lancet,* 344(8927), pp. 905-909.

Kitajima, M., Iker, B. C., Pepper, I. L. and Gerba, C. P. (2014) 'Relative abundance and treatment reduction of viruses during wastewater treatment processes — Identification of potential viral indicators', *Science of The Total Environment,* 488-489, pp. 290-296.

La Rosa, G., Proroga, Y., De Medici, D., Capuano, F., Iaconelli, M., Della Libera, S. and Suffredini, E. (2018) 'First detection of hepatitis E virus in shellfish and in seawater from production areas in Southern Italy', *Food and environmental virology,* 10(1), pp. 127-131.

Ledesma, J., Williams, D., Stanford, F. A., Hewitt, P. E., Zuckerman, M., Bansal, S., Dhawan, A., Mbisa, J. L., Tedder, R. and Ijaz, S. (2019) 'Resolution by deep sequencing of a dual hepatitis E virus infection transmitted via blood components', Journal of General Virology, 100(11), pp. 1491-1500.

Leonard, A. F., Zhang, L., Balfour, A. J., Garside, R. and Gaze, W. H. (2015) 'Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters', *Environment international,* 82, pp. 92-100.

Leonard, A. F. C., Singer, A., Ukoumunne, O. C., Gaze, W. H. and Garside, R. (2018) 'Is it safe to go back into the water? A systematic review and meta-analysis of the risk of acquiring infections from recreational exposure to seawater', *International journal of epidemiology,* 47(2), pp. 572-586.

Li, T.-C., Miyamura, T. and Takeda, N. (2007) 'Detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (Corbicula japonica) in Japan', *The American journal of tropical medicine and hygiene,* 76(1), pp. 170-172.

Li, T.-C., Yang, T., Shiota, T., Yoshizaki, S., Yoshida, H., Saito, M., Imagawa, T., Malbas, F. F., Lupisan, S. P. and Oshitani, H. (2014) 'Molecular detection of hepatitis E virus in rivers in the Philippines', *The American journal of tropical medicine and hygiene,* 90(4), pp. 764-766.

Lodder, W. J. and de Roda Husman, A. M. (2005) 'Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands', *Applied and environmental microbiology,* 71(3), pp. 1453-1461.

Lowther, J., Gustar, N., Powell, A., O'Brien, S. and Lees, D. (2018) 'A one-year survey of norovirus in UK oysters collected at the point of sale', *Food and environmental virology,* 10(3), pp. 278-287.

Lowther, J. A., Avant, J. M., Gizynski, K., Rangdale, R. E. and Lees, D. N. (2010) 'Comparison between Quantitative Real-Time Reverse Transcription PCR Results for Norovirus in Oysters and Self-Reported Gastroenteric Illness in Restaurant Customers', *Journal of Food Protection,* 73(2), pp. 305-311.

Lowther, J. A., Cross, L., Stapleton, T., Gustar, N. E., Walker, D. I., Sills, M., Treagus, S., Pollington, V. and Lees, D. N. (2019) 'Use of F-Specific RNA Bacteriophage to Estimate Infectious Norovirus Levels in Oysters', *Food and Environmental Virology,* 11(3), pp. 247-258.

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E. and Lees, D. N. (2012) 'Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom', *Applied and environmental microbiology,* 78(16), pp. 5812-5817.

Maalouf, H., Zakhour, M., Le Pendu, J., Le Saux, J.-C., Atmar, R. and Le Guyader, F. (2010) 'Distribution in Tissue and Seasonal Variation of Norovirus Genogroup I and II Ligands in Oysters', *Applied and Environmental Microbiology*, 76(16), pp. 5621-5630, *Applied and Environmental Microbiology* Available: American Society for Microbiology.

Marcheggiani, S., D'Ugo, E., Puccinelli, C., Giuseppetti, R., D'Angelo, A. M., Gualerzi, C. O., Spurio, R., Medlin, L. K., Guillebault, D. and Weigel, W. (2015) 'Detection of emerging and re-emerging pathogens in surface waters close to an urban area', *International journal of environmental research and public health,* 12(5), pp. 5505-5527.

McBride, G. B., Stott, R., Miller, W., Bambic, D. and Wuertz, S. (2013) 'Discharge-based QMRA for estimation of public health risks from exposure to stormwater-borne pathogens in recreational waters in the United States', *Water research,* 47(14), pp. 5282-5297.

McCaustland, K. A., Krawczynski, K., Ebert, J. W., Balayan, M. S.,

Andjaparidze, A. G., Spelbring, J. E., Cook, E. H., Humphrey, C., Yarbough, P. O., Favorov, M. O., Carson, D., Bradley, D. W. and Robertson, B. H. (2000) 'Hepatitis E virus infection in chimpanzees: a retrospective analysis', *Archives of Virology,* 145(9), pp. 1909-1918.

Mcleod, C., Hay, B., Grant, C., Greening, G. and Day, D. (2009) 'Localization of norovirus and poliovirus in Pacific oysters', *Journal of Applied Microbiology,* 106(4), pp. 1220-1230.

Mesquita, J. R., Oliveira, D., Rivadulla, E., Abreu-Silva, J., Varela, M. F., Romalde, J. L. and Nascimento, M. S. (2016) 'Hepatitis E virus genotype 3 in mussels (Mytilus galloprovinciallis), Spain', *Food microbiology,* 58, pp. 13-15.

Messner, M. J., Berger, P. and Nappier, S. P. (2014) 'Fractional Poisson—A Simple Dose-Response Model for Human Norovirus', *Risk Analysis,* 34(10), pp. 1820-1829.

Nordgren, J., Kindberg, E., Lindgren, P.-E., Matussek, A. and Svensson, L. (2010) 'Norovirus gastroenteritis outbreak with a secretor-independent susceptibility pattern, Sweden', *Emerging infectious diseases,* 16(1), pp. 81.

Office for National Statistics. (2020) *Population estimates for the UK, England and Wales, Scotland and Northern Ireland: mid-2019*: Population estimates for the UK, England and Wales, Scotland and Northern Ireland: mid-2019. Available at: https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/bulletins/annualmidyearpopulationestimates/mid2019estimates.

O'Hara, Z., Crossan, C., Craft, J. and Scobie, L. (2018) 'First Report of the Presence of Hepatitis E Virus in Scottish-Harvested Shellfish Purchased at Retail Level', *Food and environmental virology*, pp. 1-5.

Pouillot, R., Smith, M., Van Doren, J. M., Catford, A., Holtzman, J., Calci, K. R., Edwards, R., Goblick, G., Roberts, C., Stobo, J., White, J., Woods, J., DePaola, A., Buenaventura, E. and Burkhardt, W. (2021) 'Risk Assessment of Norovirus Illness from Consumption of Raw Oysters in the United States and in Canada', *Risk Anal*.

Prüss, A. (1998) 'Review of epidemiological studies on health effects from exposure to recreational water', *International Journal of Epidemiology,* 27(1), pp. 1-9.

Public Health England . (2018) *PHE National norovirus and rotavirus Report*: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/724329/Norovirus_update_2018_weeks_22_to_26.pdf.

Public Health England. (2019) *Hepatitis E: symptoms, transmission, treatment and prevention*. gov.uk: Public Health England. Available at: https://www.gov.uk/government/publications/hepatitis-e-symptoms-transmission-prevention-treatment/hepatitis-e-symptoms-transmission-treatment-and-prevention (Accessed: 27 August 2019).

R Core Team (2018) *R: A Language and Environment for Statistical Computing* (Version 3.4.3). Available at: https://www.R-project.org/.

Rivadulla, E., Varela, M. F., Mesquita, J. R., Nascimento, M. S. and Romalde, J. L. (2019) 'Detection of hepatitis E virus in shellfish harvesting areas from Galicia (Northwestern Spain)', *Viruses,* 11(7), pp. 618.

Ruchusatsawat, K., Nuengjamnong, C., Tawatsin, A., Thiemsing, L., Kawidam, C., Somboonna, N. and Nuanualsuwan, S. (2021) 'Quantitative Risk Assessments of Hepatitis A Virus and Hepatitis E Virus from Raw Oyster Consumption', *Risk Analysis*.

Said, B., Ijaz, S., Kafatos, G., Booth, L., Thomas, H. L., Walsh, A., Ramsay, M. and Morgan, D. (2009) 'Hepatitis E outbreak on cruise ship', *Emerging infectious diseases,* 15(11), pp. 1738.

Schets, F. M., van den Berg, H., Vennema, H., Pelgrim, M. T. M., Colle, C., Rutjes, S. A. and Lodder, W. J. (2018) 'Norovirus Outbreak Associated with Swimming in a Recreational Lake Not Influenced by External Human Fecal Sources in The Netherlands, August 2012', Int J Environ Res Public Health, 15(11).

Schijven, J. and de Roda Husman, A. M. (2006) 'A survey of diving behavior and accidental water ingestion among Dutch occupational and sport divers to assess the risk of infection with waterborne pathogenic microorganisms', Environmental Health Perspectives, 114(5), pp. 712-717.

Sharp, J. H., Clements, K., Diggens, M., McDonald, J. E., Malham, S. K. and Jones, D. L. (2021) 'E. coli Is a Poor End-Product Criterion for Assessing the General Microbial Risk Posed From Consuming Norovirus Contaminated Shellfish', *Front Microbiol,* 12, pp. 608888.

Smith, A. J., McCarthy, N., Saldana, L., Ihekweazu, C., McPhedran, K., Adak, G. K., Iturriza-Gómara, M., Bickler, G. and O'Moore, É. (2012) 'A large foodborne outbreak of norovirus in diners at a restaurant in England between January and February 2009', *Epidemiol Infect,* 140(9), pp. 1695-701.

Soller, J. A., Schoen, M. E., Bartrand, T., Ravenscroft, J. E. and Ashbolt, N. J. (2010) 'Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination', *Water research,* 44(16), pp. 4674-4691.

Stone, D. L., Harding, A. K., Hope, B. K. and Slaughter-Mason, S. (2008) 'Exposure Assessment and Risk of Gastrointestinal Illness Among Surfers', *Journal of Toxicology and Environmental Health, Part A,* 71(24), pp. 1603-1615.

Sumner, J. and Ross, T. (2002) 'A semi-quantitative seafood safety risk assessment', *International Journal of Food Microbiology,* 77(1), pp. 55-59.

Tei, S., Kitajima, N., Takahashi, K. and Mishiro, S. (2003) 'Zoonotic transmission of hepatitis E virus from deer to human beings', *The Lancet,* 362(9381), pp. 371-373.

Teunis, P. F., Moe, C. L., Liu, P., E. Miller, S., Lindesmith, L., Baric, R. S., Le Pendu, J. and Calderon, R. L. (2008) 'Norwalk virus: how infectious is it?',

Journal of medical virology, 80(8), pp. 1468-1476.

Teunis, P. F. M. and Havelaar, A. H. (2000) 'The Beta Poisson Dose-Response Model Is Not a Single-Hit Model', *Risk Analysis,* 20(4), pp. 513-520.

Tripathy, A. S., Sharma, M., Deoshatwar, A. R., Babar, P., Bharadwaj, R. and Bharti, O. K. (2019) 'Study of a hepatitis E virus outbreak involving drinking water and sewage contamination in Shimla, India, 2015-2016', *Trans R Soc Trop Med Hyg,* 113(12), pp. 789-796.

Ueki, Y., Shoji, M., Suto, A., Tanabe, T., Okimura, Y., Kikuchi, Y., Saito, N., Sano, D. and Omura, T. (2007) 'Persistence of caliciviruses in artificially contaminated oysters during depuration', *Applied and environmental microbiology,* 73(17), pp. 5698-5701.

Van Abel, N., Schoen, M. E., Kissel, J. C. and Meschke, J. S. (2017) 'Comparison of Risk Predicted by Multiple Norovirus Dose–Response Models and Implications for Quantitative Microbial Risk Assessment', *Risk Analysis,* 37(2), pp. 245-264.

van Alphen, L. B., Dorléans, F., Schultz, A. C., Fonager, J., Ethelberg, S., Dalgaard, C., Adelhardt, M., Engberg, J. H., Fischer, T. K. and Lassen, S. G. (2014) 'The application of new molecular methods in the investigation of a waterborne outbreak of norovirus in Denmark, 2012', *PloS one,* 9(9), pp. e105053-e105053.

Van Dijk, P., Lacey, R. and Pike, E. (1996) 'Health effects of sea bathing—further analysis of data from UK beach surveys', *Department of the Environment, WRc pic, Medmenham*.

Vergara, G. G. R. V., Rose, J. B. and Gin, K. Y. H. (2016) 'Risk assessment of noroviruses and human adenoviruses in recreational surface waters', *Water Research,* 103, pp. 276-282.

Viau, E. J., Lee, D. and Boehm, A. B. (2011) 'Swimmer risk of gastrointestinal illness from exposure to tropical coastal waters impacted by terrestrial dry-weather runoff', *Environmental science & technology,* 45(17), pp. 7158-7165.

Wade, T. J., Augustine, S. A. J., Griffin, S. M., Sams, E. A., Oshima, K. H., Egorov, A. I., Simmons, K. J., Eason, T. N. and Dufour, A. P. (2018) 'Asymptomatic norovirus infection associated with swimming at a tropical beach: A prospective cohort study', PLoS One, 13(3), pp. e0195056.

Wade, T. J., Pai, N., Eisenberg, J. N. S. and Colford, J. M. (2003) 'Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis', *Environmental Health Perspectives,* 111(8), pp. 1102-1109.

Williams, S. and O'Brien, S. J. (2019) *NoVAS: Assessing the contribution made by the food chain to the burden of UK acquired norovirus infection*, UK: Cefas, Fera, Leatherhead Food Research, Liverpool Clinical Laboratories (Royal Liverpool and Broadgreen University Hospital Trust), UK Health Security Agency, University of East Anglia and University of Liverpool (Accessed: 21 July 2021).

Wyer, M. D., Kay, D., Morgan, H., Naylor, S., Clark, S., Watkins, J., Davies, C. M., Francis, C., Osborn, H. and Bennett, S. (2018) 'Within-day variability in

microbial concentrations at a UK designated bathing water: Implications for regulatory monitoring and the application of predictive modelling based on historical compliance data', *Water Res X,* 1, pp. 100006.

Yin, W., Han, Y., Xin, H., Liu, W., Song, Q., Li, Z., Gao, S., Jiang, F., Cao, J., Bi, S. and Liu, H. (2019) 'Hepatitis E Outbreak in a Mechanical Factory in Qingdao City, China', *Int J Infect Dis*.

Chapter 8

General Discussion

General Overview

This PhD has endeavoured to assess the presence of hepatitis E virus (HEV) and norovirus in the aquatic environment in the UK. I aimed to identify if the aquatic environment is a likely source of HEV transmission within the UK, in comparison with norovirus, a known environmental water contaminant. Additionally, I aimed to identify the sources of HEV contamination in the aquatic environment and evaluate the risk of illness which HEV and norovirus may cause. To answer these questions, I investigated the presence of these viruses within different sample matrices, including sewage, shellfish, and cetacean and seal liver. I also investigated the sources of HEV and the common genotypes of norovirus which were present in sewage samples using next generation sequencing approaches, and I assessed the risks of illness from water activities and shellfish consumption using quantitative data for microbial risk assessment.

Enteric Viruses within Wastewater

Surveillance of sewage for viruses can be a useful tool to identify the disease burden within a population and monitor local outbreaks of a disease before cases become symptomatic in some cases, which has specifically been highlighted during the SARS-CoV-2 pandemic (Medema *et al.*, 2020a; Medema *et al.*, 2020b; Agrawal et al., 2021; Martin *et al.*, 2020). Sewage can present an onward transmission route for viruses and other pathogens during contamination of surface waters, either because recreational water activities are conducted in polluted waters or because of consumption of shellfish from contaminated harvesting areas. HEV and norovirus have been identified as contaminants of sewage, surface waters, and shellfish in other parts of the world due to the fact that they are shed in faeces and urine (La Rosa *et al.*, 2010; Romalde *et al.*, 2018; Di Bartolo *et al.*, 2015; Beyer *et al.*, 2020). However, investigations into the presence and abundance of HEV in environmental samples in the UK have been lacking, with only studies in Scotland investigating the presence of HEV in sewage (Smith et al., 2016), and shellfish samples (Crossan *et al.*, 2012; O'Hara *et al.*, 2018). The detection of

HEV and norovirus in sewage can help to inform whether there is a large HEV disease burden in the UK population and can determine whether the aquatic environment may become contaminated with these viruses, leading to possible public health risks. Not only is sewage discharged into surface waters after treatment, which classically is less effective for virus removal than for bacteria (Campos *et al.*, 2016) but sewage can also be discharged raw into surface waters through combined sewer overflows (CSOs), for example when storm conditions would otherwise cause sewers to overflow. CSOs in the UK in 2020 released raw sewage into surface waters collectively for over 3 million hours (Environment Agency, 2020), and therefore this can lead to the contamination of the aquatic environment with faecal pollution which contains viruses such as HEV and norovirus.

I wanted to identify if HEV is present in sewage in England, considering its presence in sewage in Edinburgh (Smith et al., 2016). I also wanted to determine the levels of HEV within sewage. Additionally, I tested the sewage for norovirus to provide a comparison of HEV and norovirus levels. I also aimed to identify if sewage treatment was effective for the removal of viruses. I did this using qRT-PCR detection techniques, followed by statistical analysis.

Chapter 4 identified the presence of HEV and norovirus within sewage in Southern England. Unfortunately, a study with a wider scope of locations (e.g., all of England) was not possible due to resource limitations. However, for influent and effluent respectively, a HEV prevalence of 45.7% (32/70) and 16.4% (11/70) was identified. For norovirus, a prevalence of 100% (70/70) and 90% (63/70) was identified within influent and effluent samples respectively. Levels of HEV were much lower than those of total norovirus. Influent samples contained a median of 0 copies/ml in all HEV positive influent samples (median of 2 copies/ml in HEV positive influent samples) and a maximum of 113 copies/ml. In contrast, the median norovirus in influent samples was 250 copies/ml and the maximum was 9,034 copies/ml. These results reinforce the idea that the disease burden of norovirus within England is much higher than the disease burden for HEV. It also means that norovirus and HEV from human sewage are likely to contaminate the aquatic environment in England during

release of raw sewage and treated effluent into surface waters. These results build upon those obtained by Smith et al., (2016), which showed that HEV was present within sewage samples in Edinburgh at a prevalence of 93% (14/15),and show that HEV is also present in England. The presence of these viruses within sewage indicates that they are likely to contaminate the aquatic environment in these countries.

I identified that there was a significant correlation between levels of norovirus and HEV within the sewage samples, suggesting a common source and risk factors for both viruses in human sewage. This result suggests that levels of HEV and norovirus vary in similar ways. Furthermore, some of the WWTPs within the study may have been supplied by waste from farms in the local areas, meaning that HEV of animal origin may have been detected within the sewage. This was investigated further by sequencing methods, and I identified that though the majority of the HEV sequences from the sewage samples were most similar to other sequences from humans in the UK, some were also similar to strains from wild boar, which could either have originated from the animals themselves, or from infection after consumption of meat. This means that HEV from both humans and animals is likely to contaminate the aquatic environment in England. In theory, the contribution of farm-run off to the concentration of HEV within surface waters could be significant, though there is no evidence to prove this.

For norovirus, I identified that the prevalence of GI and GII was almost identical within influent and effluent samples, yet levels of GI norovirus were much lower than GII. The high prevalence of both genogroups of norovirus suggests a high disease burden for GI and GII norovirus within the communities served by the WWTPs, but the higher levels of GII noroviruses suggest this genogroup is more prevalent within the community. This theory is supported by case data released from UK Health Security Agency (formerly PHE), which shows the majority of norovirus cases during the study period to be attributed to GII.4 norovirus (Public Health England, 2020). However, the high prevalence of GI within the sewage samples suggests that GI also has a significant disease burden within the community. Case data for GI norovirus is comparatively low,

however a theory to explain this is that GI norovirus may cause more asymptomatic infections than GII norovirus. Additionally, the reported cases of norovirus annually are expected to be vastly under-reported at various stages (e.g., the public, GP reports, etc), due to generally low severity and a short period of illness (Ondrikova *et al.*, 2021). Between 2018 and 2019, just 6,172 cases of norovirus were reported for England (Public Health England, 2021); whereas estimates of UK norovirus cases are approximately 3 million annually (Gherman *et al.*, 2020).

I identified that norovirus was present at all WWTPs, within both influent and effluent samples, and that sewage treatment only gave a significant reduction of norovirus from certain WWTPs (four of seven). WWTPs using a tertiary treatment method were more effective for norovirus removal than WWTPs using secondary treatment, showing significant reductions in norovirus RNA levels. These results agree with another study investigating the effect of different levels of treatment on norovirus, identifying that the use of UV following biological treatment was most effective for norovirus removal (Campos *et al.*, 2016). This means that many WWTPs around the country may not have an effective treatment system for the removal of viruses such as norovirus before discharge into surface waters, as secondary treatment without UV is very common. This may lead to potential public health risks from recreational water use and consumption of contaminated shellfish. A limitation of this assessment of treatment efficacy is that influent and effluent samples were collected within ten minutes of one another, meaning that the norovirus levels in influent samples may not have been representative of the norovirus levels which were present prior to the treatment of the paired effluent samples as the treatment process takes more than ten minutes. I did however consider that the samples were still useful for measuring real time reductions, as the dataset was likely to have captured times when the norovirus levels in influent were both increasing and decreasing, meaning that inflation and reduction of norovirus levels should have been cancelled out. However, this is something to consider when looking at the efficacy of the treatment processes for each WWTP. In contrast to norovirus, I was not able to assess whether HEV was effectively removed from

sewage due to the low levels of the virus which were detected in influent and effluent samples.

In summary, HEV and norovirus have been detected in both influent and effluent samples from southern England, with HEV at a much lower prevalence and concentration than norovirus. The detection of norovirus and HEV within sewage in England may have wider implications for the risks to public health due to release of raw and treated sewage into surface waters. The contamination of surface waters in the UK can lead to contamination of shellfish harvesting areas, as well as areas used for bathing and recreational water activities. It also can contaminate the habitats of aquatic organisms. As a result, I aimed to investigate the presence of HEV and norovirus in shellfish samples from the UK, as well as the presence of HEV within cetacean and seal samples from Southern England.

Viruses in the Aquatic Environment

Shellfish

There are many enteric viruses which have been detected in surface waters around the world, including norovirus, hepatitis A virus (HAV), HEV and sapovirus (Lodder and de Roda Husman, 2005; Iaconelli *et al.*, 2015; Salvador *et al.*, 2020; Sano *et al.*, 2011). These viruses are commonly shed in the faeces and urine of infected individuals, and during pollution events or release of treated sewage into surface waters, these viruses can contaminate shellfish harvesting areas.

Enteric viruses have been detected in shellfish worldwide, and outbreaks of norovirus from shellfish consumption are common. In the UK alone, norovirus is estimated to cause 11,800 cases of illness from shellfish consumption annually (Hassard et al., 2017). Outbreaks of HAV and sapovirus have also been reported in some parts of the world, with a large outbreak of HAV from clams affecting around 290,000 people in China (Tang *et al.*, 1991), and outbreaks of sapovirus from shellfish have also been identified in Japan (Iizuka *et al.*, 2010). No outbreaks of HEV have yet been conclusively linked to shellfish consumption, however it is theorised to be a transmission route due to the

identification of HEV in shellfish from many countries (Rivadulla *et al.*, 2019; Gao *et al.*, 2015; La Rosa *et al.*, 2018) and also a retrospective study of a HEV outbreak on a cruise ship identified shellfish to be the probable cause (Said *et al.*, 2009).

Though norovirus has been well established as a contaminant of the aquatic environment in the UK, investigations of the presence of HEV, HAV and sapovirus have been lacking. Previous studies in the UK have identified the presence of norovirus in shellfish from retailers and from harvesting areas. Lowther *et al.*, (2012) identified that norovirus was present in 76% (643/844) of oyster samples from production areas during a two year study, and Lowther *et al.*, (2018) identified 69% (433/630) of oyster samples from retailers contained norovirus. Until now, the only investigations of HEV contamination in the UK aquatic environment have originated from Scotland, where studies have identified HEV contamination of shellfish. The first study identified the presence of HEV in wild shellfish, some of which were located downstream from a pig slaughterhouse, and the prevalence of HEV in these shellfish was 85% (41/48) (Crossan *et al.*, 2012). A later study then identified the presence of HEV in samples of shellfish sold at retail in Scotland, with a prevalence of 3% (9/310) (O'Hara *et al.*, 2018). No studies, however, have yet investigated the presence of HEV, HAV or sapovirus in shellfish throughout the whole of UK.

Pilot Shellfish Study

To identify the prevalence and levels of enteric viruses within shellfish in the UK, two studies were performed within chapter 5. The first was a pilot study of shellfish samples obtained from harvesting areas in England which had previously shown high norovirus levels during the winter months (making it a biased selection). I tested for HEV as norovirus data was already available. I did this using qRT-PCR techniques for detection and quantification. A prevalence rate of 6% (5/91) was identified within these harvesting area shellfish samples, with levels of HEV between 16 – 121 copies/g of digestive tissue in positive samples. The health risk from both a presence and concentration standpoint appear to be low from these shellfish samples and considering that these sites are known to be contaminated with human faecal

pollution (therefore leading to norovirus contamination) this suggests the risk from an unbiased study may be even lower. However, these samples were obtained between 2009 and 2018, and therefore had long storage periods at -20°C, which may have affected the levels of RNA detected for HEV.

An important limitation to note is that the retail shellfish tested were not representative of the whole of the UK, with just 11 sites from England involved, and the study was biased to select sites which had high levels of norovirus in some samples, and therefore this should not be considered as a representative prevalence estimate for HEV in UK shellfish. Another important limitation of the study is that due to the length of time that the shellfish samples from the harvesting area study were stored at -20°C, it is possible that the prevalence and levels of HEV are lower than when the shellfish were originally collected due to RNA degradation, therefore this could possibly lead to an underestimation of the levels, prevalence, and risk of illness from HEV in shellfish. Despite this limitation, I decided that it was still worth investigating the presence of HEV within these shellfish samples as viral RNA was shown to persist for days within samples which were stored at ambient temperature (Weesendorp et al., 2010), and therefore freezing samples whilst they are still fresh can enable later testing for viral RNA. Overall, the significance of this pilot study is that HEV was detected in shellfish samples from harvesting areas, showing that HEV does contaminate the aquatic environment in the UK.

Retail Shellfish Study

The second study within chapter 5 was for shellfish samples purchased fresh from eleven retailers within England and Scotland (retailers within Wales and Northern Ireland were unavailable). Within the retail study I tested for HEV, norovirus, sapovirus and HAV. The retail shellfish were tested for norovirus to compare the prevalence and levels of the other viruses, and to determine how many samples and retailers may be affected by proposed European Union legislation on a norovirus limit of 500 copies/g. Additionally, I wanted to determine whether sites, locations or regions were significant risk factors for

detection of norovirus in UK shellfish. As the number of retailers, locations and samples was limited, I could not provide accurate UK prevalence estimates, however, the aim was to identify the prevalence and levels of these viruses within the shellfish samples to act as a baseline for what might be expected from a larger, more comprehensive study. I did this using qRT-PCR techniques for detection and quantification, followed by statistical analysis. I was unable to detect HEV within the panel of shellfish samples from retailers. There are multiple possible reasons for this. The first is that the shellfish from retailers are likely to have undergone post-harvest processing, such as depuration or relaying. This is required for shellfish from harvesting areas with a B or C harvesting site classification (according to UK hygiene regulations), which is determined by levels of *E. coli* (a faecal indicator organism) (UK Government, 2021). All of the harvesting areas within this study were class B or C sites. A class B site is defined as one where 90% of samples from a site do not exceed 230 *E. coli* per 100 grams of shellfish and a class C site is one where shellfish samples from a site contain must not contain more than 46,000 *E. coli* per 100 grams of shellfish. However, the effect which post-harvest processing (such as depuration and relaying) has on HEV is currently unknown. For norovirus, the effect of these processes is limited, however this is thought to be at least partly due to norovirus binding to histo-blood group antigen-like particles, and sialic acids within oyster digestive tissues (Almand et al., 2017; Maalouf *et al.*, 2010). Another reason why HEV may have been undetected in the retail shellfish samples could be related to sample size. If the biased pilot study generated a low prevalence within shellfish samples which were expected to be contaminated with norovirus (and therefore human sewage), then a study which utilises a non-biased sampling approach may have an even lower prevalence, and HEV may not, as a result, have been detected within the small sample size for the retail study (85 samples). These results were not unexpected, due to the low levels of HEV identified in Scottish retail shellfish samples (O'Hara *et al.*, 2018). If the same prevalence rate (3%) for the Scottish study were applied to this retail study, then only 2/85 samples would have been HEV positive. Therefore, the true HEV prevalence in these retail shellfish may have been lower. These results suggest that the aquatic environment in the UK can be

contaminated with HEV, however the levels of the contamination appear to be low, and the prevalence of HEV within shellfish also appears to be rare. This means that the risk of developing illness from HEV in shellfish from the UK appears to be low.

In addition to identifying the prevalence of HEV, we also investigated the presence of HAV and sapovirus within the retail shellfish. I was unable to detect HAV or sapovirus in the samples, suggesting that the disease burden of sapovirus and HAV within the UK is much lower than that of norovirus, and that the risk of illness from the consumption of these viruses in oysters is low. As the positive PCR DNA controls for these assays were successfully detected during the sample tests, I was confident that these results were true negative results. Furthermore, frequent detection of norovirus RNA in these same samples indicates that the RNA extracts were satisfactory for testing of viral targets.

For norovirus in the retail study in chapter 5, we identified that the prevalence of norovirus was 79% (67/85), which is a similar result to the previous UK studies. Levels of total norovirus (sum of GI and GII) in positive samples ranged from 1 – 1,525 copies/g of digestive tissues, which are also similar to levels observed in March 2016 by Lowther *et al.*, (2018). All shellfish suppliers provided at least one norovirus positive oyster sample, showing the widespread contamination of shellfish with norovirus, and therefore the widespread contamination of surface waters with human faecal pollution. Eight of the eleven suppliers provided at least one oyster sample with over 500 copies/g, which is the proposed limit for norovirus within shellfish according to the draft EU legislation (Younger, A. Personal Communication, 2021). 23% of the samples were over this proposed limit. This means that over 70% of the suppliers involved in this study would be negatively impacted by the draft EU legislation. However, considering that shellfish are estimated to cause 11,800 cases of norovirus in the UK each year (Hassard *et al.*, 2017), and that the consumption of shellfish was linked to 17.5% of international food and waterborne outbreaks between 2000 and 2007 (Baert *et al.*, 2009), such legislation may be necessary to protect public health.

Additionally, I discovered that GI norovirus had a slightly higher prevalence,

average and maximum levels than GII norovirus. This is a contrasting result both to the sewage study and the previous study by Lowther *et al.*, (2018) which found that GII had a higher maximum level than GI. It is also contrasting to data from PHE, which showed that norovirus cases were dominated by GII.4 during the time of the study (Public Health England, 2020). This may indicate that GI norovirus contamination of shellfish samples within this study have been caused by unreported GI cases, or asymptomatic norovirus infections. It could also be explained by longer retention times for GI norovirus within oysters, as GI noroviruses bind to shellfish digestive tissues with a better efficiency than GII during colder months (January to May) (Maalouf *et al.*, 2010). Another possible reason that the results are different to those seen in Lowther *et al.*, (2018) is that different genogroups of noroviruses may be circulating within the population in 2019 compared to 2015 – 2016.

In terms of risk factors for norovirus in shellfish, I identified that there was a significant difference in the norovirus levels between samples from regions southern England and northern England as well as southern England and Scotland. This suggests that human faecal pollution is a more significant problem in southern England than the other regions. This result is in agreement with previous studies, which have shown lower levels of norovirus from Scottish shellfish (Lowther, 2011; Williams and O'Brien, 2019), suggesting that Scottish waters are less affected by human faecal pollution. However, it is important to note that fewer suppliers (and therefore fewer samples) from Scotland were incorporated in the study than suppliers from Southern England, which may have skewed the data. The exact locations (where available) of the harvesting areas for the shellfish did not affect the probability of an oyster sample containing norovirus, which is likely because of how widespread norovirus contamination of surface waters is within the UK.

A limitation of the retail study is that it was conducted over a short period in March 2019 (two weeks), and though there is no identified pattern of seasonality for HEV in shellfish yet, it is possible that this short study period may have missed peak HEV levels. The study was also limited by the number of UK oyster retailers which were available to purchase from, which prevented

the inclusion of suppliers from Wales or Northern Ireland in the study. Due to these limitations, the study should not be considered as a UK-wide prevalence measure for HEV, HAV, norovirus or sapovirus, but more as a baseline for the potential levels which may be found in a more systematic study. The same applies to the harvesting area study.

Overall, the prevalence and levels of norovirus within oysters are much higher than those of HEV, HAV or sapovirus. Within the retail oyster study, high levels and prevalence of norovirus were detected, whereas no HEV was detected in these samples. Additionally, no HAV or sapovirus was detected in these samples. This indicates that there is little to no risk of HEV, HAV and sapovirus infection or illness to oyster consumers from these samples, and that the risk of norovirus infection and illness is higher. However, HEV has been detected within shellfish from harvesting areas, and therefore it can be concluded that HEV does contaminate the aquatic environment in the UK. Further work establishing the levels of HEV contamination in shellfish from both harvesters and retailers UK-wide would provide a more accurate estimate of the prevalence and levels of HEV in UK shellfish and identify any seasonal patterns of contamination (similar to norovirus). However, the results of these shellfish studies can provide a baseline for the levels of HEV which may be expected from a more comprehensive study and provide a preliminary estimate of risk to public health.

Cetaceans and Seals

In Cuba, a study identified that dolphins in captivity were experiencing symptomatic HEV infection; 32% (10/31) of captive dolphins were found to be seroprevalent for antibodies to HEV, and five of these were also positive for HEV RNA using qRT-PCR (Villalba *et al.*, 2017). One of these dolphins subsequently died of the infection. They then went on to identify genotype 3 (G3) HEV within the dolphins whose HEV RNA could be sequenced. This study highlighted for the first time that marine mammals were vulnerable to infection and symptomatic illness with HEV. However, the source of the HEV was unknown, whether it was from dolphin food (fish), poorly treated tank water, or from alternative sources. No studies have yet been conducted to

identify if human faecal pollution containing HEV could cause HEV infection within dolphins but considering that they were infected with a genotype which is commonly found in humans and other animals, this suggests that it could be possible.

Therefore, I wanted to determine whether human faecal pollution in surface waters could be having a detrimental effect on marine mammals in a study in chapter 5. I decided to investigate the presence of HEV within cetacean and seal liver samples, donated to us by the Cornwall branch of the UK Cetacean Strandings Investigation Program. I did this using qRT-PCR.

HEV was not detected within seal liver samples, however 39.7% (29/73) of common dolphin samples and 18.5% (5/27) of harbour porpoise samples tested positive for HEV by qRT-PCR. This was a higher prevalence than was expected, however the HEV levels in the samples were low, ranging from 1 – 210 copies/mg of cetacean liver in positive samples. I also identified that positive results seemed to be significantly more common in females than in males. If these qRT-PCR results are true HEV detections, then they indicate the circulation of HEV within cetacean species in their wild habitats. However, when attempting to sequence 18 of the cetacean samples with the highest detected levels of HEV using G3 and genotype 1 (G1) specific HEV assays, no sequencing data could be obtained. I theorised that this may be because of a variant or genotype of HEV which may circulate within cetaceans, instead of false negatives, due to the levels of HEV which were detected in the samples. Of the 18 cetacean samples selected for sequencing, the C_T values ranged from 31 – 33. Shellfish and sewage samples with lower detected levels of HEV were capable of being sequenced using the G3 assay (C_T range 37 – 39), and therefore the chances of the cetacean samples being false positives seems unlikely. Additionally, it seems improbable that the positive results were caused by large contamination issues as this would probably have caused higher C_T values across PCR plates. To avoid contamination by positive controls, samples were also processed, extracted, PCR mastermixes prepared, and samples added to PCR mastermix plates all in different rooms to avoid introducing positive controls into the samples at any stage. All negative PCR

and extraction controls were negative for HEV amplification also, meaning that cross contamination of the samples was unlikely. Some samples were also retested to double check the HEV results, and the results were the same for the second qPCR. The HEV qRT-PCR targets a small region of the genome (approximately 69 bases) and was designed to target genotypes 1-4, however an alignment of genotypes 1-8 shows that the qPCR would be capable of targeting these genotypes also, as it is a highly conserved region (based on alignment results). Therefore, it is possible that it could detect another genotype which is circulating within cetaceans. The sequencing nested PCRs (nPCRs), on the other hand, are targeted to specific genotypes as it is difficult to identify primer regions which encompass all genotypes for larger amplicons. This may explain why the G1 and G3 nPCRs were not capable of generating amplicons for the cetacean samples to sequence them, if the HEV within the cetacean samples is of a different genotype (possibly one which has not been discovered yet). This would mean that the HEV circulating within cetaceans is unlikely to have sourced from human or animal faecal pollution in the UK, as the dominant genotype in the UK is G3, which would have been sequenced using the nPCRs.

Another limitation of the cetacean testing was that during testing of the extraction efficiency, some cetacean samples had over 100% extraction efficiency. This may be due to some reference extractions extracting more poorly than samples themselves. This could perhaps be due to the more complex matrix within the sample extractions than the reference extractions, as this pattern has been identified during extraction of SARS-CoV-2 from water compared to viral transfer media (Carter, D. Personal Communication, 2022). It may also suggest that the RNeasy Mini kit (Qiagen) may not be the best RNA extraction method, however this could not have been known until PCR testing for mengo virus and HEV had already begun, and the kit seemed to be the best virus extraction method for the sample type (liver). An RNA integrity control also could not be utilised for the cetacean samples; however, as discussed previously for the older shellfish samples, I still believed that older samples may contain valuable data, with the caveat that HEV quantification and prevalence may not be as accurate with older samples due to RNA

degradation.

These studies have shown that HEV does contaminate the aquatic environment and lead to the contamination of shellfish, and potentially also cetaceans, possibly because of faecal pollution from humans or animals infected with HEV. I subsequently looked to use Oxford Nanopore Technologies (ONT) sequencing to identify the possible sources of the contamination.

Sources of Viral Contamination

HEV

HEV is a zoonotic virus which can infect many different host species. Within the UK, G3 HEV is the most common genotype within human infections and has also been detected within pigs in the UK. However, G3 HEV is also capable of infecting other hosts, such as deer and rabbits (Kenney, 2019), and it is possible that animal reservoirs of HEV lead to transmission to humans through the consumption of meat and milk from infected animals (Grierson *et al.*, 2015; Huang *et al.*, 2016). For example, consumption of a spit roasted piglet at a wedding lead to an outbreak of HEV within 17 people (Guillois *et al.*, 2015). Furthermore, an outbreak of HEV from consumption of raw deer meat lead to HEV illness within seven individuals (Tei *et al.*, 2003). It is possible that the HEV which contaminates the aquatic environment in the UK could source from both human sewage, animal farm run-off, slaughterhouse waste streams, or from wild animals themselves.

To determine where the HEV from both the shellfish and sewage samples originated, I used nPCRs which targeted either G1 or G3 to amplify small regions of the virus genome before sequencing it using a barcoding sequencing approach on the ONT MinION.

During the study in chapter 6, twelve sequences were obtained from nine wastewater influent samples and one shellfish sample from 2009, using the G3 HEV nPCR assay followed by metabarcode sequencing. Using a typing tool, I determined that at least eleven of the twelve sequences belonged to G3, with the final sequence (obtained from the shellfish sample) not capable of being assigned. Using BLAST, I determined that published sequences with

the closest homology to those from this study originated mainly from humans, with some also closely related to sequences originating from wild boar in Italy.

Unfortunately, the size of the sequenced amplicon could not give enough resolution to enable four sequences to be conclusively linked to just one previously published sequence. These results suggest that faecal pollution from both humans and animals may contaminate the aquatic environment through release of raw sewage into surface waters (through use of CSOs), and possibly from farm run-off; or it could show that people who have consumed imported pork products from infected boar have become infected and excreted the virus. The only sequence obtained from shellfish was more distantly related to other HEV sequences identified in the past and was shown through distance matrix analysis to possibly be considered a new subtype of G3. As there are many possible sources of the shellfish contamination, including farms (from run off), wild animals, and human sewage, it is difficult to know what the source of this contamination would have been without more sequencing data from animals in the UK.

Most of the sequences which were related to those from this study in chapter 6 were identified in the UK, but some were also identified from France, Italy, and the Netherlands, suggesting that HEV strains in the UK may also circulate elsewhere, or these may have been travel-associated cases. Using a final phylogenetic analysis, I determined that all the sewage sequences belonged to G3 (as expected) and that they fell into the subtypes G3c, and G3e, with one sewage sample remaining inconclusive. The shellfish sample genotyping and subtyping results suggested that it may be considered to belong to a new subtype within G3. Some previous studies in the UK had identified that many of the UK cases of HEV between years 2003 – 2011 belonged to one phylogenetic clade (group 1), and then in 2011 a new phylogenetic clade (group 2) started to become more dominant (Ijaz *et al.*, 2013; Grierson *et al.*, 2015; Oeser *et al.*, 2019). Group 1 includes subtypes 3e, 3f, 3g and group 2 includes subtypes 3a, 3b, 3c, 3d, 3h, 3i, 3j (Ijaz *et al.*, 2013; Smith *et al.*, 2021). This was theorised to have resulted from European HEV strains being imported into the UK within European pork products as evidence of infection of

UK pigs with group 2 HEV infections was lacking (Grierson *et al.*, 2015). I observed HEV sequences from both phylogenetic clades within the sewage samples and the sample sizes were too low to determine whether one clade occurred more commonly than another.

A limitation of the phylogenetic data is that within the G3 clade, some branches within subtypes had low bootstrap support (<70%), which may limit the accuracy of the subtype clustering; though subtype sequences were seen to cluster well together generally, and the phylogenetic results agreed with the subtyping results for the most closely related strains using the RIVM Hepatitis E Virus Genotyping tool. The low bootstrap support for some branches is likely to be a result of low phylogenetic resolution due to small amplicon sizes. The small amplicon sizes may also have prevented detection of new norovirus and HEV variants, however using smaller amplicons did allow limited data to be obtained from more samples for norovirus.

No PCR products were generated from the G1 HEV nPCR assay, and there are two possible reasons for this, the first being that the nPCR was not fit for purpose as we were unable to obtain a G1 positive control for the assay, and the other is that G1 HEV is less common in the UK than G3 HEV (Oeser *et al.*, 2019). As G1 HEV is normally a travel-associated infection in the UK, and the PhD was focussing more on endemic HEV within the UK (G3 HEV (Ijaz *et al.*, 2013)), I did not pursue sequencing of G1 HEV products any further. I did, however, investigate norovirus sequences from sewage.

Norovirus

Norovirus is an enteric virus which has been detected in several different animal hosts, such as pigs, cattle, and dogs (Villabruna et al., 2019). However, the genotypes of norovirus which infect humans appear to infect humans exclusively. This means that human faecal pollution is the source of norovirus GI and GII within the aquatic environment. Sequencing of viruses within sewage can be a useful tool for tracking the variants of a virus through a pandemic, or through endemic outbreaks, as has been demonstrated by the SARS-CoV-2 pandemic (Agrawal et al., 2021; Martin *et al.*, 2020). Sequencing

of norovirus within sewage can also provide insights into the circulating strains or genotypes within the community and could be used to identify new strains before they are able to become highly abundant, and possibly lead to outbreaks (Matsushima *et al.*, 2015).

I sequenced GII norovirus from sewage to identify the circulating norovirus strains within the community at the time, and how well these reflected the strains from reported cases published by PHE. To do this, I used an nPCR which generated small amplicons (approximately 344 bp in size including primers) followed by barcode sequencing on the ONT MinION.

Sequencing data was obtained for 23 sewage samples. In total, 120 sequences belonging to eight genotypes were generated. The genotype was determined using the RIVM norovirus typing tool (Kroneman *et al.*, 2011). The most common genotype was GII.4, which is the genotype most associated with outbreaks of norovirus (Lindesmith *et al.*, 2008). This was unsurprising as GII.4 has been the most detected norovirus genotype in reported cases worldwide since 2014 (Public Health England, 2020). However, the genotype with the most reads was GII.3, and GII.2 and GII.3 were the only genotypes detected in the influent and effluent of every WWTP which provided sequencing data (five of seven). Additionally, cases from genotypes GII.9 and GII.13 were not reported by PHE during the time of the sewage study in chapter 4, despite their presence in sewage. These results indicate that many norovirus cases from different genotypes go unreported by the public, or that the rate of asymptomatic illness is high. Under- reporting is supported by estimates of annual norovirus cases. PHE reported 6172 norovirus cases between 2018 and 2019 (Public Health England, 2021), yet norovirus is estimated to cause 3 million cases in the UK annually by the Food Standards Agency (Gherman *et al.*, 2020). Additionally, a study of the ratio of symptomatic to asymptomatic norovirus cases identified that 32% were asymptomatic (Miura *et al*., 2018), which could explain why some sequences are less prominent than others in recorded cases in comparison to within sewage.

Some limitations of using the ONT sequencing chemistry are that, determining whether a sequencing run is of good quality and has little to no cross-

contamination can be difficult. Many studies using NGS sequencing have assigned an arbitrary cut-off for a negative control of 1,000 reads as the point where a run has failed quality control. However, there is no actual scientific basis for picking 1,000 reads, and in fact this cut off may have limited the results available to analyse in this study in chapter 6. Additionally, ONT is known to produce more error prone reads than technologies such as Illumina sequencing. ONT has previously been shown to have an error rate of approximately 5-20%, and ONT only provides the modal accuracy for high accuracy reads, at 97.8%. However, applying this percentage accuracy would mean that just four to five bases of the 202 bp HEV sequences were incorrect, or six to seven bases of the 302 bp norovirus sequences.

These results have shown that G3 HEV is present in sewage and shellfish, meaning that contamination of the aquatic environment with human or animal faecal pollution can lead to health risks for the public. I have also shown that monitoring of viruses such as norovirus within sewage can provide information on strains and genotypes of norovirus which are under-reported, perhaps due to asymptomatic illness, or due to a lack of reporting. Though I have shown that sewage and shellfish in England are contaminated with HEV and norovirus, the risks to health have not yet been investigated in a UK context. I therefore looked to investigate the risk of infection and illness from the consumption of shellfish, and from recreational water activities, using the quantitative data obtained for the shellfish and sewage samples within these studies.

Assessment of the Risks to Human Health by Viruses in the Aquatic Environment

This PhD has focussed on the presence of viruses within the aquatic environment, and their sources. Norovirus has been known to contaminate the aquatic environment in the UK for many years, having been detected through surveillance of norovirus in shellfish (Lowther *et al.*, 2012; Lowther *et al.*, 2018). Shellfish are known to be sensitive to water pollution due to their ability to bioaccumulate pathogens during filter feeding. However, despite the detection of norovirus in the aquatic environment and shellfish in the UK, risk

assessments have not been performed to identify the risk of norovirus from recreational water activities in the UK. Additionally, as HEV research in the UK has typically been limited, no risk assessment for HEV illness has been done for shellfish or recreational water activities in the UK either.

Chapter 7 used previously published dose response models and data from reviews to identify calculations and parameters to find the doses of norovirus and HEV which may result from consumption of shellfish and from recreational water activities. For surface water ingestion doses, I used the median and maximum virus copies/ml alongside dilution factors to simulate dilution of influent and effluent within surface waters prior to ingestion. I then used ingestion volumes of water from Leonard *et al.*, (2015) to calculate the doses received during each recreational water activity for each sewage dilution factor. For the shellfish doses, I used the median and maximum virus copies/g of digestive tissue from the shellfish studies and applied a calculation which corrected for the likely dose from the whole oyster (minus the shell). I then multiplied the dose in one oyster by a range of oyster meal sizes. Dose data for HEV and norovirus were then used to identify the risk of illness using dose response models, with an assumption of exposure. The risk of illness was calculated for shellfish consumption and from ingestion of surface water during recreational water activities. For norovirus, two different scenarios were investigated with the dose response models, the first being where the virions were aggregated (virions clumped together) and the second being where the dose was disaggregated (virions separate to each other). These scenarios affected the risk of illness from the norovirus dose.

HEV and norovirus within sewage

Quantitative data for the median and maximum copies/ml of virus in influent or effluent was obtained during chapter 4. Using the HEV copies/ml from the influent, I identified that the median dose of HEV from influent and effluent would have been 0 copies/ml, and therefore only the maximum dose of HEV was used for calculating the risk of illness from HEV during recreational water activities. Doses were not calculated for the effluent as the levels of HEV were too low. Using the maximum dose of HEV within influent, I identified the risk of

HEV illness from recreational water activities by applying the dose-response models to the calculated doses for each dilution. The risk of HEV illness was low, with a 10-fold dilution of the influent HEV dose giving a risk of 0.69% for surfers, and a negligible risk for all other water activities. For all subsequent dilutions, the risk was negligible for all water activities. This means that the chance of becoming ill with HEV from recreational water activities using these sewage doses is low. This is because levels of HEV within influent were low, and because the calculated dose at which 50% of the population are susceptible to infection is high for HEV (3.03×10^{7}). Additionally, the chance of becoming ill with HEV from effluent release into the environment would be negligible as the maximum concentration of HEV in effluent was just 5 copies/ml.

For norovirus, I identified that the risk of illness was dependent on the type of dose received, whether norovirus was in an aggregated state within the shellfish and environmental water or not. The risk of infection and illness from a disaggregated dose of norovirus was high for both the median and maximum doses in comparison to those from aggregated doses. Assuming exposure, a 1,000-fold dilution of the median disaggregated norovirus dose from influent gave a 15.0 – 43.2% chance of illness for all water activities. This makes the contamination of the aquatic environment with raw sewage particularly more of a public health risk. Even 1,000-fold dilutions of the median disaggregated dose from treated effluent gave a risk of illness of 1.3 – 34.0% for all water activities, with the highest risks seen in surfing (due to higher ingestion volumes). The risks from a maximum disaggregated dose of influent and effluent were even higher, with a 1,000-fold dilution of influent or effluent giving a range of 42.8 – 43.2%. Norovirus within water is suspected to be in a disaggregated form as the pH of the water is normally higher than the isoelectric point of the virus (Van Abel *et al.*, 2017), which means that the risks to public health from faecal pollution containing norovirus is likely to be quite high. However, as the norovirus originates within sewage, and that and environmental water may represent more of a complex matrix due to the presence of organic matter (and a more complex matrix can cause virus aggregation (Teunis *et al.*, 2008)), it is

possible that norovirus may exist within a more aggregated form in environmental waters. Therefore, as best practise, I calculated the risk using an aggregated dose model also.

The risk of illness, assuming exposure, from an aggregated norovirus dose was much lower, with the median effluent norovirus dose at a 1,000-fold dilution providing a risk of 0.17% for surfers, but a negligible risk for all other water activities. For the median dose of norovirus in influent at a 1,000-fold dilution, the probability of illness was 0.05 – 2.2%, which is much lower than the estimates from the disaggregated doses. The maximum influent and effluent aggregated doses provided higher risks, again signifying the importance of reducing environmental water pollution; with 1,000-fold dilutions of the maximum effluent dose giving probabilities of illness between 0.5 – 18.6%, and 1,000-fold dilutions of the maximum influent dose giving probabilities of illness between 1.7 – 36.9%. The results from the aggregated dose calculations can be compared to a UK study by Kay *et al.*, (1994), which identified that the likelihood of gastroenteritis was 14.8% in bathers compared to non-bathers (9.7%). The cause of gastroenteritis in these cases was not specifically identified, however the almost ubiquitous nature of norovirus in sewage (see chapter 4 and Campos *et al.*, (2016)) suggests that it may have been a contributing factor to the reported gastroenteritis cases. In comparison, the risk calculations from the disaggregated doses are much higher, and may be less realistic, suggesting that perhaps norovirus is aggregated within sewage diluted by environmental waters. Vergara et al., (2016) also identified that the risk from water activities which involved submersing the head was just 0.61%, however this study used a Monte Carlo simulation to estimate the exposure to the population, whereas these calculations assumed exposure to the dose.

These results mean that there is a high risk of contracting norovirus illness from recreational water activities where the recreational waters are impacted by treated or untreated sewage. Even 1,000-fold dilutions of the sewage within environmental waters may not be enough to make the risk of illness negligible, and release of raw sewage through CSOs poses a particular risk due to the high levels of norovirus which can be found in raw influent. To reduce the risk

of norovirus from recreational water activities, investment in sewage treatment infrastructure should be prioritised which will limit the use of CSOs and enable more effective removal of viruses from influent.

However, it is important to note that quantitative microbial risk assessment (QMRA) is not a perfect science due to the lack of knowledge which surrounds certain parameters which are used in the risk assessment process. This lack of knowledge can introduce uncertainty into the results of QMRA. During this study in chapter 7, there were many limitations to the calculation of risk of illness from HEV and norovirus during recreational water activities. The first is that some of the ingestion volumes used from Leonard *et al.*, (2015) were obtained by survey, meaning that the volumes may be subjective to the individual, which may influence whether dose calculations are correct. A second limitation is that during sample processing and viral RNA extraction, some virus is likely to be lost, which may result in a lower dose than the exposed dose would have been. Additionally, this study utilised dilution factors with sewage data from chapter 4 which made direct comparison of risk data to other studies difficult. The viability constants used for the calculations were also taken from a dose-response study for norovirus with a different sample matrix, and without viability data on norovirus and HEV in these samples, it is difficult to say whether this viability factor would indeed be correct, or even the same for HEV. Another factor which may influence virus viability is treatment at WWTPs, if these treatment processes can reduce norovirus viability within sewage, this suggests that norovirus viability in effluent may be lower than in influent. Another significant issue is that the state of aggregation of norovirus and HEV within the sewage and diluted sewage is unknown, and this may affect the dose response. These factors may all influence the risk calculations observed, but another assumption which may make the data more uncertain is that exposure has been assumed within all water activities in order to calculate the risk of illness, and this may not be truly representative of reality because though norovirus is fairly ubiquitous within sewage (see chapter 4 and Campos *et al.*, (2016)), HEV is not, and the presence of these viruses in environmental waters is also not likely to be ubiquitous.

HEV and norovirus within shellfish

For shellfish, the dose of virus was calculated by multiplying the copies/g of digestive tissue by a correction factor to enable calculation of the amount of virus within whole oysters (minus the shell). For HEV, I calculated that the probability of illness (assuming exposure) from the consumption of a single oyster was 0.0074%, and for twelve oysters was 0.089%. Consumption of 24 oysters gave a 0.18% risk of illness. Therefore, the risk of HEV from these shellfish samples was low, especially for consumption of a small number of oysters. This means that the risk to the public from HEV in shellfish is likely to be very low, however the harvesting area shellfish study from chapter 5 was only representative of eleven English harvesting locations, and therefore cannot be ruled as a decisive risk probability for the public. The HEV doses were calculated from the quantified HEV from qRT-PCR of the shellfish samples from 2009 – 2018, and there is therefore a limitation that the quantification of HEV in these samples may be lower than when the shellfish were originally harvested, due to long-term storage at -20°C. This could mean that the HEV dose and the risk of illness may be underestimated. Additionally, the shellfish were obtained from harvesting areas and had not yet undergone any form of post-harvest processing (depuration or relaying). It is unknown whether these techniques may be helpful for the removal of HEV from shellfish, but if these techniques are effective, it may mean that the risk to the public is lower as most shellfish sold in the UK are subject to post harvest processing before sale (due to *E. coli* classification systems mentioned previously).

For norovirus, the risk was dependent on whether the dose of norovirus was aggregated or disaggregated. A median and a maximum dose of norovirus was used to calculate the probability of infection and illness from shellfish consumption. A disaggregated dose from shellfish consumption gave a risk of illness of 43.2% for all oyster meal sizes for the median and maximum doses. Whether norovirus would be in a disaggregated state within shellfish is unknown, though norovirus has been shown to aggregate within infected host cells (Green *et al.*, 2020) (though norovirus does not infect oyster tissues), and

(Williams and O'Brien, 2019) predict that norovirus is likely to aggregate within shellfish tissues. For aggregated doses, the risk of illness was much lower, with the risk from one oyster at a median aggregated norovirus dose at 0.98%, and the risk from twelve oysters at 10.4%. The risk from one oyster with a maximum aggregated dose was 12.1%. For 12 oysters, the risk of illness from a maximum aggregated dose was 42.4%. The results from a median aggregated dose are closer to those from previous studies, for example Lowther *et al.*, (2010) observed an illness reporting rate from oysters containing norovirus of 0.21%, and Williams and O'Brien, (2019) identified a risk of illness per oyster meal of 0.68%. The similarity to the estimates from these studies may suggest that norovirus is within an aggregated state whilst in oysters.

There are limitations for the calculation of the risk of illness from oyster consumption. Similar to the recreational water dose estimates, the shellfish dose estimates may also be low due to loss of virus during sample processing and extraction. For HEV, the shellfish doses were also calculated from oysters kept in storage at -20^{o}C for up to ten years, and therefore the detected virus genome copies within the samples may be lower than original contamination levels, which may lead to an underestimation of risk. These shellfish were also collected from shellfish harvesting areas rather than retailers (as no shellfish were HEV positive during the retail study, see chapter 5) and had therefore not undergone post-harvest processing techniques such as depuration or relaying. It is unknown whether these processing techniques could enable a reduction in HEV within the shellfish as depuration studies on HEV have not been performed, to my knowledge. In the case that HEV concentration can be reduced in shellfish using these techniques, this may mean that the risk of illness from oysters available to the consumer is lower than estimated here. The viability constant used for the calculations was taken from the ratio of PCR genome copies and cultured F-specific bacteriophage (GII) in oysters, but although the data comes from similar oyster samples, it is impossible to tell whether it directly represents the viability of norovirus and HEV within these shellfish samples. Without viability data on norovirus and HEV in these samples, it is difficult to say whether this viability factor would indeed be

correct, or even the same for HEV. Another issue is that the state of aggregation of norovirus and HEV within the oysters is unknown, though Green *et al.*, (2020) observed norovirus aggregation within infected host cells (though shellfish tissues are not infected with norovirus) and Williams and O'Brien, (2019) predict that norovirus is likely to be in an aggregated form in shellfish. Aggregation affects the dose response and therefore the risk of illness, and therefore information on the aggregation of norovirus in shellfish would be useful. Another assumption which may make the data more uncertain is that exposure has been assumed within all oyster meals in order to calculate the risk of illness, and this may not be truly representative of reality because norovirus and HEV are not ubiquitous within shellfish (see chapter 5 and Lowther *et al.*, (2012) and Lowther *et al.*, (2018)), and therefore this may lead to overestimation of the risk of illness for both aggregated and disaggregated data, and it makes it difficult to compare these results to other studies.

Exposure estimates for norovirus

Unfortunately, exposure estimates for the number of water activities which may lead to HEV illness annually and the number of meals which may cause HEV illness annually could not be calculated as the median HEV dose in sewage and shellfish samples was 0 copies/ml and 0 copies/g respectively and using the maximum levels would potentially overestimate the risk of illness from HEV in shellfish. Additionally, the HEV data from shellfish was obtained from a biased study in chapter 5. However, these calculations were possible for norovirus in water activities and shellfish.

I identified that the number of water activity sessions annually which may lead to norovirus illness was again very dependent on the dose type, whether aggregated or disaggregated. To calculate the number of water activity sessions resulting in illness annually, the illness risk data obtained from a 1,000-fold dilution of a median norovirus dose was used. For effluent, assuming 100% of water activities were exposed to this median effluent dose of norovirus, the number of water activity sessions resulting in illness ranged from 53,533 – 13,272,718 (with the higher estimate for disaggregated doses). Using the risk data from a 1,000-fold dilution of the median influent

dose (again assuming 100% exposure), the number of water activity sessions resulting in illness ranged from 725,994 – 47,501,918 (with higher estimate for disaggregated dose). This represents 0.03 – 32.0% of water activity sessions occurring annually in England and Wales. The lower estimates were calculated from aggregated doses and seem to be more realistic in comparison to other study estimates. A study by (EFTEC, 2002) estimated that the number of gastrointestinal illnesses from water activities in 2002 was 2.84 million; higher than the aggregated dose estimates but lower than the disaggregated dose estimates. The reason for the discrepancy may be that gastrointestinal illnesses can be caused by several pathogens within water (e.g., *Giardia*, astrovirus), and therefore the burden of norovirus is lower than that estimate. Considering that norovirus causes an estimated 3 million cases in the UK annually (Gherman *et al.*, 2020), the exposure estimates for a disaggregated dose seem more unrealistic. However, there is an assumption that 100% of the water activities are exposed to this median norovirus dose, which is unlikely to be correct. Therefore, the disaggregated risk of illness is possible, were a lower rate of exposure considered.

Additionally, I identified that 947,754 – 3,949,695 oyster meals could cause norovirus annually, depending on whether the dose is aggregated or disaggregated and assuming that all oyster meals would lead to norovirus exposure. Again the estimates from a disaggregated dose are very high and may seem unrealistic when compared to previous studies. Williams and O'Brien, (2019) estimated that oyster consumption would cause 14,000 cases of norovirus in the UK annually, whilst Hassard *et al.*, (2017) estimated 11,800 cases. These values are also much lower than the exposure estimates for an aggregated norovirus dose. This may be due to the assumption that 100% of consumed oysters contain the median dose of norovirus, which is unlikely to be true, and therefore if exposure were factored into these estimates, the predicted number of oyster meals which could cause norovirus annually would be lower for both the aggregated and disaggregated doses. As they stand, the exposure estimates for the disaggregated doses are very high, and would suggest that more than 3 million people may become ill with norovirus in the UK annually (which is the current estimate (Gherman *et al.*, 2020)) due to the

contribution from shellfish consumption and water activities alone. Norovirus is known to be under-reported in the UK (Tam *et al.*, 2012), however it seems unlikely that 13 million cases of norovirus are caused by water activities, and 4 million cases are caused by shellfish consumption annually. It is also important to note that the risk of illness for calculating the population exposure for shellfish was taken from an oyster meal size of twelve, which may not represent the meal size most consumed in the UK, as data on common oyster meal sizes was taken from Florida as UK data was unavailable (Degner and Petrone, 1994; Pouillot *et al.*, 2021).

The results of the risk assessment suggest that shellfish and recreational water contamination with norovirus and sewage in general are under-appreciated threats to human health. Sewage contamination of surface waters is a significant problem in the UK, with wastewater treatment plants discharging over 3 million hours worth of raw sewage in 2020 (Environment Agency, 2020). In order to tackle the infections associated with shellfish consumption and recreational water activities, both the government and water companies must invest in improving wastewater treatment practices, standardizing practices across the industry to ensure the maximum efficacy of wastewater treatment, and increasing the capacity of WWTPs to meet the demands of, not only the current catchment populations, but anticipated increases in population levels over time. The use of Combined Sewer Overflows must be reduced as much as possible to prevent discharging of raw sewage into water courses, and there must be investment in sewerage infrastructure to reduce the effect of stormwater pressure on the water system. However, as acknowledged in Figure 8.1, the biggest risk of transmission of norovirus is unlikely to be from consumption of shellfish or recreational water activities, but instead comes from person-to-person transmission during an outbreak. Much of the public are unaware of norovirus and other viruses until they are affected by them, and simple policies like ensuring that hands are washed after using the toilet, and before eating or preparing food are simple ways to reduce norovirus spread. Better education of the public, perhaps through simple NHS advertising campaigns, could also help to reduce the spread of norovirus and other viruses, and perhaps reduce the burden of norovirus infections within hospitals

each year (Public Health England, 2021a).

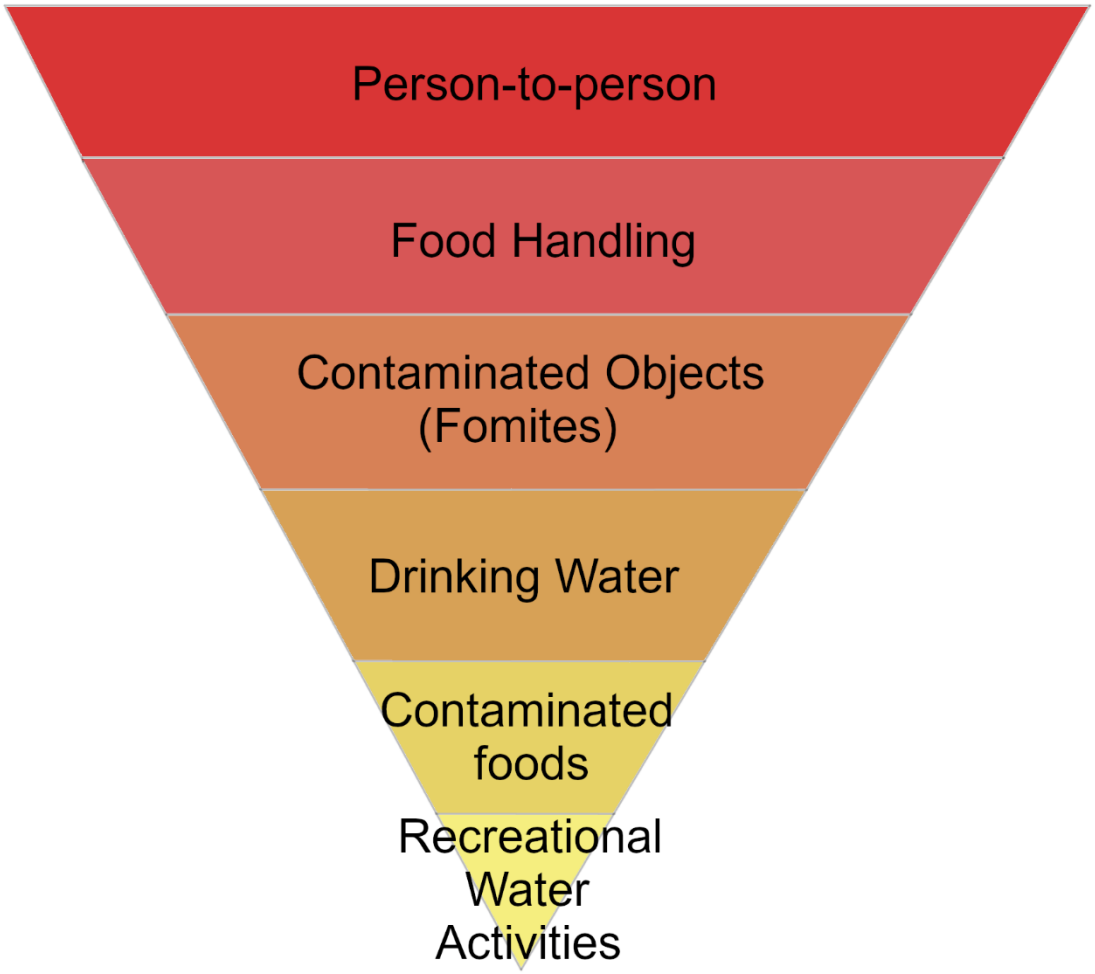


Figure 8.1 A risk hierarchy for the transmission routes of norovirus to humans

A risk hierarchy showing the transmission routes with the highest risk at the top and the lowest risk at the bottom. Person-to-person transmission is the route which causes most norovirus cases, but other transmission routes such as contaminated foods can lead to outbreaks which are then amplified by secondary person-to-person transmission.

Despite these recommendations, it is important to note that the effect of sewage treatment and environmental conditions on norovirus and HEV viability is unknown, and that estimates of viability cannot be confirmed for these samples. Though qRT-PCR can detect norovirus and HEV RNA, I was unable to tell if the viruses which provide this RNA were viable prior to testing. The culturing of norovirus within human intestinal enteroids is a relatively new technique, which some laboratories have had limited success with (Ettayebi *et al.*, 2016). Currently, it appears that only certain strains are capable of being

cultured, and the culturing process requires high titres of virus to start. The method is also labour-intensive and requires much time and resources (Ettayebi *et al.*, 2016). HEV culture has also proven to be difficult for certain strains, and also requires high titres of virus to begin the culturing process (Johne *et al.*, 2014). Unfortunately, norovirus and HEV cell culturing was not possible during this PhD due to laboratory and resource limitations, as well as low viral concentrations for many of the samples, so it was not possible to test for viability within these sewage and shellfish samples. Surrogate viruses have typically been used to estimate norovirus viability previously (Lowther *et al.*, 2019; Farkas *et al.*, 2019; Farkas *et al.*, 2020), but none have yet been considered the ideal surrogate for this purpose as it has not been possible to compare it to the viability of norovirus. Therefore, it is possible that viability estimates within shellfish may not be correct. Additionally, the viability estimate used for the sewage samples was obtained from a dose response study (Teunis *et al.*, 2008), which may not accurately represent the sample matrix or environmental conditions which norovirus would be exposed to within sewage and environmental waters, and therefore may over- or under- estimate risk.

Additionally, the sewage samples which were used to perform the risk assessment were all collected during the winter period between 2018 and 2019. This is a limitation of the data because norovirus is seasonal, with higher cases and levels of the virus in sewage in colder months of the year (Public Health England, 2021a; Campos *et al.*, 2016; Lowther *et al.*, 2012). Therefore, the risk data can only be assumed to be correct for the winter months and should not be applied to the summer months when the levels and therefore dosage of norovirus in both sewage and shellfish would be lower. Monthly sampling and QMRA would enable more accurate risk assessment to better inform policy changes which could reduce the risk of norovirus transmission from recreational water activities and shellfish consumption.

There are additional limitations due to the models or assumptions which formed the models. Unfortunately, the HEV dose-response model was only able to utilise one study by McCaustland *et al.*, (2000), which had a very limited sample size of chimpanzees to measure dose response data from. This may

mean that the risk of illness has been incorrectly estimated, but only with more dose- response data for HEV will more accurate estimates be achieved. Both the HEV and the disaggregated norovirus models also lacked data on the response to low doses of HEV and norovirus, leading to possible inaccuracies for the risks

at these lower doses. Additionally, the value of P within the norovirus model (which is the proportion of the population fully susceptible to norovirus) may be too low, as this value has been calculated based on the assumption that secretor negative people (those who do not secrete histo-blood group antigen complexes within the gut) cannot be infected with norovirus. However, an outbreak of norovirus was shown to affect secretor negative people, and therefore the risk of infection may be under-estimated (Nordgren *et al.*, 2010). Additionally, this estimate is unlikely to consider acquired immunity (meaning lower susceptibility) or waning of acquired immunity (meaning greater susceptibility), and this is due to the complexity of individual immune responses to norovirus. Other significant limitations of the study in chapter 7 are that the exposure scenarios used within the study may not be representative of real-life exposures. Exposure calculations were made with the assumption that faecal contamination of waters was caused by either influent or effluent; however, in reality, when CSOs are used to discharge influent into waters, effluent will still also be discharging. Additionally, influent will not be discharged from CSOs constantly. Therefore, the real risk of illness to an exposed individual may be between the risk calculated for the effluent and influent doses, and therefore the population exposure events would also fall between these calculations. Another issue with the population exposure estimates is that the 1,000-fold dilution illness risk data was used to calculate the number of water activity sessions which may cause illness annually, assuming exposure, and this dilution may not best represent all recreational water sites in the UK. Therefore, conducting this form of QMRA would be more beneficial if it were conducted on a site-by-site basis, using a specific calculated dilution factor for each individual recreational water site to obtain more specific risk estimates. Data on the specific sewage dilutions in recreational waters surrounding the WWTPs within the sewage study in chapter 5 were unavailable.

In summary, this study has identified the difficulties which can be associated with QMRA, in that many parameters which are needed to accurately assess the risk are unknown, or little information is known about them, and so assumptions must be made. This can cause uncertainty within risk estimates. We have identified that the risk of HEV illness from shellfish consumption and recreational water activity sessions is low within the UK, however the risk of norovirus illness is much higher, which makes it important to invest in infrastructure which enables effective removal of viruses from sewage and prevents the use of CSOs.

Further Work

Though this PhD has sought to provide some answers about the presence and transmission of HEV within the UK, limited resources and time mean that there is still much to learn. Given more time, and unlimited resources and equipment, there are several studies which could help researchers to better understand the transmission of HEV in the UK.

Wastewater and Surface Waters

A study which was to investigate the prevalence of HEV and norovirus in wastewater throughout the UK using qPCR techniques could be incredibly beneficial to identify the true burden of disease in the population as this would capture HEV from both symptomatic and asymptomatic individuals and enable us to identify if there are correlations between HEV and norovirus presence. Sequencing the positive PCR samples could also allow an identification of the different genotypes and subtypes which are commonly identified in the UK, which could enable us to identify patterns in sources of contamination, monitor outbreaks, and even track variants, much like the SARS-CoV-2 pandemic. Combining a study such as this with another which assesses the presence and levels of HEV and norovirus in surface waters at outfall and storm overflow points, within nearby surface waters and nearby bathing areas could also help to inform risk assessment models more accurately on the risks of becoming ill from HEV and norovirus in surface waters.

Additionally, whilst pigs have been established as the main reservoir of HEV,

little research in the UK has been performed on whether pig farm run off and abattoir waste into surface waters may lead to contamination of the aquatic environment. It would be interesting to investigate HEV presence and sequences within surface waters from outside of abattoirs and nearby to pig farms to investigate if such areas may be more affected by HEV contamination to others with no local pig farms or abattoirs. Indeed, the study by Crossan *et al.*, (2012) identified shellfish downstream from a slaughterhouse to be contaminated with HEV, and therefore this could be an interesting avenue to investigate.

Shellfish

To determine whether the consumption of shellfish from the UK may constitute a significant risk of contracting HEV, a more thorough and representative study of HEV in UK shellfish is needed. Different types of shellfish (e.g. oysters, mussels, cockles) should be collected from farms around the UK and, much like the UK norovirus baseline survey from Lowther *et al.*, (2012), tested for the presence and levels of HEV. If a prevalence of 5% were to be anticipated, with high statistical power, approximately 2,473 samples would be required to estimate the true prevalence of HEV with significant statistical power. This would require a lot of financial and administrative resources, as well as a lot of time. However, spreading the study across some years would enable an identification of any changes in HEV levels, as well as a possible identification of any seasonal patterns (much like norovirus has). Additionally, the shellfish could be tested for norovirus for monitoring purposes and to compare HEV presence with norovirus presence and identify any correlations between the data. Using the HEV sequencing technique from this thesis, it could then be possible to sequence the HEV samples and identify possible contamination sources by comparing the sequences to others from animals and humans, though HEV sequences from most animals are generally lacking.

Additionally, a study investigating the effect of depuration and relaying on HEV in shellfish would be interesting and useful. It is well known that norovirus is capable of binding to organic molecules within the digestive glands of oysters,

causing it to bioaccumulate, but whether a similar process also occurs for HEV is unknown. Studies in Scotland have shown high levels of contamination of individual wild shellfish (85%) (Crossan *et al.*, 2012), yet a low prevalence of HEV in retail shellfish samples (3%) (O'Hara *et al.*, 2018), and perhaps depuration or relaying techniques could be one explanation for this.

HEV in animals and animal products

Though UK studies have identified a high seroprevalence of HEV antibodies in pigs, and established that many pigs were positive for HEV at slaughter (Grierson *et al.*, 2015), little research has been conducted into the presence of HEV in other animals in the UK. Deer are well known to be reservoirs of HEV, and many other animals have also been identified as capable of carrying the virus. It would be interesting to plan a study which investigated the seroprevalence of HEV antibodies and the presence and levels of HEV RNA within cattle and deer. It would also be interesting to investigate the presence and levels of HEV RNA within cow's milk. Considering that Slot *et al.*, (2017) identified 14% of vegetarians within the study were seroprevalent for HEV antibodies in The Netherlands, this brings the question of how vegetarians who do not consume meat, but may consume other animal products, may have been infected with HEV. Therefore, an investigation of cow's milk would be interesting, especially considering that previous studies have found high levels of HEV in milk from cows which were also infected with HEV (Huang *et al.*, 2016; Yan *et al.*, 2016; Demirci *et al.*, 2019). An investigation of the prevalence of HEV in deer in the UK may also be interesting, as though it is a less commonly consumed meat in the UK, outbreaks have been known to occur after the consumption of it (Tei *et al.*, 2003). Identifying whether HEV is present in cattle, cow's milk and deer and then sequencing any positive samples may allow researchers to piece together the sources of some HEV cases, as sequences from animals in the UK are significantly lacking.

In addition to investigating the prevalence of HEV in other animals and animal products, given more time and resources I would like to investigate the cetacean samples which were positive for HEV by qRT-PCR. I would like to investigate, using shotgun sequencing, if there is indeed a HEV-like virus

within the cetacean samples, or whether perhaps there is a new genotype of HEV within them. Considering that the C_T values for some samples were low in comparison to some of the shellfish and sewage samples, it would be interesting to see why the attempts to sequence the virus initially using the metabarcoding approach were unsuccessful. The results of such a study would better inform researchers of whether a HEV-like virus circulates in cetaceans; and how related this virus is to existing HEV genotypes may better inform the host diversity and evolutionary history of the virus.

Final Conclusion

During this PhD I have identified that HEV is present infrequently, and at low levels within sewage and shellfish in the UK, and that it may also be present within cetaceans. I also identified that norovirus is present at much higher levels and prevalence within sewage and shellfish in the UK in comparison with HEV. I was also able to determine that G3 HEV sequences from within sewage and shellfish were closely related to other sequences which had been obtained from humans and pigs, and that some strains of norovirus which were detected in sewage were not commonly reported within the population despite their presence within samples. Additionally, I identified that the risk of illness from HEV during oyster consumption or water activities was low, but that the risk of illness from norovirus was high, though this was dependent on whether the norovirus was aggregated within the dose. The importance of this research is that I have identified that HEV can contaminate the aquatic environment in the UK from sewage release into surface waters, and that HEV has been detected within shellfish from the aquatic environment. However, the concentration and prevalence of HEV is low, and so, therefore, is the risk of illness from HEV in the aquatic environment, though it is not negligible. However, the same cannot be said for norovirus, which was detected in high levels in sewage and shellfish and was shown to constitute a high risk of norovirus illness during water activities and shellfish consumption.

These results highlight the risks to human health from faecal pollution of the

aquatic environment and show that more must be done to reduce and monitor faecal pollution within environmental waters, with a particular focus on significantly reducing the use of CSOs which release raw sewage into environmental waters. This may require significant investment in the infrastructure of sewage treatment in the UK.

References

Agrawal, S., Orschler, L. and Lackner, S. (2021) 'Long-term monitoring of SARS-CoV-2 RNA in wastewater of the Frankfurt metropolitan area in Southern Germany', *Scientific reports,* 11(1), pp. 5372-5372.

Almand, E. A., Moore, M. D. and Jaykus, L.-A. (2017) 'Norovirus binding to ligands beyond histo-blood group antigens', *Frontiers in microbiology,* 8, pp. 2549.

Baert, L., Uyttendaele, M., Stals, A., Van Coillie, E., Dierick, K., Debevere, J. and Botteldoorn, N. (2009) 'Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context', *Epidemiology & Infection,* 137(3), pp. 316-325.

Beyer, S., Szewzyk, R., Gnirss, R., Johne, R. and Selinka, H.-C. (2020) 'Detection and characterization of hepatitis E Virus genotype 3 in wastewater and urban surface waters in Germany', *Food and environmental virology*, pp. 1- 11.

Campos, C. J., Avant, J., Lowther, J., Till, D. and Lees, D. N. (2016) 'Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes', *Water research,* 103, pp. 224-232.

Crossan, C., Baker, P. J., Craft, J., Takeuchi, Y., Dalton, H. R. and Scobie, L. (2012) 'Hepatitis E virus genotype 3 in shellfish, United Kingdom', *Emerging infectious diseases,* 18(12), pp. 2085.

Degner, R. L. and Petrone, C. (1994) 'Consumer and restaurant manager reaction to depurated oysters and clams', *FAMRC industry report/(USA)*.

Demirci, M., Yiğin, A., Ünlü, Ö. and Kılıç, S. A. (2019) 'Detection of HEV RNA amounts and genotypes in raw milks obtained from different animals', *Mikrobiyoloji bulteni,* 53(1), pp. 43-52.

Di Bartolo, I., Pavoni, E., Tofani, S., Consoli, M., Galuppini, E., Losio, M. N., Ruggeri, F. M. and Varisco, G. (2015) 'Waterborne norovirus outbreak during a summer excursion in Northern Italy', *New Microbiol,* 38(1), pp. 109-12.

EFTEC (2002) 'Valuation of benefits to England and Wales of a revised bathing water quality directive and other beach characteristics using the choice experiment methodology', *Final Rep.*

Environment Agency. (2020) 'Event Duration Monitoring - Storm Overflows - 2020' [Database of CSO use throughout UK in 2020]. Available at: https://environment.data.gov.uk/dataset/21e15f12-0df8-4bfc-b763-45226c16a8ac (Accessed 23 Jun 2021).

Ettayebi, K., Crawford, S. E., Murakami, K., Broughman, J. R., Karandikar, U., Tenge, V. R., Neill, F. H., Blutt, S. E., Zeng, X. L., Qu, L., Kou, B., Opekun, A. R., Burrin, D., Graham, D. Y., Ramani, S., Atmar, R. L. and Estes, M. K. (2016) 'Replication of human noroviruses in stem cell-derived human enteroids', *Science,* 353(6306), pp. 1387-1393.

Farkas, K., Adriaenssens, E. M., Walker, D. I., McDonald, J. E., Malham, S. K.

and Jones, D. L. (2019) 'Critical Evaluation of CrAssphage as a Molecular Marker for Human-Derived Wastewater Contamination in the Aquatic Environment', *Food and environmental virology,* 11(2), pp. 113-119.

Farkas, K., Walker, D. I., Adriaenssens, E. M., McDonald, J. E., Hillary, L. S., Malham, S. K. and Jones, D. L. (2020) 'Viral indicators for tracking domestic wastewater contamination in the aquatic environment', *Water Res,* 181, pp. 115926.

Gao, S., Li, D., Zha, E., Zhou, T., Wang, S. and Yue, X. (2015) 'Surveillance of hepatitis E virus contamination in shellfish in China', *International journal of environmental research and public health,* 12(2), pp. 2026-2036.

Gherman, I., Holland, D., Clifford, R., Mahmoudzadeh, N., Uy, F., Adkin, A., Kainth, B., Cook, P., Day, A. and Wilson, A. (2020) 'Technical Report: Review of Quantitative Risk Assessment of foodborne norovirus transmission', *Science,* 1, pp. 1.

Green, K. Y., Kaufman, S. S., Nagata, B. M., Chaimongkol, N., Kim, D. Y., Levenson, E. A., Tin, C. M., Yardley, A. B., Johnson, J. A., Barletta, A. B. F., Khan, K. M., Yazigi, N. A., Subramanian, S., Moturi, S. R., Fishbein, T. M., Moore, I. N. and Sosnovtsev, S. V. (2020) 'Human norovirus targets enteroendocrine epithelial cells in the small intestine', *Nature communications,* 11(1), pp. 2759-2759.

Grierson, S., Heaney, J., Cheney, T., Morgan, D., Wyllie, S., Powell, L., Smith, D., Ijaz, S., Steinbach, F. and Choudhury, B. (2015) 'Prevalence of hepatitis E virus infection in pigs at the time of slaughter, United Kingdom, 2013', *Emerging infectious diseases,* 21(8), pp. 1396.

Guillois, Y., Abravanel, F., Miura, T., Pavio, N., Vaillant, V., Lhomme, S., Le Guyader, F. S., Rose, N., Le Saux, J.-C. and King, L. A. (2015) 'High proportion of asymptomatic infections in an outbreak of hepatitis E associated with a spit-roasted piglet, France, 2013', *Clinical Infectious Diseases,* 62(3), pp. 351-357.

Hassard, F., Sharp, J. H., Taft, H., LeVay, L., Harris, J. P., McDonald, J. E., Tuson, K., Wilson, J., Jones, D. L. and Malham, S. K. (2017) 'Critical review on the public health impact of norovirus contamination in shellfish and the environment: a UK perspective', *Food and environmental virology,* 9(2), pp. 123-141.

Huang, F., Li, Y., Yu, W., Jing, S., Wang, J., Long, F., He, Z., Yang, C., Bi, Y. and Cao, W. (2016) 'Excretion of infectious hepatitis E virus into milk in cows imposes high risks of zoonosis', *Hepatology,* 64(2), pp. 350-359.

Iaconelli, M., Purpari, G., Della Libera, S., Petricca, S., Guercio, A., Ciccaglione, A., Bruni, R., Taffon, S., Equestre, M. and Fratini, M. (2015) 'Hepatitis A and E viruses in wastewaters, in river waters, and in Bivalve Molluscs in Italy', *Food and environmental virology,* 7(4), pp. 316-324.

Iizuka, S., Oka, T., Tabara, K., Omura, T., Katayama, K., Takeda, N. and Noda, M. (2010) 'Detection of sapoviruses and noroviruses in an outbreak of gastroenteritis linked genetically to shellfish', *Journal of medical virology,* 82(7), pp. 1247-1254.

Ijaz, S., Said, B., Boxall, E., Smit, E., Morgan, D. and Tedder, R. S. (2013) 'Indigenous hepatitis E in England and Wales from 2003 to 2012: evidence of an emerging novel phylotype of viruses', *The Journal of infectious diseases,* 209(8), pp. 1212-1218.

Johne, R., Reetz, J., Ulrich, R., Machnowska, P., Sachsenröder, J., Nickel, P. and Hofmann, J. (2014) 'An ORF 1-rearranged hepatitis E virus derived from a chronically infected patient efficiently replicates in cell culture', *Journal of viral hepatitis,* 21(6), pp. 447-456.

Kay, D., Jones, F., Wyer, M. D., Fleisher, J. M., Salmon, R. L., Godfree, A. F., Zelenauch-Jacquotte, A. and Shore, R. (1994) 'Predicting likelihood of gastroenteritis from sea bathing: results from randomised exposure', *The Lancet,* 344(8927), pp. 905-909.

Kenney, S. P. (2019) 'The current host range of hepatitis E viruses', *Viruses,* 11(5), pp. 452.

Kroneman, A., Vennema, H., Deforche, K., v d Avoort, H., Peñaranda, S., Oberste, M. S., Vinjé, J. and Koopmans, M. (2011) 'An automated genotyping tool for enteroviruses and noroviruses', *J Clin Virol,* 51(2), pp. 121-5.

La Rosa, G., Pourshaban, M., Iaconelli, M., Vennarucci, V. S. and Muscillo, M. (2010) 'Molecular detection of hepatitis E virus in sewage samples', *Applied and environmental microbiology,* 76(17), pp. 5870-5873.

La Rosa, G., Proroga, Y., De Medici, D., Capuano, F., Iaconelli, M., Della Libera, S. and Suffredini, E. (2018) 'First detection of hepatitis E virus in shellfish and in seawater from production areas in Southern Italy', *Food and environmental virology,* 10(1), pp. 127-131.

Leonard, A. F., Zhang, L., Balfour, A. J., Garside, R. and Gaze, W. H. (2015) 'Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters', *Environment international,* 82, pp. 92-100.

Lindesmith, L. C., Donaldson, E. F., Lobue, A. D., Cannon, J. L., Zheng, D. P., Vinje, J. and Baric, R. S. (2008) 'Mechanisms of GII.4 norovirus persistence in human populations', *PLoS Med,* 5(2), pp. e31.

Lodder, W. J. and de Roda Husman, A. M. (2005) 'Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands', *Applied and environmental microbiology,* 71(3), pp. 1453-1461.

Lowther, J. (2011) 'Investigation into the prevalence, distribution and levels of norovirus titre in oyster harvesting areas in the UK', *Weymouth: CEFAS Weymouth laboratory*.

Lowther, J., Gustar, N., Powell, A., O'Brien, S. and Lees, D. (2018) 'A one-year survey of norovirus in UK oysters collected at the point of sale', *Food and environmental virology,* 10(3), pp. 278-287.

Lowther, J. A., Avant, J. M., Gizynski, K., Rangdale, R. E. and Lees, D. N. (2010) 'Comparison between Quantitative Real-Time Reverse Transcription PCR Results for Norovirus in Oysters and Self-Reported Gastroenteric Illness in Restaurant Customers', *Journal of Food Protection,* 73(2), pp. 305-311.

Lowther, J. A., Cross, L., Stapleton, T., Gustar, N. E., Walker, D. I., Sills, M., Treagus, S., Pollington, V. and Lees, D. N. (2019) 'Use of F-Specific RNA Bacteriophage to Estimate Infectious Norovirus Levels in Oysters', *Food and Environmental Virology,* 11(3), pp. 247-258.

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E. and Lees, D. N. (2012) 'Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom', *Applied and environmental microbiology,* 78(16), pp. 5812-5817.

Maalouf, H., Zakhour, M., Le Pendu, J., Le Saux, J.-C., Atmar, R. and Le Guyader, F. (2010) 'Distribution in Tissue and Seasonal Variation of Norovirus Genogroup I and II Ligands in Oysters', *Applied and Environmental Microbiology*, 76(16), pp. 5621-5630, *Applied and Environmental Microbiology* Available: American Society for Microbiology.

Martin, J., Klapsa, D., Wilton, T., Zambon, M., Bentley, E., Bujaki, E., Fritzsche, M., Mate, R. and Majumdar, M. (2020) 'Tracking SARS-CoV-2 in Sewage: Evidence of Changes in Virus Variant Predominance during COVID-19 Pandemic', *Viruses,* 12(10), pp. 1144.

Matsushima, Y., Ishikawa, M., Shimizu, T., Komane, A., Kasuo, S., Shinohara, M., Nagasawa, K., Kimura, H., Ryo, A., Okabe, N., Haga, K., Doan, Y. H., Katayama, K. and Shimizu, H. (2015) 'Genetic analyses of GII.17 norovirus strains in diarrheal disease outbreaks from December 2014 to March 2015 in Japan reveal a novel polymerase sequence and amino acid substitutions in the capsid region', *Euro Surveill,* 20(26).

McCaustland, K. A., Krawczynski, K., Ebert, J. W., Balayan, M. S., Andjaparidze, A. G., Spelbring, J. E., Cook, E. H., Humphrey, C., Yarbough, P. O., Favorov, M. O., Carson, D., Bradley, D. W. and Robertson, B. H. (2000) 'Hepatitis E virus infection in chimpanzees: a retrospective analysis', *Archives of Virology,* 145(9), pp. 1909-1918.

Medema, G., Been, F., Heijnen, L. and Petterson, S. (2020a) 'Implementation of environmental surveillance for SARS-CoV-2 virus to support public health decisions: Opportunities and challenges', *Current Opinion in Environmental Science & Health*.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R. and Brouwer, A. (2020b) 'Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands', *Environmental Science & Technology Letters,* 7(7), pp. 511-516.

Miura, F., Matsuyama, R. and Nishiura, H. (2018) 'Estimating the Asymptomatic Ratio of Norovirus Infection During Foodborne Outbreaks With Laboratory Testing in Japan', *Journal of epidemiology,* 28(9), pp. 382-387.

Nordgren, J., Kindberg, E., Lindgren, P.-E., Matussek, A. and Svensson, L. (2010) 'Norovirus gastroenteritis outbreak with a secretor-independent susceptibility pattern, Sweden', *Emerging infectious diseases,* 16(1), pp. 81.

Oeser, C., Vaughan, A., Said, B., Ijaz, S., Tedder, R., Haywood, B., Warburton, F., Charlett, A., Elson, R. and Morgan, D. (2019) 'Epidemiology of Hepatitis E in England and Wales: A 10-Year Retrospective Surveillance Study, 2008–2017',

The Journal of Infectious Diseases, 220(5), pp. 802-810.

Ondrikova, N., Clough, H. E., Cunliffe, N. A., Iturriza-Gomara, M., Vivancos, R. and Harris, J. P. (2021) 'Understanding norovirus reporting patterns in England: a mixed model approach', *BMC Public Health,* 21(1), pp. 1245.

O'Hara, Z., Crossan, C., Craft, J. and Scobie, L. (2018) 'First Report of the Presence of Hepatitis E Virus in Scottish-Harvested Shellfish Purchased at Retail Level', *Food and environmental virology*, pp. 1-5.

Pouillot, R., Smith, M., Van Doren, J. M., Catford, A., Holtzman, J., Calci, K. R., Edwards, R., Goblick, G., Roberts, C., Stobo, J., White, J., Woods, J., DePaola, A., Buenaventura, E. and Burkhardt, W. (2021) 'Risk Assessment of Norovirus Illness from Consumption of Raw Oysters in the United States and in Canada', *Risk Anal*.

Public Health England. (2020) *PHE National norovirus and rotavirus Report*, GOV.UK: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/871753/Norovirus_update_2020_weeks_08_to_09.pdf.

Public Health England. (2021) *2018 to 2019 Season Norovirus Report National norovirus laboratory and outbreak data in England*, gov.uk: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/966775/2021-01-29-Norovirus_annual_report_2018_to_2019.pdf.

Rivadulla, E., Varela, M. F., Mesquita, J. R., Nascimento, M. S. and Romalde, J. L. (2019) 'Detection of hepatitis E virus in shellfish harvesting areas from Galicia (Northwestern Spain)', *Viruses,* 11(7), pp. 618.

Romalde, J., Rivadulla, E., Varela, M. and Barja, J. (2018) 'An overview of 20 years of studies on the prevalence of human enteric viruses in shellfish from Galicia, Spain', *Journal of applied microbiology,* 124(4), pp. 943-957.

Said, B., Ijaz, S., Kafatos, G., Booth, L., Thomas, H. L., Walsh, A., Ramsay, M. and Morgan, D. (2009) 'Hepatitis E outbreak on cruise ship', *Emerging infectious diseases,* 15(11), pp. 1738.

Salvador, D., Neto, C., Benoliel, M. J. and Caeiro, M. F. (2020) 'Assessment of the Presence of Hepatitis E virus in Surface Water and Drinking Water in Portugal', Microorganisms, 8(5), pp. 761.

Sano, D., Perez-Sautu, U., Guix, S., Pinto, R. M., Miura, T., Okabe, S. and Bosch, A. (2011) 'Quantification and genotyping of human sapoviruses in the Llobregat river catchment, Spain', *Applied and environmental microbiology,* 77(3), pp. 1111-1114.

Slot, E., Zaaijer, H. L., Molier, M., Van den Hurk, K., Prinsze, F. and Hogema, B. M. (2017) 'Meat consumption is a major risk factor for hepatitis E virus infection', *PloS one,* 12(4), pp. e0176414.

Smith, D. B., Paddy, J. O. and Simmonds, P. (2016) 'The use of human sewage screening for community surveillance of hepatitis E virus in the UK', *Journal of medical virology,* 88(5), pp. 915-918.

Smith, I., Said, B., Vaughan, A., Haywood, B., Ijaz, S., Reynolds, C., Brailsford, S., Russell, K. and Morgan, D. (2021) 'Case–Control Study of Risk Factors for Acquired Hepatitis E Virus Infections in Blood Donors, United Kingdom, 2018–2019', *Emerging Infectious Diseases,* 27(6), pp. 1654-1661.

Tam, C., Viviani, L., Adak, B., Bolton, E., Dodds, J., Cowden, J., Evans, M., Gray, J., Hunter, P. and Jackson, K. (2012) 'The second study of infectious intestinal disease in the community (IID2 study)', *UK Food Standards Agency*.

Tang, Y., Wang, J., Xu, Z., Guo, Y., Qian, W. and Xu, J. (1991) 'A serologically confirmed, case-control study, of a large outbreak of hepatitis A in China, associated with consumption of clams', *Epidemiology & Infection,* 107(3), pp. 651-657.

Tei, S., Kitajima, N., Takahashi, K. and Mishiro, S. (2003) 'Zoonotic transmission of hepatitis E virus from deer to human beings', *The Lancet,* 362(9381), pp. 371-373.

Teunis, P. F., Moe, C. L., Liu, P., E. Miller, S., Lindesmith, L., Baric, R. S., Le Pendu, J. and Calderon, R. L. (2008) 'Norwalk virus: how infectious is it?', *Journal of medical virology,* 80(8), pp. 1468-1476.

UK Government. EUR-Lex. (2021) *Commission Implementing Regulation (EU) 2019/627 of 15 March 2019 laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) No 2074/2005 as regards official controls*: UK Government.

Van Abel, N., Schoen, M. E., Kissel, J. C. and Meschke, J. S. (2017) 'Comparison of Risk Predicted by Multiple Norovirus Dose–Response Models and Implications for Quantitative Microbial Risk Assessment', *Risk Analysis,* 37(2), pp. 245-264.

Vergara, G. G. R. V., Rose, J. B. and Gin, K. Y. H. (2016) 'Risk assessment of noroviruses and human adenoviruses in recreational surface waters', *Water Research,* 103, pp. 276-282.

Villabruna, N., Koopmans, M. P. G. and de Graaf, M. (2019) 'Animals as Reservoir for Human Norovirus', *Viruses,* 11(5).

Villalba, M. C. M., Martínez, D. C., Ahmad, I., Lay, L. A. R., Corredor, M. B., March, C. G., Martínez, L. S., Martínez-Campo, L. S. and Jameel, S. (2017) 'Hepatitis E virus in bottlenose dolphins Tursiops truncatus', *Diseases of aquatic organisms,* 123(1), pp. 13-18.

Williams, S. and O'Brien, S. J. (2019) *NoVAS: Assessing the contribution made by the food chain to the burden of UK acquired norovirus infection*, UK: Cefas, Fera, Leatherhead Food Research, Liverpool Clinical Laboratories (Royal Liverpool and Broadgreen University Hospital Trust), UK Health Security Agency, University of East Anglia and University of Liverpool (Accessed: 21 July 2021).

Yan, B., Zhang, L., Gong, L., Lv, J., Feng, Y., Liu, J., Song, L., Xu, Q., Jiang, M. and Xu, A. (2016) 'Hepatitis E virus in yellow cattle, Shandong, Eastern China',

Emerging infectious diseases, 22(12), pp. 2211.

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Appendix

Letter from Conal Wright

Conal Wright

Flat 4, 76 Poole Road

Bournemouth

Dorset

BH4 9DZ

05/02/2022

Samantha Treagus

CEFAS

The Nothe

Barrack Road

Weymouth

Dorset

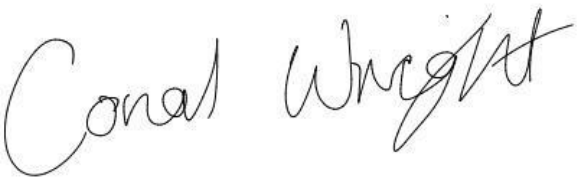
DT4 8UB

Dear Samantha,

I hereby allow the use of the figure "The geographical distribution of HEV genotypes 1-4" in the thesis "The Transmission of Enteric Viruses through the Aquatic Environment in the UK" as this was a collaborative project between us for the publication "The Foodborne Transmission of Hepatitis E Virus to Humans".

Best wishes,

Conal Wright



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The foodborne transmission of Hepatitis E virus to humans

Samantha Treagus[1, 3], Conal Wright[2], Craig Baker-Austin[3], Ben Longdon[1], James Lowther[3]

Author for editorial correspondence:

Samantha Treagus,

Centre for Environment Fisheries and Aquaculture Science

Barrack Road

Weymouth

Dorset

DT4 8UB

Phone: +44 (0) 1305-206635

samantha.treagus@cefas.co.uk

ORCID iD: 0000-0002-1905-9024

Author affiliations: [1] Biosciences, college of Life and Environmental Sciences, University of Exeter, Penryn campus, Penryn, Cornwall, United Kingdom. [2]Seacorp Technologies Limited, Bournemouth, United Kingdom. [3] Centre for Environment, Fisheries and Aquaculture Science, Weymouth, United Kingdom.

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Abstract

Globally, Hepatitis E virus (HEV) causes over 20 million cases worldwide. HEV is an emerging and endemic pathogen within economically developed countries, chiefly resulting from infections with genotype 3 (G3) HEV. G3 HEV is known to be a zoonotic pathogen, with a broad host range. The primary source of HEV within more economically developed countries is considered to be pigs, and consumption of pork products is a significant risk factor and known transmission route for the virus to humans. However, other foods have also been implicated in the transmission of HEV to humans. This review consolidates the information available regarding transmission of HEV and looks to identify gaps where further research is required to better understand how HEV is transmitted to humans through food.

Keywords

Hepatitis E virus, foodborne, zoonotic, transmission, meat, shellfish

Introduction

Globally, hepatitis E virus (HEV), of the family *Hepeviridae*, is considered the most common cause of acute viral hepatitis. There were an estimated 20 million infections worldwide annually in 2005 from genotype 1 (G1) and 2 (G2) HEV combined (Rein *et al.*, 2012), and 44,000 recorded fatalities due to the virus in 2015 (World Health Organisation, 2019). Generally, HEV causes an acute, self-limiting infection which resolves within a few weeks; however, in some persons (such as the immunocompromised) it can cause chronic infections, fulminant hepatitis (acute liver failure) and extrahepatic manifestations (infections in other organs), which can be fatal. Table 1 shows a summary of the pattern of infection of the different HEV genotypes, demonstrating the variable clinical manifestations and factors such as average age of infection and gender, where these are known. There is limited data to be able to estimate the number of infections worldwide, but with a high level of asymptomatic infections seen in numerous outbreaks it is probable that more infections occur worldwide than estimated in 2005 (Guillois *et al.*, 2015; Yin *et al.*, 2019); especially considering that this estimate was made only considering G1 and G2.

HEV was once thought only to be endemic to certain economically developing countries within Asia and Africa, but research over the past decade has highlighted the emergence of HEV within higher income countries. The virus spreads through a faecal oral route, making it easily transmissible through faecally contaminated water. Indeed, this is thought to be the main transmission route within China, where G1 and genotype 4 (G4) HEV are dominant (Wang *et al.*, 2001). However, it is also possible for the virus to be transmitted through foodstuffs such as pork, due to the ability for some HEV genotypes to infect non-human animals. Currently, the virus is classified into eight genotypes, with G1-4 and genotype 7 (G7) capable of infecting humans. There is, however, a diverse host range for the different genotypes, with G1 and G2 generally only infecting humans and non-human primates, but genotypes 3-8 infecting many other animals, such as pigs, deer, camels, rabbits, and dolphins. A summary of the different host species of HEV was published recently (Kenney, 2019), and is summarised briefly in table 2. Genotype 3 (G3) HEV has been found to be the

most geographically diverse of the viruses thus far (Pérez-Gracia *et al.*, 2015), and is the genotype which has emerged in the past two decades in many developed countries. The geographical distribution of genotypes 1-4 can be seen in Fig 1.

G3 HEV is thought to be spread primarily through the consumption of undercooked pork products from infected pigs, however it is unknown if all transmission routes to humans have been identified. The current theories and known routes of transmission can be seen in Fig 2. This review will discuss theoretical routes of HEV transmission to humans through foodstuffs and identify areas which require further research for better understanding of the virus.

HEV in Pigs and Pork Products

Over the past two decades, evidence has accumulated implicating pigs and other animals in the zoonotic transmission of G3 HEV to humans (Tei *et al.*, 2003; Guillois *et al.*, 2015; Lhomme *et al.*, 2013; Rivero-Juarez *et al.*, 2017). In 1998 it was shown that a HEV strain isolated from an acute HEV patient in the USA was capable of infecting pigs, and that a genetically similar strain isolated from pigs was capable of infecting non-human primates; suggesting a significant possibility that pigs could act as a zoonotic source of HEV (Meng *et al.*, 1998). Since this study, many countries have noted the emergence of HEV cases. At this stage, it was thought that HEV was only endemic in developing countries; (such as those in Asia, Africa, and central America) and that HEV cases in non-endemic areas were obtained through travel. However, studies such as those by Dalton *et al.*, (2007) and Fraga *et al.*, (2017) identified that indigenous cases of HEV were occurring in economically developed countries such as the UK and Switzerland. It is now widely accepted that pigs are a zoonotic source of HEV transmission to humans and may be at least partially responsible for increasing cases worldwide annually, though increased detection and awareness of HEV may also play a small role in the observed increase in cases. Outbreaks of HEV have directly been linked to pork product consumption, including an outbreak in Spain linked to consumption of wild boar (Rivero-Juarez *et al.*, 2017), and another outbreak associated with consumption of a spit-roasted

93 piglet in France (Guillois *et al.*, 2015).

94 Consumption of pork products is now considered a significant risk factor for developing HEV
95 infection, which is concerning considering the seroprevalence levels in European pigs (Said *et al.*,
96 2014; Slot *et al.*, 2017). Table 3 shows a non-exhaustive list of countries that have detected anti-HEV
97 antibodies in pigs, and HEV RNA in pork products.

98 Considering Table 3, it is possible that the HEV prevalence in pork sausages may be over- or under-
99 estimated by the fact that the sample sizes for some of these studies are relatively small. It is also
100 important to note that different methods have been used between studies, some of which have
101 been shown to be less sensitive for detecting HEV than others. Interestingly, the seroprevalence of
102 HEV in pigs is significantly higher than the prevalence of HEV RNA in pork products in most cases;
103 this discrepancy is expected as not all pigs would be viraemic at slaughter.

104 It has been found that HEV generally infects swine asymptomatically at an early point in their life
105 (prior to 6 months of age (de Oya *et al.*, 2011)), and that because of this, many pigs are seropositive
106 at the time of slaughter (Grierson *et al.*, 2015; Rose *et al.*, 2011; de Oya *et al.*, 2011). The slaughter
107 age of pigs is normally slightly before one year of age. The transmission between pigs is suspected to
108 be through a faecal-oral route and this is likely to be due to the high shedding that is seen in pig
109 faeces and urine (Bouwknegt *et al.*, 2009; Halbur *et al.*, 2001). Infection with HEV early in life means
110 that there is a lower chance of the pigs being viraemic at slaughter, and they are therefore less likely
111 to be capable of HEV transmission to humans through the pork food chain. However, whether HEV
112 causes life-long immunity in swine after recovery has been open to debate. In rhesus macaques and
113 humans, the anti-HEV IgG antibodies (characteristic of long term immunity) wane over a variable
114 number of years until they are undetectable, and the period for which individuals may be IgG-
115 positive for varies (Arankalle et al., 1999; Lee *et al.*, 1994). This may not be an issue in pigs as they
116 are normally slaughtered before a year of age, so early life infections would likely allow immunity
117 against subsequent HEV challenge over their lifetime. However, it has been shown that animals can
118 be re-infected by different HEV strains (De Deus *et al.*, 2008), and whether or not one strain can

confer protective immunity to all other HEV strains has also been contested. It has been shown that after infection with one strain of G3 HEV, pigs developed some protective immunity against other strains within the same genotype, and also within G4 (Sanford *et al.*, 2011). However, in rhesus monkeys, it was shown that infection with one strain from a genotype could not confer protective immunity to a strain in a different genotype upon subsequent challenge; and that in some cases, infection with a different strain from the same genotype could also not confer protective immunity (Huang *et al.*, 2008). If there is genetic or environmental variation in host development of immunity to HEV, and some strains of HEV may not provide protection against others, then farms with multiple circulating HEV strains could be more likely to have viraemic pigs at the time of slaughter. This would therefore mean that there could be a higher likelihood of contracting HEV from undercooked pork products or food products containing raw pig components from these sources.

It has been reported that 21% of pigs in the UK tested positive for HEV RNA at the time of slaughter (Grierson *et al.*, 2015), and that in the USA 6.3% of pigs from slaughterhouses were HEV RNA positive (Sooryanarain *et al.*, 2020). As such it is probable that the consumption of raw and undercooked pork products is acting as a transmission route of HEV to humans.

Table 4 summarises studies that have investigated the thermal inactivation of HEV in non-food matrix samples. Inactivation condition combinations found sufficient to inactivate virus in these studies are shown in Fig 3. Results vary between the different studies, and several factors make comparison difficult. The studies use a variety of different units or expressions of reduction/inactivation. In the study by Huang *et al.*, (1999) a temperature of 56°C for 30 minutes was reported to completely inactivate the virus; however the virus was only left to grow for a relatively short period of time (72 hours). However, it was shown that HEV was still viable following similar heat treatments in cell culture studies with longer growth periods (Emerson et al., 2005; Tanaka *et al.*, 2007). Schielke *et al.*, (2011) used RNase treatment in an attempt to remove viral RNA that had broken from the capsid after heat treatment, assuming this would remove RNA from non-viable virus. However, it is unknown if any RNA from viable virus could have been lost during this

145 treatment, and the standard deviations seen within the results were relatively large. Without a cell
146 culture component, it is not possible to say with 100% certainty that remaining detected RNA was
147 from viable virus. In addition to these limitations, it is known that HEV is difficult to culture
148 effectively *in vitro*, often requiring large titres of virus to begin the culture, and therefore it is
149 possible that the inactivation requirements for HEV have been underestimated as treatments
150 sufficient to eliminate infectivity *in vitro* may not completely eliminate *in vivo* infectivity. Some
151 researchers have investigated different cell lines and strains of HEV which appear to be more
152 efficiently cultured *in vitro* due to insertions within the HEV genome, however culturing these strains
153 still requires large titres of virus to begin the culturing process (10^6 copies/ml) (Johne *et al.*, 2014;
154 Schemmerer *et al.*, 2016).
155 It is possible that interactions of HEV with organic molecules in food matrices may cause the thermal
156 inactivation temperature to be higher. Some studies have investigated the thermal inactivation of
157 HEV within food stuffs, such as liver and pork sausage (summarised in Table 5). These studies have
158 all taken different approaches to heat inactivation of the virus and the food types used, and
159 therefore it is difficult to compare the results and form a definitive answer for the heat treatment
160 required to inactivate the virus within foodstuffs. Feagins *et al.*, (2007) identified that boiling or stir-
161 frying infected pig liver to an internal temperature of 71°C for 5 minutes could prevent infection
162 when the liver was then fed to pigs. However, though the pigs were not infected by this oral dose, it
163 is known that pigs commonly require a high dose of HEV to become infected through the faecal-oral
164 route (Kasorndorkbua *et al.*, 2004), of approximately 10^6 genome copies (Andraud *et al.*, 2013), and
165 therefore it is possible that lower doses of active virus were still present in these food stuffs. The
166 oral infectious dose for humans is unknown. Intravenous inoculation of pigs with the cooked
167 foodstuffs as carried out by Barnaud *et al.*, (2012) is likely to have provided a more accurate
168 estimate of whether viable virus still existed, especially as intravenous inoculation of pigs has been
169 reported to require much lower HEV doses to cause infection (Dähnert *et al.*, 2018). Imagawa *et al.*,
170 (2018) reported similar inactivation requirements to Feagins *et al.*, (2007). However, the limit of

detection for the cell culture system was 10^4-10^5 genome copies, and therefore, as with the previous study, viable virus remaining in the minced pork may not have been detected in the cell culture system. In addition, the different food preparations between the studies may have influenced viral stability, as could the different strains of G3 HEV used. The initial viral titres used could also have influenced the results and explain why a longer treatment time was needed in some studies. Further research is clearly required to investigate HEV inactivation within foods. This may require a more efficient cell culturing method and an assessment of different foods, cooking methods and HEV strains. However, taking the results of studies conducted in non-food and food (pork product) samples together, a conservative measure would be to cook pork products for longer than 20 minutes at temperatures higher than 72°C.

In addition to the potential for HEV to survive some cooking processes, raw pork products are used in some consumables. Raw blood products are commonly used in ready-cooked foods such as processed ham (fibrinogen) and other blood proteins are used as food additives such as emulsifiers. Spray-dried plasma powder (SDPP) is also used in domestic and farm animal foods but this has some heat processing prior to use. SDPP is commonly fed to weaned piglets; although a previous study reported no transmission of HEV in pigs fed spray dried plasma products that were positive for HEV RNA (Pujols *et al.*, 2014), and therefore the heating of spray dried plasma may be sufficient to inactivate the virus present. However, as other porcine products are not subjected to heat processes before they are used, they could constitute a transmission risk to humans through use in food. A study conducted in 2017 found that of 36 liquid porcine products derived from blood, 33 were positive for HEV RNA, and seven of 24 spray dried plasma products were also positive for HEV RNA (Boxman *et al.*, 2017). This is especially significant when blood products from multiple animals are commonly pooled together, meaning that products from one viraemic animal could contaminate a batch and lead to widespread HEV transmission through many different food products.

Pork product consumption has been considered to be a major risk factor in the development of HEV due to the connection to foodborne outbreaks and the fact that HEV in pork products can reach high

levels (e.g. $7x10^4$ genome copies/g in liver pâté in the Netherlands, (Boxman *et al.*, 2019)). However, pigs are not the only animals consumed which can act as reservoirs for the virus.

HEV in other Land Animals and Animal Products

In addition to pigs, deer have been reported to be infected with HEV in many different countries (summarised in Table 6). It is important to identify the transmission risk that deer may have to humans, as HEV outbreaks have been directly linked to the consumption of raw deer meat. For example, in Japan, multiple people who had consumed raw Sika deer meat contracted HEV 6-7 weeks later (typical of the HEV incubation period), with the HEV sequence confirmed as 100% identical between the meat and infected patients (Tei *et al.*, 2003). With the presence of HEV in deer being so variable between studies (perhaps due to study limitations such as sample size), further research is required to identify the level of active HEV infection within deer populations through larger prevalence studies. However, with the number of countries that have detected HEV in deer, and the occurrence of foodborne outbreaks from deer meat, deer could be acting as another reservoir for HEV.

There have been reports of HEV infections in cattle (*Bos taurus*) from China, where both antibody seroprevalence and HEV RNA of G4 has been identified in multiple studies. Hu and Ma, (2010) showed the presence of G4 HEV RNA in 8.8% of cattle from Xinjiang Autonomous Region. A subsequent study then identified that 37.1% of tested dairy cows in Yunnan Province were HEV RNA positive, and that 100% of the HEV-positive cows were producing milk that contained HEV RNA (Huang *et al.*, 2016). A further study in Shandong Province also found 3% of yellow cattle to be G4 HEV RNA positive, with 47% seroprevalent for anti-HEV antibodies (Yan *et al.*, 2016). A study in Turkey identified HEV from G1, G3 and G4 in 20.3% of raw milk samples from various domestic animals (cows, sheep, goats, donkeys) (Demirci *et al.*, 2019). Other studies investigating HEV in cattle have produced negative or mixed results; a study in Beijing, China identified 29.4% of cattle were seroprevalent for HEV, but no HEV RNA could be detected (Chang *et al.*, 2009). A study in Burkina

Faso also found 26.4% of 72 cattle to be seroprevalent for HEV (Ouoba *et al.*, 2019). Another study in Germany testing 400 milk samples found no evidence of HEV RNA; although G4 HEV is much less commonly reported in Europe than in Asia (Baechlein and Becher, 2017). Likewise, a study in Belgium also found no evidence of HEV RNA in cow milk or faeces (Vercouter *et al.*, 2018). In the USA, Yugo *et al.*, (2019) identified that of 983 cows, 20.4% were seroprevalent for anti-HEV antibodies; however HEV RNA could not be detected in any of the cows. The authors concluded that this may have been because of an antigenically similar relative to HEV rather than due to HEV itself, which could be possible as G4 HEV is not thought to be endemic to the USA; however this would call into question the specificity of ELISA assays and studies investigating HEV seroprevalence. Notwithstanding the significant number of studies with negative findings, these results are concerning as meat and dairy from cows are consumed worldwide by humans, and the possibility that cows could be a HEV reservoir could have a significant impact on our understanding of HEV transmission to humans. More research worldwide is therefore needed to identify which HEV genotypes are capable of infecting cattle, and to find the prevalence of HEV in cattle and dairy products. This will help to identify the risk of transmission of HEV from cattle to humans.

Goats have also been shown to be potential reservoirs for HEV infection, which is important due to goat meat, milk, and cheese production. In Italy in 2016, 9.2% of goat faecal samples from six farms were found to be positive for HEV RNA, belonging to G3 strains which were highly related to strains found in pigs and humans (Di Martino *et al.*, 2016). Also, in Yunnan province, China, Long *et al.*, (2017) found 70.3% of goat faecal samples to be positive for HEV RNA, with milk samples from these animals also positive for HEV RNA. The strains obtained from these animals were from G4 HEV; and the Huang *et al.*, (2016) and Long *et al.*, (2017) studies highlight that farms with mixed animals may demonstrate a higher risk of HEV transmission. Another study in the Tai'an Region in China identified 4% of goat livers to be HEV positive, with G4 HEV that was similar to cow HEV detected in the same region (Li *et al.*, 2017). In Turkey, 18.5% of goat milk samples were reported positive for HEV RNA

(Demirci *et al.*, 2019). Meanwhile in Burkina Faso, 28.4% of 81 goats were found to have anti-HEV antibodies (Ouoba *et al.*, 2019). In the USA however, (Sanford *et al.*, 2013) suggested that a HEV-related agent was causing HEV in goats after discovering a seroprevalence of 16% but a lack of any HEV RNA; however this again calls into question the accuracy of HEV ELISAs. In addition, no HEV RNA was detected by conventional RT-PCR in goats that had been experimentally infected with three different HEV strains from G1, G3 and G4 in this study; however the sensitivity of this PCR may be lower than previously reported HEV PCR assays, as assays which target a larger amplicon are generally observed to have lower sensitivity (Debode *et al.*, 2017), and qRT-PCR has generally been observed to be more sensitive for amplicon detection of HEV and other amplicon targets (Zhao *et al.*, 2007; Zemtsova et al., 2015).

Dromedary camels have been implicated in the transmission of G7 HEV to humans. In one case, a patient who regularly consumed camel meat and milk contracted chronic G7 HEV after a liver transplant (Lee *et al.*, 2016). This chronicity is likely to have been opportunistic and influenced by immunosuppressive medication to prevent organ rejection. In a separate paper, HEV was demonstrated to be seroprevalent in 23.1% of dromedary camels which originated in Sudan and Saudi Arabia (El-Kafrawy *et al.*, 2020). Due to the recent discovery of this genotype of HEV, and its implication in human infection, further research is warranted to investigate how widespread camel HEV is within countries which regularly consume camel products to determine the risk such products may have for the foodborne transmission of HEV.

Rabbits and related species e.g. hares are also gaining increasing attention for their potential to transfer HEV to humans through consumption of meat. In France, five cases of rabbit HEV (defined within Orthohepevirus A, G3ra) were identified in confirmed HEV-positive patients out of 919 from 2015-2016 (Abravanel *et al.*, 2017). Several countries have identified rabbits to be seroprevalent and RNA positive for HEV (table 7). The number of observations and the apparent ability of humans to contract rabbit HEV suggests that it is a source of zoonotic HEV transmission. However, with most

human cases worldwide belonging to other genotypes and sub-genotypes, rabbits are likely to be the cause of only a minority of cases. Studies have shown mixed results in terms of the ability of rabbits to carry other sub-genotypes of HEV. Zhang *et al.*, (2017) has shown that though rabbits are capable of carrying sub-genotype 3ra, attempts to cause infections with another sub-genotype (3b) were unsuccessful; however, Hammerschmidt *et al.*, (2017) identified a wild rabbit with HEV sub-genotype 3g. Further research is therefore needed to identify which genotypes or sub-genotypes of HEV are capable of infecting rabbits. An interesting observation from the studies summarised in table 7 is that concordance in HEV prevalence between different samples from the same animals is often lacking. For example, Burt *et al.*, (2016) found that 60% of liver samples from 32 animals were HEV positive, however only 16% of these 32 animals were faecally shedding the virus. It may therefore be wise to identify standardised testing methods worldwide for identification of infected animals, with a decision made on what samples to test and which assays are best to use, to avoid underestimating HEV prevalence.

Despite identifying that animal product consumption is a risk factor in the transmission of HEV it is quite possible that there are other food transmission routes. One study in the Netherlands showed that though seroprevalence of HEV antibodies was higher in meat-eaters (22.8%), vegetarians still displayed a relatively high seroprevalence (13.8%) (Slot *et al.*, 2017). This suggests that either they became HEV-positive before becoming vegetarian through animal meat, or they were infected through other transmission routes. Fig 2 shows the known and theorised routes of HEV transmission. One transmission route, which is much more tightly controlled now, was the transmission of HEV through blood transfusion (Hewitt *et al.*, 2014), which may be one way to explain the seroprevalence levels in vegetarians. Another explanation could be consumption of dairy products such as milk. It is also possible to contract HEV through organ transplant with an infected organ (Pas *et al.*, 2012), and organ transplant-associated cases commonly result in chronic infections due to immunosuppression medication.

Contamination of the Aquatic Environment

In addition to medical routes of transmission, it is important to consider the impact that HEV within animal farm run off and sewage has on the aquatic environment. It has been suggested that human and farm sewage may have a part to play in other HEV transmission routes, potentially through farm run-off from animal slurry stores or application of animal slurry to crops; and through contamination of surface waters used for irrigation and shellfish farms. Raw human sewage collected at two week intervals in 2014-15 from a sewer which serves the whole of Edinburgh was found to contain HEV in 93% of the samples collected (Smith et al., 2016), and many other countries have also detected HEV in human sewage influent, such as Spain, Switzerland, Portugal and France (Clemente-Casares *et al.*, 2009; Clemente-Casares *et al.*, 2003; Rodriguez-Manzano *et al.*, 2010; Matos *et al.*, 2018; Masclaux *et al.*, 2013). This could therefore mean that when storm overflows discharge into water courses such as rivers and seas, HEV contamination can occur. Because there are many different types of wastewater treatment practises, and many combinations of practices between wastewater treatment plants, it is difficult to know which wastewater treatment plants will be more effective at removing viruses from wastewater. However, other viruses such as adenovirus and norovirus are commonly found in treated sewage (Bofill-Mas *et al.*, 2006; Campos *et al.*, 2016). It is therefore possible that in addition to storm overflows, inadequate treatment of sewage could result in HEV pollution of the aquatic environment, especially considering that HEV is a single stranded RNA non-enveloped virus like norovirus. Pig farm and slaughterhouse sewage has also been found to be positive for HEV in multiple countries, for example, HEV RNA was detected in sewage from one of twelve slaughterhouses in Spain (Pina *et al.*, 2000), whilst 75% of swine slurry samples collected from Italian pig farms were HEV positive (La Rosa *et al.*, 2017), and both fresh swine faecal material and pooled stored slurry from pig farms in the USA were shown to contain HEV (Kasorndorkbua *et al.*, 2005). Human sewage, pig farm run off and abattoir outflows could also therefore be contaminating water courses with HEV, which is supported by studies in Italy, the Philippines and Cambodia, showing river water contamination with HEV (Iaconelli *et al.*, 2015; Li *et al.*, 2014; Baez *et*

al., 2017; Rodriguez-Manzano *et al.*, 2010).

Countries within the European Union must conform to EU regulations on how farm manure, animal carcasses and digestive tract content are processed, transported, stored, used as crop fertiliser, and disposed of. The sewage and wastewater that originates on farms must either be discharged to public sewers or treated in a sewage treatment plant on the farm before the effluent can be discharged to surface waters, and a permit is required for the processing and disposal of sewage and wastewater in this way. However, it is possible for farms within EU countries to use sewage and slurry that has been produced on a farm to be spread on crops at the same farm, without prior processing, for the sake of fertiliser or soil quality improvement (European Commission, 2001). Accordingly, the United States also allows manure originating on one farm to be spread on crops from that farm (Environment Protection Agency, NA). However, manure use and farm practises are likely to be more diverse and potentially problematic in countries within Asia, Africa, and South America.

Previous studies have shown that sewage treatment processes such as long term fermentation and composting are likely to be capable of removing HEV from sewage (García *et al.*, 2014). A study in Switzerland also identified HEV positive influent samples from wastewater treatment plants, but no HEV positive effluent samples, suggesting effective wastewater treatment using a cleaning and activated sludge process (Masclaux *et al.*, 2013). However, Loisy-Hamon and Leturnier, (2015) detected HEV in treated pig sewage samples from France that had been treated using one of: sawdust composting, slurry dehydration or anaerobic digestion. Other studies have found that river water close to pig farms and pig processing plants had been contaminated with HEV, for example, in Scotland and Italy (Crossan *et al.*, 2012; Idolo *et al.*, 2013; Marcheggiani *et al.*, 2015). Therefore, it is possible that leachate (liquid leaching from solids into the environment) from stored manure and yard run off from farms and abattoirs may be polluting surface waters such as rivers. However, it is unknown whether all of the virus leaching into the environment is viable – for example, viral RNA detected in treated sewage may not indicate viable virus, but remaining RNA.

Crop Contamination

Surface waters from sources such as rivers and groundwater are commonly used as crop irrigation sources throughout the world (Food and Agriculture Organisation, 2011; Food and Agriculture Organisation, 2016). Due to the potential contamination of such water with HEV and other pathogens from faecal matter (whether from human or animal sources), this could cause contamination of irrigated crops. Animal waste (that can potentially be contaminated with HEV as shown above) is also used as crop fertiliser for farms. A small number of studies have found some evidence of crop contamination with HEV. In France, two out of 230 herb and spice samples were positive for HEV RNA (Loisy-Hamon and Leturnier, 2015), a study testing 125 lettuce samples from Greece, Serbia and Poland detected four positive samples (Kokkinos *et al.*, 2012), and in Italy, six of 911 "pre-washed and ready to eat" vegetable samples tested positive for HEV RNA (Terio *et al.*, 2017). Another study in four European countries (Czech Republic, Finland, Poland and Serbia) also detected HEV RNA in one frozen raspberry sample of 38 tested (Maunula *et al.*, 2013). However, it is important to note that no foodborne outbreaks of HEV from contaminated crops have been reported, and the quantities of virus found on the crops is also low enough to call into question whether they would cause illness when consumed. It is also unknown whether the HEV RNA detected originated from viable virus.

HEV in Bivalve Shellfish and other Aquatic Animals

Bivalve molluscs are filter feeding organisms, meaning that they can accumulate and concentrate pathogens from their environment within their tissues. In the EU, bivalve shellfish are tested regularly for faecal contamination, using a faecal indicator, *Escherichia coli*, in accordance with food safety regulations. However, studies have shown that though it functions well as a bacterial faecal indicator, *E. coli* can be a poor indicator of the presence of faecally-derived viruses. Lowther *et al.*, (2012) found that norovirus RNA was present in 76.2% of total UK oyster samples

from commercial harvesting areas, with 73.9% of those samples giving *E. coli* results compliant with the end product standard of ≤230 *E. coli*/100g shellfish flesh. Norovirus within oysters is linked to human faecal pollution that has originated from storm overflows and CSOs, or sewage that has received insufficient treatment (Campos *et al.*, 2013; Campos *et al.*, 2016). CSOs release untreated sewage into surface water to prevent overflows within mains drainage, but outfall events can last for several hours or days and are often poorly monitored (Marine Conservation Society, 2011). Considering that farm or abattoir run off, combined sewer overflows, and inadequately treated sewage could be polluting watercourses with HEV, it is also possible for aquatic organisms, such as shellfish, to be affected by HEV contamination. Indeed, studies around the world have found HEV to be present within bivalve shellfish, these are summarised in table 8. The study by Rivadulla *et al.*, (2019) also showed shellfish to have as much as 1.1×10^5 RNA copies per gram of shellfish tissue, which is close to the pig ID50, but the human infectious dose is still unknown. It is important to note however, that not all RNA found in the shellfish may have been associated with viable virus. To date, there have been no proven foodborne outbreaks of HEV from shellfish consumption, although an outbreak of HEV on a cruise ship was theorised to have been caused by consumption of shellfish on the basis of a retrospective risk analysis (Said *et al.*, 2009).

HEV has also been found in other aquatic organisms, including dolphins, who present clinical symptoms of HEV infection. A study of 31 dolphins at the National Aquarium, Cuba, found that 32.2% of their dolphins were seroprevalent for HEV during two different studies (Villalba et al., 2017). The cause of the infections within the dolphins was unknown, however, it is possible that contamination of food items such as fish may be the cause, making an investigation of the presence of HEV in such animals important to determine whether there is any risk of HEV to humans from the consumption of fish. It may also be important to investigate the presence of HEV in aquatic mammals as they are used as a food source in some countries.

Conclusion

In summary, the host range of HEV appears to be diverse, having been found within pig, deer, rabbit, cattle, goat, and camels, amongst other animals. HEV has also been detected in shellfish meat as a result of contamination of their growing waters. Therefore, there is a risk of contracting HEV from undercooked products from these animals (although it is important to note that epidemiological evidence of foodborne transmission for many of these is currently lacking), and there is also potential for other livestock species to be unidentified hosts for the virus. Generally, foodstuffs containing raw meat or shellfish products are more likely to cause a foodborne infection than cooked foods or crops due to no thermal inactivation of the virus through cooking. Cooking in such a way that a minimum internal temperature of 72°C is reached for at least twenty minutes is likely to completely inactivate any HEV present, however this is likely to produce unwanted deterioration of organoleptic qualities in some risky food types e.g. shellfish.

In addition to animal meat, milk from cows, sheep, goats, donkeys, and camels has also been found to contain HEV in some countries, but studies investigating the presence of HEV in milk are much more limited. Because of this, the true risk of HEV transmission from animal milk is yet unknown and requires further research. However, if proven to be a prominent transmission route for the virus, a worrying consideration is that high temperature short time (HTST) pasteurisation of milk products, which is commonly used in the UK and USA, may be insufficient to reduce infectious HEV within milk, as generally the heat treatment used for HTST pasteurisation is only 72°C for 15 seconds. Other pasteurisation methods, such as ultra-high temperature pasteurisation which utilise treatments of around 135°C for 2-4 seconds, should be more capable of removing viable virus from milk products due to the higher temperature.

Though crops can also become contaminated with HEV, it seems that the risk of contracting HEV from them is much less likely, as confirmed outbreaks from crops have not been identified, and the HEV RNA prevalence and copies of viral RNA present are lower for these foods. However, it may be

safe to conduct further research into the contamination of irrigation water, and the presence of HEV in crops from other countries to better assess the risk of contracting HEV from crop contamination.

It has also been shown that marine mammals can be infected with HEV, which is concerning both from an ecosystem and a seafood point of view. If marine mammals are becoming infected naturally, it could be possible that fish and other seafood also become contaminated or infected. Considering that shellfish in many countries have been found to be contaminated with HEV, this is perhaps something that warrants further investigation.

Due to mixed conclusions between and within countries about HEV presence within different hosts or matrices, it appears that there needs to be not only standardised and improved methods for the purpose of HEV detection, but also that further research through larger studies around the world are required to identify the full host range of HEV and the risk of each potential host to transmit the virus to humans (through food or other means). In particular, the suggestion that a HEV-related virus may be causing seroprevalence estimates to be higher than they genuinely are requires investigation.

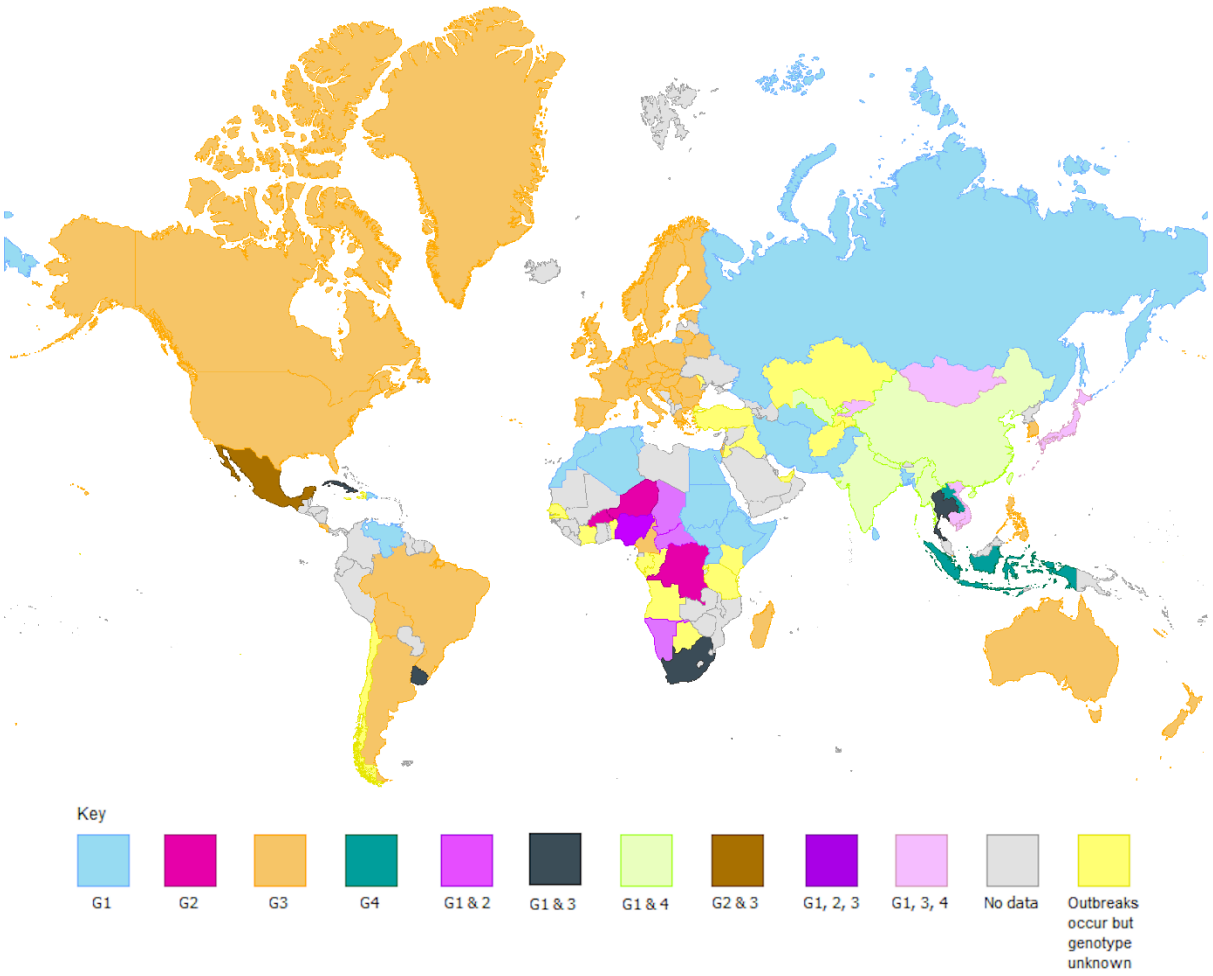
Further studies identifying both the seroprevalence and the presence of HEV through ELISA and RT-PCR techniques respectively (or similar techniques identifying RNA presence) would be best equipped to identify both the prevalence of the virus within animal populations and the amount of active infections within the populations at that point in time. However, sequencing technologies such as nanopore RNA sequencing within human and animal populations would also be useful to identify similarities between HEV sequences, enabling the identification of infection sources. Some studies investigating the evolution of the virus have already been performed, but are often biased by the large amount of HEV sequences derived from humans (Forni *et al.*, 2018).

Investigations of HEV within food and environmental matrices using whole genome sequencing

approaches have been limited so far due to the general observation of low genome copy numbers and fragmented HEV RNA within these matrices. However, techniques utilising methods such as multiplexing RNA extracted samples to obtain a full genome from multiple amplicons, followed by MinION next generation sequencing, which has been successfully applied to sequencing of low levels of zika virus (Quick *et al.*, 2017), could be instrumental in future efforts to identify low levels of HEV in a variety of matrices, including foods.

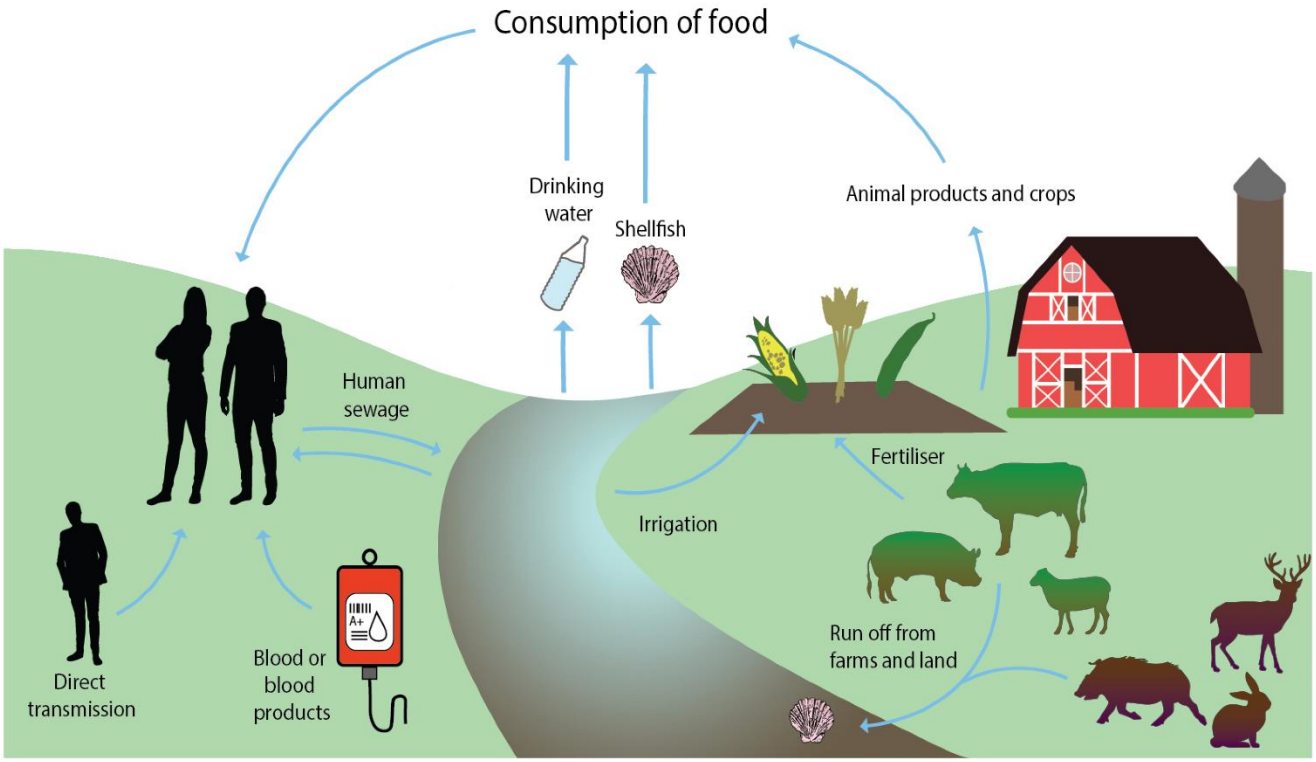
Globally, HEV is an under-recognised viral threat, which causes an increasing case incidence annually. The best way to tackle a virus is to understand its sources and modes of transmission. Therefore, further research and better understanding of HEV will allow a better assessment of the risk that animal products and other foods may have in the transmission of HEV to humans. In turn, this may allow the introduction of legislative controls to prevent and control the spread of the virus.

Fig 1 The geographical distribution of HEV genotypes 1-4



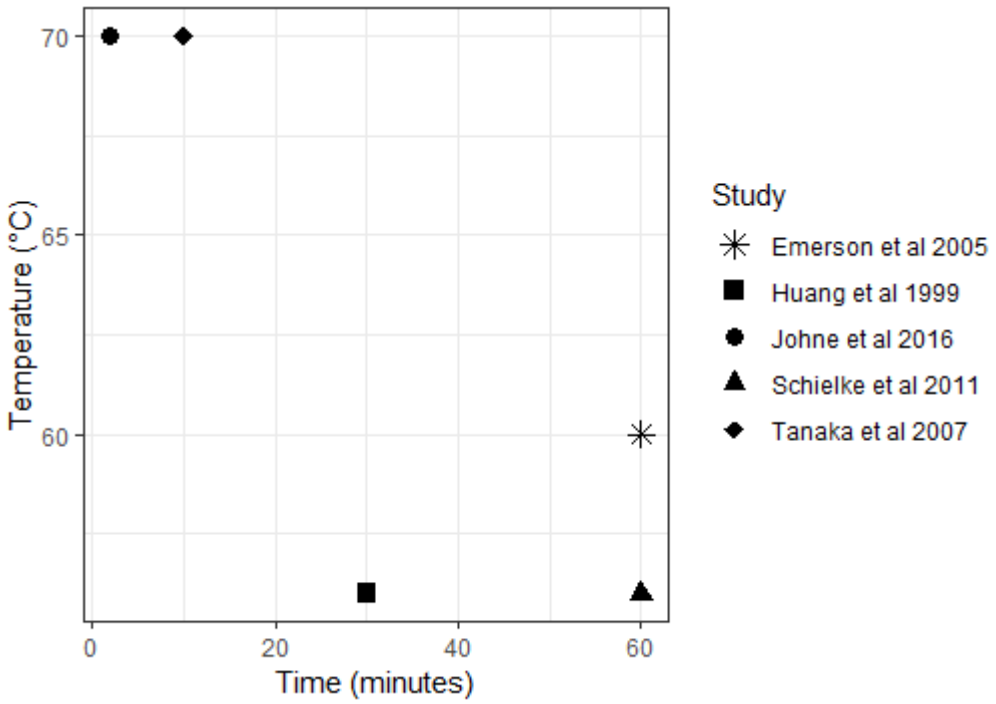
This figure shows the genotypes of HEV which are endemic to each country, where enough data was available. For graphs which are compatible with the conditions protanopia, deuteranopia and achromatopsia please see online resources 1 and 2. Maps created in ArcMap using the World Countries (generalized) layer package by esri_dm and visualised in GIMP.

462 **Fig 2** Theoretical and confirmed transmission routes of HEV



463 The figure shows confirmed and theoretical routes of HEV transmission to humans. The theoretical
464 routes of transmission include HEV infections contracted from the consumption of shellfish, sheep,
465 and cows, as well as crops and drinking water, as no confirmed outbreaks from these sources have
466 yet been identified. Illustration created using Adobe Illustrator and edited using GIMP.

Fig 3 A summary of the reported thermal inactivation requirements for HEV from different studies



This graph summarises the observed HEV inactivation requirements for five different studies investigating the effect of heat treatment over time on HEV viability, with the highest reported inactivation requirements being 70°C for 10 minutes, and the lowest being 56°C for 30 minutes. Graph created in R studio.

Table 1 Pattern of infection for the different genotypes of HEV, adapted from Centers for Disease Control and Prevention, (2020)

Genotype	Transmission in humans?	Transmission Routes	Geographical distribution pattern	Extrahepatic manifestations	Age groups at higher risk	Gender more commonly affected	Lethality
1	Yes	Faecal-oral; Waterborne; Blood transfusion; Organ donation	Economically developed and developing countries	Pancreatic	Differs by country [a,b]	Differs by country [a,b]	0.5-1%[c]; 20% in pregnant women [d,e,f]
2	Yes	Faecal-oral; Waterborne; Blood transfusion; Organ donation	Economically developing countries	Unknown	Young adults	Unknown	0.5-1%[c]
3	Yes	Foodborne; Blood transfusion; Organ donation	Economically developed and developing countries	Chronic infections in immune-compromised patients. Neurological, haematological, immunological and renal manifestations[g]	Older adults (>40 years)	Males	0.5-1%[c]
4	Yes	Foodborne; Blood transfusion; Organ donation	Economically developed and developing countries	Unknown	Young adults	Possibly males (limited data)[h]	0.5-1%[c]
5	No	Faecal-oral	Unknown	Unknown	Unknown	Unknown	Unknown
6	No	Faecal-oral	Unknown	Unknown	Unknown	Unknown	Unknown
7	Yes	Foodborne; Faecal-oral; Blood transfusion?	Unknown	Unknown	Unknown	Unknown	Unknown
8	No	Faecal-oral	Unknown	Unknown	Unknown	Unknown	Unknown

[a]Pathak and Barde, (2017), [b]Spina *et al.*, (2017), [c]Peron *et al.*, (2007), [d]Kumar *et al.*, (2017), [e]Jin *et al.*, (2016), [f]Kamar *et al.*, (2014), [g]Horvatits *et al.*, (2019), [h]Mizuo *et al.*, (2005)

Table 2 Summary of HEV host species by genotype adapted from Kenney, 2019

Genotype	Hosts identified (common names)	Species infected
1	Humans, Chimpanzees, Monkeys, Horses	*Homo sapiens, Pan troglodytes, Chlorocebus sabaeus*, Chlorocebus pygerythrus*, Erythrocebus patas*, Macaca mulatta, Macaca radiata, Macaca fascicularis, Semnopithecus entellus, Aotus trivirgatus*, Saguinus mystax mystax*, Saimiri sciureus*, Equus caballus ferus*
2	Humans, Monkeys	*Homo sapiens, Chlorocebus pygerythrus*, Erythrocebus patas*, Macaca mulatta, Macaca fascicularis, Aotus trivirgatus*, Saguinus mystax mystax*, Saimiri sciureus**
3	Humans, Monkeys, Hares and Rabbits, Rats, Minks, Mongooses, Pigs, Goats and Sheep, Deer, Dolphins, Horses, Vultures	*Homo sapiens, Erythrocebus patas, Macaca mulatta, Macaca fascicularis, Macaca fuscata, Lepus europaeus, Oryctolagus cuniculus domesticus, Rattus norvegicus, Neovison vison, Herpestes javanicus, Sus scrofa, Sus scrofa domestica, Capra hircus aegagrus, Ovis aries orientalis, Cervus elaphus, Cervus nippon, Capreolus, Tursiops truncatus, Equus africanus, Equus caballus ferus, Gyps himalayensis*
4	Humans, Monkeys, Gerbils, Dogs, Bears, Leopards, Pigs, Cows, Goats, Deer, Cranes, Pheasants	*Homo sapiens, Macaca fascicularis, Macaca mulatta, Meriones unguiculatus*, Canis lupus familiaris, Ursus thibetanus, Neofelis nebulosa, Sus scrofa, Sus scrofa domesticus, Bos taurus primigenuis, Bos grunniens, Capra hircus aegagrus, Ovis aries orientalis, Cervus nippon, Elaphodus cephalophus, Muntiacus reevesi, Balearica regulorum, Lophura nycthemera*
5	Monkeys, Pigs	*Macaca fascicularis, Sus scrofa*
6	Pigs	*Sus scrofa*
7	Humans, Camels	*Homo sapiens, Camelus dromedarius,*
8	Camels	*Camelus bactrianus*

*Infections instigated through experimental conditions

Table 3 A list of the seroprevalence levels of anti-HEV antibodies in pigs and the percentage of pork products found to be HEV-positive by RT-PCR in those locations

Location	Pig sample population anti-HEV antibody seroprevalence	Percentage of tested foodstuffs HEV-positive by RT-PCR
Brazil	63.6% of 357 pigs (Vitral *et al.*, 2005)	1.7% of 118 slaughterhouse livers (Gardinali *et al.*, 2012)
Canada	59.4% of 998 pigs (Yoo *et al.*, 2001)	8.8% of 283 livers, 1.0% of 599 pork chops (Wilhelm *et al.*, 2014)
		47.0% of 76 pork pâtés and 10.5% of 19 retail raw pork livers (Mykytczuk *et al.*, 2017)
France	31.0% of 6565 pigs (Rose *et al.*, 2011); 60% of 1034 pigs (Feurer *et al.*, 2018)	4.0% of 3715 slaughterhouse livers (Rose *et al.*, 2011)
		2.8% of 1034 slaughterhouse livers (Feurer *et al.*, 2018)
		30.0% of 140 figatelli and fitone, 29.0% of 169 liver sausages, 25.0% of 55 quenelles or quenelle paste, 3.0% of 30 dried salted livers (Pavio et al., 2014)
		58.3% of 12 raw liver sausage (Colson *et al.*, 2010)
Germany	49.8% of 1072 pigs (Baechlein *et al.*, 2010)	4.0% of 200 retail livers (Wenzel *et al.*, 2011)
		20.0% of 70 raw sausages and 22.0% of 50 liver sausages (Szabo *et al.*, 2015)
Italy	45.1% of 2700 pigs (Mughini-Gras *et al.*, 2017)	20.8% of 48 slaughterhouse livers (Di Bartolo *et al.*, 2011)

		6.0% of 33 slaughterhouse livers (Di Bartolo *et al.*, 2012)
		13.3% of 15 fresh liver sausages, 7.1% of 14 dried liver sausages (Di Bartolo *et al.*, 2015)
Japan	57.9% of 2500 pigs (Takahashi *et al.*, 2003)	1.9% of 363 retail livers (Yazaki *et al.*, 2003)
Netherlands	89.0% of 417 organic pigs, 72% of 265 conventionally farmed pigs, 76% of 164 free range pigs (Rutjes *et al.*, 2014)	6.5% of 62 commercial pork livers (Bouwknegt *et al.*, 2007)
		12.7% of 79 livers, 70.7% of 99 liver sausages, 68.9% of 90 liver pâté samples (Boxman *et al.*, 2019)
Spain	20.4% of 1441 pigs (de Oya *et al.*, 2011)	6.0% of 93 sausages, 3.0% of 39 slaughterhouse livers (Di Bartolo *et al.*, 2012)
Switzerland	62.3% of 1001 pigs in 2006, 53.8% of 999 pigs in 2011 (Burri *et al.*, 2014)	1.3% of 160 slaughterhouse livers (Müller *et al.*, 2017)
		11.8% of 102 raw liver sausages (Giannini *et al.*, 2017)
		11.7% of 90 pork liver and raw meat sausages (Moor *et al.*, 2018)
UK	92.8% of 629 pigs, with 20.5% viraemic at slaughter (Grierson *et al.*, 2015)	10.0% of 63 sausages, 3.0% of 40 slaughterhouse livers (Berto *et al.*, 2012)
USA	21.9% of 182 pigs (Owolodun *et al.*, 2013)	11.0% of 127 retail liver (Feagins *et al.*, 2007)

Table 4 A summary of studies investigating thermal inactivation treatments for HEV in non-food matrix samples

Study	Cell culture or molecular detection?	Genotype	Heat treatment		Growth period	Inactivation/ Reduction
			Temperature (°C)	Time (minutes)		
Emerson et al., (2005)	Cell culture, HepG2/C3A cells	1 (strain Akluj[1])	56	60	5-6 days	>80% reduction
		1 (strain SAR55[2])	56	60	5-6 days	~50% reduction
		2 (strain Mex14[3])	60	60	5-6 days	96% reduction
Huang *et al.*, (1999)	Cell culture, A549 cells	3 (strains G93-1*, G93-2*, G93-3*, G93-4*)	56	30	72 hours	<1.0 (TCID50/0.025 ml)
Johne *et al.*, (2016)	Cell culture, A549/D3 cells	3 (strain 47832c[6])	55	1	35 days	~1 log reduction in focus forming units
			70	2		"no infectious virus"
Schielke *et al.*, (2011)	Molecular detection	3 (strain wbGER27[5])	56	15	N/A	74.07% reduction
		3 (strain wbGER27[5])	56	60	N/A	99.90% reduction
Tanaka *et al.*, (2007)	Cell culture, PLC/PRF/5 cells	3 (strain JE03-1760F[4])	70	10	35 days	"no infectious virus"
		3 (strain JE03-1760F[4])	56	30	50 days	"still infectious"

*Accession numbers unknown; [1]AF107909; [2]M80581.1; [3]KX578717.1; [4]AB437319.1; [5]FJ705359.1; [6]KC618403.1

Table 5 A summary of studies investigating thermal inactivation treatments for HEV in foodstuffs

Study	Food stuff	Cooking method	Temperature (°C)	Time (minutes)	Measurement of inactivation	HEV inactivated?
Barnaud *et al.*, (2012)	Pâté preparation (spiked with 10^8 HEV genome copies)	Water bath	62	5	Intravenous administration to pigs	No
				20		No
				120		No
			68	5		No
				10		No
				20		No
			71	5		No
				10		No
				20		Yes
Feagins *et al.*, (2008)	Pig liver (naturally infected)	Incubation	56	60	Oral administration to pigs	No
		Boiling	≥71 (internal)	5		Yes
		Stir fry	≥71 (internal)	5		Yes
Imagawa *et al.*, (2018)	Minced meat (spiked with 10^{10} HEV genome copies)	Boiling or roasting	63	1	Cell culture	No
				5		No
				30		Yes
			65	1		No
				5		Yes
			70	1		No
				5		Yes

Table 6 Summary of studies investigating the prevalence of HEV in deer

Study	Location	Deer species	ELISA observed seroprevalence	RT-PCR prevalence
Weger *et al.*, (2017)	Canada	*Odocoileus virginianus* (White-tailed deer)	8.8%	ND[†]
		Odocoileus hemionus (Mule deer)	4.5%	ND[†]
		Rangifer tarandus groenlandicus (Barren-ground caribou)	1.7%	ND[†]
		Rangifer tarandus (Woodland caribou)	5.2%	ND[†]
Zhang *et al.*, (2015)	China	*Cervus nippon* (Sika deer)	5.4%	ND[†]
Anheyer-Behmenburg *et al.*, (2017)	Germany	*Capreolus* (Roe deer)	0.0%	6.4%
		Cervus elaphus (Red deer)	0.0%	3.5%
Neumann *et al.*, (2016)		*Cervus elaphus* (Red deer)	2.5%	3.7%
		Capreolus (Roe deer)	6.5%	0.0%
Reuter *et al.*, (2009)	Hungary	*Capreolus* (Roe deer)	ND[†]	12.2%
Di Bartolo *et al.*, (2017)	Italy	*Cervus elaphus* (Red deer)	13.6%	11.0%
Sonoda *et al.*, (2004)	Japan	*Cervus nippon* (Sika deer)	2.0%	0.0%
Matsuura *et al.*, (2007)		*Cervus nippon* (Sika deer)	2.6%	0.0%
Tomiyama *et al.*, (2009)		*Cervus nippon yesoensis* (Yezo deer)	34.8%	ND[†]
Spancerniene *et al.*, (2018)	Lithuania	*Capreolus* (Roe deer)	ND[†]	22.6%
		Cervus elaphus (Red deer)	ND[†]	6.7%

Medrano *et al.*, (2012)	Mexico	*Odocoileus virginianus* (White-tailed deer)	62.7%	ND[†]
Rutjes *et al.*, (2010)	The Netherlands	*Cervus elaphus* (Red deer)	8.0%	15.0%
		Capreolus (Roe deer)	12.5%	0.0%
Boadella *et al.*, (2010)	Spain	*Cervus elaphus* (Red deer)	10.4%	N/A*
Kukielka *et al.*, (2016)		*Cervus elaphus* (Red deer)	12.9%	11.1%
Roth *et al.*, (2016)	Sweden	*Capreolus* (Roe deer)	7.0%	0.0%
		Cervus elaphus (Red deer)	7.0%	0.0%

* did not test full sample population which were tested for seropositivity; ND[†] not done

Table 7 A summary of studies identifying HEV (genotype 3ra) in rabbits and hares

Country	Study	Seroprevalence	RNA prevalence
Burkina Faso	Ouoba *et al.*, (2019)	60.0% of 100 rabbits, 52.6% of 19 hares	ND†
Canada	Xie *et al.*, (2017)	ND†	5.0% of 63 companion rabbit faecal samples, 0.90% of 114 commercial rabbit faecal samples
China	Geng *et al.*, (2011a)	54.6% of 119 farmed rabbits	7.0% of 119 farmed rabbit serum samples
	Geng *et al.*, (2011b)	15.4% of 1094 farmed rabbits	2.0% of 1094 farmed rabbit serum samples
	Xia *et al.*, (2015)	ND†	5.0% of 492 rabbit faecal samples
	Li *et al.*, (2020a)	ND†	15.0% of 120 rabbit faecal samples
	Li *et al.*, (2020b)	7.1% of 70 farmed rabbits	11.4% of 70 farmed rabbit faecal samples
France	Izopet *et al.*, (2012)	ND†	7.0% of 200 farmed rabbit bile samples, 23.0% of 205 wild rabbit liver samples
Germany	Eiden *et al.*, (2016)	30.8% of 13 wild rabbits	30.8% of 13 wild rabbit serum samples
	Hammerschmidt *et al.*, (2017)	37.3% of 164 wild rabbits, 2.2% of 669 wild hares	17.1% of wild rabbit serum samples, 0.0% of wild hare serum samples
	Ryll *et al.*, (2018)	25% of 72 wild rabbits	34.7% of 72 wild rabbit liver samples
	Corman *et al.*, (2019)	0.04% of 2389 wild hares	2.6% of 2389 wild hare serum samples
Italy	Di Bartolo *et al.*, (2016)	3.4% of 206 farmed rabbits, 6.6% of 122 pet rabbits	0.0% of 7 IgG positive farmed rabbit serum

			samples, 0.0% of 122 pet rabbit serum samples
The Netherlands	Burt *et al.*, (2016)	ND†	23.0% of 35 petting farm rabbit faecal samples, 0% of 10 farmed rabbit liver and faecal samples, 60.0% of 32 wild rabbit liver samples and 16% of wild rabbit faecal samples
Poland	Bigoraj *et al.*, (2020)	6.0% of 482 farmed rabbits	14.9% of 482 farmed rabbit liver samples
South Korea	Ahn *et al.*, (2017)	ND†	6.4% of 264 rabbit faecal samples
USA	Cossaboom *et al.*, (2011)	36.5% of 85 rabbits	16.5% of 85 serum samples, 15.3% of 85 faecal samples

ND† not done

Table 8 The presence of HEV in shellfish in different countries

Location	Study	Percentage of shellfish HEV-positive
China	Gao *et al.*, (2015)	17.5% of 126 shellfish samples* of various species from production areas
Denmark	Krog et al., (2014)	0% of 29 mussel samples* from 19 production areas
France	Grodzki *et al.*, (2014)	0% of 286 shellfish samples* of various species from two production areas
Italy	La Rosa *et al.*, (2018)	2.6% of 384 shellfish samples* of various species from production areas
Japan	Li et al., (2007)	6.3% of 32 Yamato-Shijimi clam samples*
Scotland	Crossan *et al.*, (2012)	85.4% of 48 individual wild mussels
	O'Hara *et al.*, (2018)	2.9% of 310 retail shellfish samples* (mussels and oysters)
Spain	Mesquita *et al.*, (2016)	14.8% of 81 mussel samples* from a production area
	Rivadulla *et al.*, (2019)	24.4% of 164 mussel, clam, and cockle samples*

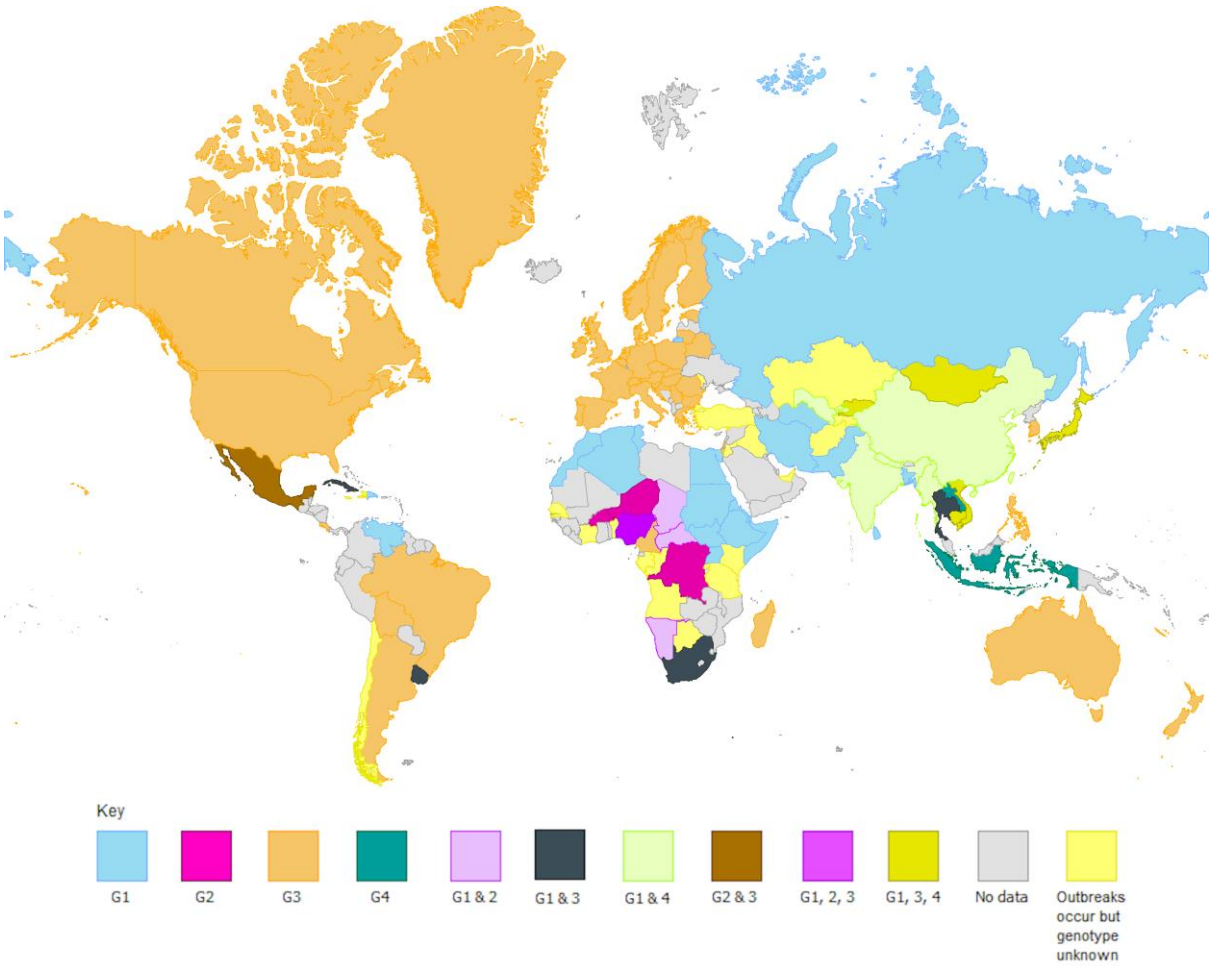
*Where the study states that samples of shellfish were tested, it was either stated or assumed in each publication that each "sample" would have been formed by ten or more shellfish individuals and is therefore technically a pooled sample.

Supplementary Data

Supplementary data provided includes alternative versions of Fig 1 which are compatible for people with the conditions deuteranopia, protanopia and achromatopsia. The Fig 1 displayed in the article is compatible for the conditions protanomaly, deuteranomaly, tritanopia, tritanomaly, and achromatomaly, tested using colour blindness filters in GIMP.

Supplementary data 1: Fig 1 compatible for the conditions deuteranopia and protanopia

Fig 1 The geographical distribution of HEV genotypes 1-4



This figure shows the genotypes of HEV which are endemic to each country, where enough data was available. Maps created in ArcMap using the World Countries (generalized) layer package by esri_dm and visualised in GIMP.

Supplementary data 2: Fig 1 compatible for the condition achromatopsia

Fig 1 The geographical distribution of HEV genotypes 1-4 (achromatopsia compatible)

This figure shows the genotypes of HEV which are endemic to each country, where enough data was available. Maps created in ArcMap using the World Countries (generalized) layer package by esri_dm and visualised in GIMP.

Declarations

Authors' contributions

Samantha Treagus was responsible for the idea, did the literature search and data analysis for the writing, as well as drafting and critically revising the work. Conal Wright was involved in illustration creation and editing. Craig Baker-Austin, Ben Longdon and James Lowther were responsible for editing and critically revising the work.

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The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethics approval

This is an observational study. The University of Exeter Research Ethics Committee has confirmed that no ethical approval is required.

Consent to participate

This is an observational study. No human participants were involved in this work.

Consent to publish

This is an observational study. No human participants were involved in this work.

Availability of data and material

All data obtained from publicly available information.

Code availability

Not applicable

References

Abravanel, F., Lhomme, S., El Costa, H., Schvartz, B., Peron, J.-M., Kamar, N. and Izopet, J. (2017) 'Rabbit hepatitis E virus infections in humans, France', *Emerging infectious diseases,* 23(7), pp. 1191.

Agency, E. P. *Agriculture Nutrient Management and Fertilizer*. USA. Available at: https://www.epa.gov/agriculture/agriculture-nutrient-management-and-fertilizer#Manure (Accessed: 08 September 2020).

Ahn, H. S., Park, B. J., Han, S. H., Kim, Y. H., Kim, D. H., Kim, B. S., Lee, J. B., Park, S. Y., Song, C. S. and Lee, S. W. (2017) 'Prevalence and genetic features of rabbit hepatitis E virus in Korea', *Journal of medical virology,* 89(11), pp. 1995-2002.

Andraud, M., Dumarest, M., Cariolet, R., Aylaj, B., Barnaud, E., Eono, F., Pavio, N. and Rose, N. (2013) 'Direct contact and environmental contaminations are responsible for HEV transmission in pigs', *Veterinary research,* 44(1), pp. 102.

Anheyer-Behmenburg, H. E., Szabo, K., Schotte, U., Binder, A., Klein, G. and Johne, R. (2017) 'Hepatitis E virus in wild boars and spillover infection in red and roe deer, Germany, 2013–2015', *Emerging infectious diseases,* 23(1), pp. 130.

Arankalle, V. A., Chadha, M. S. and Chobe, L. P. (1999) 'Long-term serological follow up and cross-challenge studies in rhesus monkeys experimentally infected with hepatitis E virus', *Journal of hepatology,* 30(2), pp. 199-204.

Baechlein, C. and Becher, P. (2017) 'No evidence for zoonotic hepatitis E virus infection through dairy milk in Germany', *Hepatology,* 65(1), pp. 394-395.

Baechlein, C., Schielke, A., Johne, R., Ulrich, R. G., Baumgaertner, W. and Grummer, B. (2010) 'Prevalence of Hepatitis E virus-specific antibodies in sera of German domestic pigs estimated by using different assays', *Veterinary microbiology,* 144(1), pp. 187-191.

Baez, P. A., Lopez, M. C., Duque-Jaramillo, A., Pelaez, D., Molina, F. and Navas, M.-C. (2017) 'First evidence of the Hepatitis E virus in environmental waters in Colombia', *PloS one,* 12(5), pp. e0177525.

Barnaud, E., Rogée, S., Garry, P., Rose, N. and Pavio, N. (2012) 'Thermal inactivation of infectious hepatitis E virus in experimentally contaminated food', *Applied and environmental microbiology,* 78(15), pp. 5153-5159.

Berto, A., Martelli, F., Grierson, S. and Banks, M. (2012) 'Hepatitis E virus in pork food chain, United Kingdom, 2009–2010', *Emerging infectious diseases,* 18(8), pp. 1358.

Bigoraj, E., Kozyra, I., Kwit, E. and Rzeżutka, A. (2020) 'Detection of hepatitis E virus (rabbit genotype) in farmed rabbits entering the food chain', *International Journal of Food Microbiology,* 319, pp. 108507.

Boadella, M., Casas, M., Martín, M., Vicente, J., Segalés, J., De la Fuente, J. and Gortázar, C. (2010) 'Increasing contact with hepatitis E virus in red deer, Spain', *Emerging infectious diseases,* 16(12), pp. 1994.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M. and Girones, R. (2006) 'Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices', *Appl. Environ. Microbiol.,* 72(12), pp. 7894-7896.

Bouwknegt, M., Lodder-Verschoor, F., Van Der Poel, W. H., Rutjes, S. A. and De Roda Husman, A. M. (2007) 'Hepatitis E virus RNA in commercial porcine livers in The Netherlands', *Journal of food protection,* 70(12), pp. 2889-2895.

Bouwknegt, M., Rutjes, S. A., Reusken, C. B., Stockhofe-Zurwieden, N., Frankena, K., de Jong, M. C., de Roda Husman, A. M. and van der Poel, W. H. (2009) 'The course of hepatitis E virus infection in pigs after contact-infection and intravenous inoculation', *BMC veterinary research,* 5(1), pp. 7.

Boxman, I. L., Jansen, C. C., Hägele, G., Zwartkruis-Nahuis, A., Cremer, J., Vennema, H. and Tijsma, A. S. (2017) 'Porcine blood used as ingredient in meat productions may serve as a vehicle for hepatitis E virus transmission', *International journal of food microbiology,* 257, pp. 225-231.

Boxman, I. L., Jansen, C. C., Hägele, G., Zwartkruis-Nahuis, A., Tijsma, A. S. and Vennema, H. (2019) 'Monitoring of pork liver and meat products on the Dutch market for the presence of HEV RNA', *International journal of food microbiology,* 296, pp. 58-64.

Burri, C., Vial, F., Ryser-Degiorgis, M. P., Schwermer, H., Darling, K., Reist, M., Wu, N., Beerli, O., Schöning, J. and Cavassini, M. (2014) 'Seroprevalence of hepatitis E virus in domestic pigs and wild boars in Switzerland', *Zoonoses and public health,* 61(8), pp. 537-544.

Burt, S. A., Veltman, J., Hakze-van der Honing, R., Schmitt, H. and van der Poel, W. H. (2016) 'Hepatitis E virus in farmed rabbits, wild rabbits and petting farm rabbits in the Netherlands', *Food and environmental virology,* 8(3), pp. 227-229.

Campos, C. J., Avant, J., Lowther, J., Till, D. and Lees, D. N. (2016) 'Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes', *Water research,* 103, pp. 224-232.

Campos, C. J., Kershaw, S. R. and Lee, R. J. (2013) 'Environmental influences on faecal indicator organisms in coastal waters and their accumulation in bivalve shellfish', *Estuaries and coasts,* 36(4), pp. 834-853.

Chang, Y., Wang, L., Geng, J., Zhu, Y., Fu, H., Ren, F., Li, L., Wang, X. and Zhuang, H. (2009) 'Zoonotic risk of hepatitis E virus (HEV): A study of HEV infection in animals and humans in suburbs of Beijing', *Hepatology Research,* 39(12), pp. 1153-1158.

Clemente-Casares, P., Pina, S., Buti, M., Jardi, R., Martín, M., Bofill-Mas, S. and Girones, R. (2003) 'Hepatitis E virus epidemiology in industrialized countries', *Emerging infectious diseases,* 9(4), pp. 449.

Clemente-Casares, P., Rodriguez-Manzano, J. and Girones, R. (2009) 'Hepatitis E virus genotype 3 and sporadically also genotype 1 circulate in the population of Catalonia, Spain', *Journal of water and health,* 7(4), pp. 664-673.

Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal, V., Gallian, P., Heyries, L., Raoult, D. and Gerolami, R. (2010) 'Pig liver sausage as a source of hepatitis E virus transmission to humans', *The Journal of infectious diseases,* 202(6), pp. 825-834.

Commission, E. (2001) *Survey of wastes spread on land*: European Commission. Available at: https://ec.europa.eu/environment/waste/studies/compost/landspreading_4-6.pdf.

Corman, V. M., Hilgensloh, L., Voigt, U., Marklewitz, M., Siebert, U., Drosten, C. and Drexler, J. F. (2019) 'Hepatitis E Virus Infection in European Brown Hares, Germany, 2007–2014', *Emerging infectious diseases,* 25(6), pp. 1233.

Cossaboom, C. M., Córdoba, L., Dryman, B. A. and Meng, X.-J. (2011) 'Hepatitis E virus in rabbits, Virginia, USA', *Emerging infectious diseases,* 17(11), pp. 2047.

Crossan, C., Baker, P. J., Craft, J., Takeuchi, Y., Dalton, H. R. and Scobie, L. (2012) 'Hepatitis E virus genotype 3 in shellfish, United Kingdom', *Emerging infectious diseases,* 18(12), pp. 2085.

Dalton, H., Thurairajah, P., Fellows, H., Hussaini, H., Mitchell, J., Bendall, R., Banks, M., Ijaz, S., Teo, C. G. and Levine, D. (2007) 'Autochthonous hepatitis E in southwest England', *Journal of viral hepatitis,* 14(5), pp. 304-309.

De Deus, N., Casas, M., Peralta, B., Nofrarías, M., Pina, S., Martín, M. and Segalés, J. (2008) 'Hepatitis E virus infection dynamics and organic distribution in naturally infected pigs in a farrow-to-finish farm', *Veterinary Microbiology,* 132(1), pp. 19-28.

de Oya, N. J., de Blas, I., Blázquez, A.-B., Martín-Acebes, M. A., Halaihel, N., Gironés, O., Saiz, J.-C. and Escribano-Romero, E. (2011) 'Widespread distribution of hepatitis E virus in Spanish pig herds', *BMC research notes,* 4(1), pp. 412.

Debode, F., Marien, A., Janssen, É., Bragard, C. and Berben, G. (2017) 'The influence of amplicon length on real-time PCR results', *Base*.

Demirci, M., Yiğin, A., Ünlü, Ö. and Kılıç, S. A. (2019) 'Detection of HEV RNA amounts and genotypes in raw milks obtained from different animals', *Mikrobiyoloji bulteni,* 53(1), pp. 43-52.

Di Bartolo, I., Angeloni, G., Ponterio, E., Ostanello, F. and Ruggeri, F. M. (2015) 'Detection of hepatitis E virus in pork liver sausages', *International journal of food microbiology,* 193, pp. 29-33.

Di Bartolo, I., De Sabato, L., Marata, A., Martinelli, N., Magistrali, C., Monini, M., Ponterio, E., Ostanello, F. and Ruggeri, F. (2016) 'Serological survey of hepatitis E virus infection in farmed and pet rabbits in Italy', *Archives of virology,* 161(5), pp. 1343-1346.

Di Bartolo, I., Diez-Valcarce, M., Vasickova, P., Kralik, P., Hernandez, M., Angeloni, G., Ostanello, F., Bouwknegt, M., Rodríguez-Lázaro, D. and Pavlik, I. (2012) 'Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010', *Emerging infectious diseases,* 18(8), pp. 1282.

Di Bartolo, I., Ponterio, E., Angeloni, G., Morandi, F., Ostanello, F., Nicoloso, S. and Ruggeri, F. (2017) 'Presence of hepatitis E virus in a RED deer (Cervus elaphus) population in Central Italy', *Transboundary and emerging diseases,* 64(1), pp. 137-143.

Di Bartolo, I., Ponterio, E., Castellini, L., Ostanello, F. and Ruggeri, F. M. (2011) 'Viral and antibody HEV prevalence in swine at slaughterhouse in Italy', *Veterinary microbiology,* 149(3-4), pp. 330-338.

Di Martino, B., Di Profio, F., Melegari, I., Sarchese, V., Robetto, S., Marsilio, F. and Martella, V. (2016) 'Detection of hepatitis E virus (HEV) in goats', *Virus research,* 225, pp. 69-72.

Dähnert, L., Eiden, M., Schlosser, J., Fast, C., Schröder, C., Lange, E., Gröner, A., Schäfer, W. and Groschup, M. H. (2018) 'High sensitivity of domestic pigs to intravenous infection with HEV', *BMC veterinary research,* 14(1), pp. 381.

Eiden, M., Vina-Rodriguez, A., Schlosser, J., Schirrmeier, H. and Groschup, M. H. (2016) 'Detection of hepatitis E virus in archived rabbit serum samples, Germany 1989', *Food and environmental virology,* 8(1), pp. 105-107.

El-Kafrawy, S. A., Hassan, A. M., El-Daly, M. M., Qadri, I., Tolah, A. M., Al-Subhi, T. L., Alzahrani, A. A., Alsaaidi, G. A., Al-Abdullah, N. and Kaki, R. M. (2020) 'Seroprevalence of Dromedary Camel HEV in Domestic and Imported Camels from Saudi Arabia', *Viruses,* 12(5), pp. 553.

Emerson, S. U., Arankalle, V. A. and Purcell, R. H. (2005) 'Thermal stability of hepatitis E virus', *The Journal of infectious diseases,* 192(5), pp. 930-933.

Feagins, A., Opriessnig, T., Guenette, D., Halbur, P. and Meng, X. (2008) 'Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States', *International journal of food microbiology,* 123(1-2), pp. 32-37.

Feagins, A., Opriessnig, T., Guenette, D., Halbur, P. and Meng, X.-J. (2007) 'Detection and characterization of infectious Hepatitis E virus from commercial pig livers sold in local grocery stores in the USA', *Journal of general virology,* 88(3), pp. 912-917.

Feurer, C., Le Roux, A., Rossel, R., Barnaud, E., Dumarest, M., Garry, P. and Pavio, N. (2018) 'High load of hepatitis E viral RNA in pork livers but absence in pork muscle at French slaughterhouses', *International journal of food microbiology,* 264, pp. 25-30.

Forni, D., Cagliani, R., Clerici, M. and Sironi, M. (2018) 'Origin and dispersal of hepatitis E virus', *Emerging microbes & infections,* 7(1), pp. 1-13.

Fraga, M., Doerig, C., Moulin, H., Bihl, F., Brunner, F., Müllhaupt, B., Ripellino, P., Semela, D., Stickel, F. and Terziroli Beretta-Piccoli, B. (2017) 'Hepatitis E virus as a cause of acute hepatitis acquired in Switzerland', *Liver International*.

Gao, S., Li, D., Zha, E., Zhou, T., Wang, S. and Yue, X. (2015) 'Surveillance of hepatitis E virus contamination in shellfish in China', *International journal of environmental research and public health,* 12(2), pp. 2026-2036.

García, M., Fernández-Barredo, S. and Pérez-Gracia, M. (2014) 'Detection of hepatitis E virus (HEV) through the different stages of pig manure composting plants', *Microbial biotechnology,* 7(1), pp. 26-31.

Gardinali, N. R., Barry, A. F., Otonel, R. A. A., Alfieri, A. F. and Alfieri, A. A. (2012) 'Hepatitis E virus in liver and bile samples from slaughtered pigs of Brazil', *Memorias do Instituto Oswaldo Cruz,* 107(7), pp. 935-939.

Geng, J., Wang, L., Wang, X., Fu, H., Bu, Q., Zhu, Y. and Zhuang, H. (2011a) 'Study on prevalence and genotype of hepatitis E virus isolated from Rex Rabbits in Beijing, China', *Journal of viral hepatitis,* 18(9), pp. 661-667.

Geng, Y., Zhao, C., Song, A., Wang, J., Zhang, X., Harrison, T. J., Zhou, Y., Wang, W. and Wang, Y. (2011b) 'The serological prevalence and genetic diversity of hepatitis E virus in farmed rabbits in China', *Infection, Genetics and Evolution,* 11(2), pp. 476-482.

Giannini, P., Jermini, M., Leggeri, L., Nüesch-Inderbinen, M. and Stephan, R. (2017) 'Detection of hepatitis E virus RNA in raw cured sausages and raw cured sausages containing pig liver at retail stores in Switzerland', *Journal of food protection,* 81(1), pp. 43-45.

Grierson, S., Heaney, J., Cheney, T., Morgan, D., Wyllie, S., Powell, L., Smith, D., Ijaz, S., Steinbach, F. and Choudhury, B. (2015) 'Prevalence of hepatitis E virus infection in pigs at the time of slaughter, United Kingdom, 2013', *Emerging infectious diseases,* 21(8), pp. 1396.

Grodzki, M., Schaeffer, J., Piquet, J.-C., Le Saux, J.-C., Chevé, J., Ollivier, J., Le Pendu, J. and Le Guyader, F. S. (2014) 'Bioaccumulation efficiency, tissue distribution, and environmental occurrence of hepatitis E virus in bivalve shellfish from France', *Applied and environmental microbiology,* 80(14), pp. 4269-4276.

Guillois, Y., Abravanel, F., Miura, T., Pavio, N., Vaillant, V., Lhomme, S., Le Guyader, F. S., Rose, N., Le Saux, J.-C. and King, L. A. (2015) 'High proportion of asymptomatic infections in an outbreak of

hepatitis E associated with a spit-roasted piglet, France, 2013', *Clinical Infectious Diseases,* 62(3), pp. 351-357.

Halbur, P., Kasorndorkbua, C., Gilbert, C., Guenette, D., Potters, M., Purcell, R., Emerson, S., Toth, T. and Meng, X. (2001) 'Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human', *Journal of clinical microbiology,* 39(3), pp. 918-923.

Hammerschmidt, F., Schwaiger, K., Dähnert, L., Vina-Rodriguez, A., Höper, D., Gareis, M., Groschup, M. and Eiden, M. (2017) 'Hepatitis E virus in wild rabbits and European brown hares in Germany', *Zoonoses and public health,* 64(8), pp. 612-622.

Hewitt, P. E., Ijaz, S., Brailsford, S. R., Brett, R., Dicks, S., Haywood, B., Kennedy, I. T., Kitchen, A., Patel, P. and Poh, J. (2014) 'Hepatitis E virus in blood components: a prevalence and transmission study in southeast England', *The Lancet,* 384(9956), pp. 1766-1773.

Horvatits, T., Schulze zur Wiesch, J., Lütgehetmann, M., Lohse, A. W. and Pischke, S. (2019) 'The clinical perspective on hepatitis E', *Viruses,* 11(7), pp. 617.

Hu, G. and Ma, X. (2010) 'Detection and sequences analysis of bovine hepatitis E virus RNA in Xinjiang Autonomous Region', *Bing du xue bao= Chinese journal of virology,* 26(1), pp. 27-32.

Huang, F., Li, Y., Yu, W., Jing, S., Wang, J., Long, F., He, Z., Yang, C., Bi, Y. and Cao, W. (2016) 'Excretion of infectious hepatitis E virus into milk in cows imposes high risks of zoonosis', *Hepatology,* 64(2), pp. 350-359.

Huang, R., Li, D., Wei, S., Li, Q., Yuan, X., Geng, L., Li, X. and Liu, M. (1999) 'Cell culture of sporadic hepatitis E virus in China', *Clinical and diagnostic laboratory immunology,* 6(5), pp. 729-733.

Huang, W., Zhang, H., Harrison, T. J., Lang, S., Huang, G. and Wang, Y. (2008) 'Cross-protection of hepatitis E virus genotypes 1 and 4 in rhesus macaques', *Journal of medical virology,* 80(5), pp. 824-832.

Iaconelli, M., Purpari, G., Della Libera, S., Petricca, S., Guercio, A., Ciccaglione, A., Bruni, R., Taffon, S., Equestre, M. and Fratini, M. (2015) 'Hepatitis A and E viruses in wastewaters, in river waters, and in Bivalve Molluscs in Italy', *Food and environmental virology,* 7(4), pp. 316-324.

Idolo, A., Serio, F., Lugoli, F., Grassi, T., Bagordo, F., Guido, M., Privitera, G., Lobreglio, G. and De Donno, A. (2013) 'Identification of HEV in symptom-free migrants and environmental samples in Italy', *Journal of viral hepatitis,* 20(6), pp. 438-443.

Imagawa, T., Sugiyama, R., Shiota, T., Li, T.-C., Yoshizaki, S., Wakita, T. and Ishii, K. (2018) 'Evaluation of heating conditions for inactivation of hepatitis e virus genotypes 3 and 4', *Journal of food protection,* 81(6), pp. 947-952.

Izopet, J., Dubois, M., Bertagnoli, S., Lhomme, S., Marchandeau, S., Boucher, S., Kamar, N., Abravanel, F. and Guérin, J.-L. (2012) 'Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France', *Emerging infectious diseases,* 18(8), pp. 1274.

Jin, H., Zhao, Y., Zhang, X., Wang, B. and Liu, P. (2016) 'Case-fatality risk of pregnant women with acute viral hepatitis type E: a systematic review and meta-analysis', *Epidemiology & Infection,* 144(10), pp. 2098-2106.

Johne, R., Reetz, J., Ulrich, R., Machnowska, P., Sachsenröder, J., Nickel, P. and Hofmann, J. (2014) 'An ORF 1-rearranged hepatitis E virus derived from a chronically infected patient efficiently replicates in cell culture', *Journal of viral hepatitis,* 21(6), pp. 447-456.

Johne, R., Trojnar, E., Filter, M. and Hofmann, J. (2016) 'Thermal stability of hepatitis E virus as estimated by a cell culture method', *Applied and environmental microbiology,* 82(14), pp. 4225-4231.

Kamar, N., Dalton, H. R., Abravanel, F. and Izopet, J. (2014) 'Hepatitis E virus infection', *Clinical microbiology reviews,* 27(1), pp. 116-138.

Kasorndorkbua, C., Guenette, D., Huang, F., Thomas, P., Meng, X.-J. and Halbur, P. (2004) 'Routes of transmission of swine hepatitis E virus in pigs', *Journal of clinical microbiology,* 42(11), pp. 5047-5052.

Kasorndorkbua, C., Opriessnig, T., Huang, F., Guenette, D., Thomas, P., Meng, X.-J. and Halbur, P. (2005) 'Infectious swine hepatitis E virus is present in pig manure storage facilities on United States farms, but evidence of water contamination is lacking', *Applied and environmental microbiology,* 71(12), pp. 7831-7837.

Kenney, S. P. (2019) 'The current host range of hepatitis E viruses', *Viruses,* 11(5), pp. 452.

Kokkinos, P., Kozyra, I., Lazic, S., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A. M., Kaupke, A. and Legaki, E. (2012) 'Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries', *Food and environmental virology,* 4(4), pp. 179-191.

Krog, J. S., Larsen, L. E. and Schultz, A. C. (2014) 'Enteric porcine viruses in farmed shellfish in Denmark', *International journal of food microbiology,* 186, pp. 105-109.

Kukielka, D., Rodriguez-Prieto, V., Vicente, J. and Sánchez-Vizcaíno, J. (2016) 'Constant hepatitis E virus (HEV) circulation in wild boar and red deer in Spain: an increasing concern source of HEV zoonotic transmission', *Transboundary and emerging diseases,* 63(5), pp. e360-e368.

Kumar, N., Das, V., Agarwal, A., Pandey, A. and Agrawal, S. (2017) 'Fetomaternal outcomes in pregnant women with hepatitis E infection; still an important fetomaternal killer with an unresolved mystery of increased virulence in pregnancy', *Turkish Journal of Obstetrics and Gynecology,* 14(2), pp. 106.

La Rosa, G., Della Libera, S., Brambilla, M., Bisaglia, C., Pisani, G., Ciccaglione, A., Bruni, R., Taffon, S., Equestre, M. and Iaconelli, M. (2017) 'Hepatitis E virus (genotype 3) in slurry samples from swine farming activities in Italy', *Food and environmental virology,* 9(2), pp. 219-229.

La Rosa, G., Proroga, Y., De Medici, D., Capuano, F., Iaconelli, M., Della Libera, S. and Suffredini, E. (2018) 'First detection of hepatitis E virus in shellfish and in seawater from production areas in Southern Italy', *Food and environmental virology,* 10(1), pp. 127-131.

Lee, G.-H., Tan, B.-H., Teo, E. C.-Y., Lim, S.-G., Dan, Y.-Y., Wee, A., Aw, P. P. K., Zhu, Y., Hibberd, M. L. and Tan, C.-K. (2016) 'Chronic infection with camelid hepatitis E virus in a liver transplant recipient who regularly consumes camel meat and milk', *Gastroenterology,* 150(2), pp. 355-357. e3.

Lee, S. D., Wang, Y. J., Lu, R. H., Chan, C. Y., Lo, K. J. and Moeckli, R. (1994) 'Seroprevalence of antibody to hepatitis E virus among Chinese subjects in Taiwan', *Hepatology,* 19(4), pp. 866-870.

Lhomme, S., Dubois, M., Abravanel, F., Top, S., Bertagnoli, S., Guerin, J.-L. and Izopet, J. (2013) 'Risk of zoonotic transmission of HEV from rabbits', *Journal of Clinical Virology,* 58(2), pp. 357-362.

Li, M., Zhang, H., Wang, L., Li, Z., Wang, J., Xu, B., Hao, R., Liu, C., Fu, H. and Rao, H. (2020a) 'The investigation of hepatitis A virus and hepatitis E virus co-infection in humans and animals in China', *Acta Virologica,* 64(1), pp. 20-27.

Li, S., Li, M., He, Q., Liang, Z., Shu, J., Wang, L. and Wang, L. (2020b) 'Characterization of hepatitis E virus natural infection in farmed rabbits', *Journal of Viral Hepatitis*.

Li, S., Liu, M., Cong, J., Zhou, Y. and Miao, Z. (2017) 'Detection and characterization of hepatitis E virus in goats at slaughterhouse in Tai'an region, China', *BioMed research international,* 2017.

Li, T.-C., Miyamura, T. and Takeda, N. (2007) 'Detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (Corbicula japonica) in Japan', *The American journal of tropical medicine and hygiene,* 76(1), pp. 170-172.

Li, T.-C., Yang, T., Shiota, T., Yoshizaki, S., Yoshida, H., Saito, M., Imagawa, T., Malbas, F. F., Lupisan, S. P. and Oshitani, H. (2014) 'Molecular detection of hepatitis E virus in rivers in the Philippines', *The American journal of tropical medicine and hygiene,* 90(4), pp. 764-766.

Loisy-Hamon, F. and Leturnier, G. (2015) 'Autochthonous cases of hepatitis E: where does the virus come from? Impact of pig slurry treatment on reduction of the viral load and prevalence of the virus in food substrates', *EuroReference,* 18, pp. 13-18.

Long, F., Yu, W., Yang, C., Wang, J., Li, Y., Li, Y. and Huang, F. (2017) 'High prevalence of hepatitis E virus infection in goats', *Journal of medical virology,* 89(11), pp. 1981-1987.

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E. and Lees, D. N. (2012) 'Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom', *Applied and environmental microbiology,* 78(16), pp. 5812-5817.

Marcheggiani, S., D'Ugo, E., Puccinelli, C., Giuseppetti, R., D'Angelo, A. M., Gualerzi, C. O., Spurio, R., Medlin, L. K., Guillebault, D. and Weigel, W. (2015) 'Detection of emerging and re-emerging pathogens in surface waters close to an urban area', *International journal of environmental research and public health,* 12(5), pp. 5505-5527.

Masclaux, F. G., Hotz, P., Friedli, D., Savova-Bianchi, D. and Oppliger, A. (2013) 'High occurrence of hepatitis E virus in samples from wastewater treatment plants in Switzerland and comparison with other enteric viruses', *Water research,* 47(14), pp. 5101-5109.

Matos, A., Mesquita, J. R., Gonçalves, D., Abreu-Silva, J., Luxo, C. and Nascimento, M. S. (2018) 'First detection and molecular characterization of hepatitis E virus in water from wastewater treatment plants in Portugal', *Annals of Agricultural and Environmental Medicine,* 25(2), pp. 364-367.

Matsuura, Y., Suzuki, M., Yoshimatsu, K., Arikawa, J., Takashima, I., Yokoyama, M., Igota, H., Yamauchi, K., Ishida, S. and Fukui, D. (2007) 'Prevalence of antibody to hepatitis E virus among wild sika deer, Cervus nippon, in Japan', *Archives of virology,* 152(7), pp. 1375-1381.

Maunula, L., Kaupke, A., Vasickova, P., Söderberg, K., Kozyra, I., Lazic, S., van der Poel, W. H., Bouwknegt, M., Rutjes, S. and Willems, K. A. (2013) 'Tracing enteric viruses in the European berry fruit supply chain', *International journal of food microbiology,* 167(2), pp. 177-185.

Medrano, C., Boadella, M., Barrios, H., Cantu, A., Garcia, Z., de la Fuente, J. and Gortazar, C. (2012) 'Zoonotic pathogens among white-tailed deer, northern Mexico, 2004–2009', *Emerging Infectious Diseases,* 18(8), pp. 1372.

Meng, X.-J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., Purcell, R. H. and Emerson, S. U. (1998) 'Genetic and experimental evidence for cross-species infection by swine hepatitis E virus', *Journal of virology,* 72(12), pp. 9714-9721.

Mesquita, J. R., Oliveira, D., Rivadulla, E., Abreu-Silva, J., Varela, M. F., Romalde, J. L. and Nascimento, M. S. (2016) 'Hepatitis E virus genotype 3 in mussels (Mytilus galloprovinciallis), Spain', *Food microbiology,* 58, pp. 13-15.

Mizuo, H., Yazaki, Y., Sugawara, K., Tsuda, F., Takahashi, M., Nishizawa, T. and Okamoto, H. (2005) 'Possible risk factors for the transmission of hepatitis E virus and for the severe form of hepatitis E acquired locally in Hokkaido, Japan', *Journal of medical virology,* 76(3), pp. 341-349.

Moor, D., Liniger, M., Baumgartner, A. and Felleisen, R. (2018) 'Screening of ready-to-eat meat products for hepatitis E virus in Switzerland', *Food and environmental virology,* 10(3), pp. 263-271.

Mughini-Gras, L., Angeloni, G., Salata, C., Vonesch, N., D'amico, W., Campagna, G., Natale, A., Zuliani, F., Ceglie, L. and Monne, I. (2017) 'Hepatitis E virus infection in North Italy: high seroprevalence in swine herds and increased risk for swine workers', *Epidemiology & Infection,* 145(16), pp. 3375-3384.

Mykytczuk, O., Harlow, J., Bidawid, S., Corneau, N. and Nasheri, N. (2017) 'Prevalence and molecular characterization of the hepatitis E virus in retail pork products marketed in Canada', *Food and environmental virology,* 9(2), pp. 208-218.

Müller, A., Collineau, L., Stephan, R., Müller, A. and Stärk, K. D. (2017) 'Assessment of the risk of foodborne transmission and burden of hepatitis E in Switzerland', *International journal of food microbiology,* 242, pp. 107-115.

Neumann, S., Hackl, S. S., Piepenschneider, M., Vina-Rodriguez, A., Dremsek, P., Ulrich, R. G., Groschup, M. H. and Eiden, M. (2016) 'Serologic and molecular survey of hepatitis E virus in German deer populations', *Journal of wildlife diseases,* 52(1), pp. 106-113.

Food and Agriculture Organisation. (2011) *Irrigation in Southern and Eastern Asia in figures*: Food and Agriculture Organisation. Available at: http://www.fao.org/3/i2809e/i2809e.pdf.

Food and Agriculture Organisation. (2016) *Irrigation in Eastern Europe in figures*: Food and Agriculture Organisation of the United Nations. Available at: http://www.fao.org/3/CA3380EN/ca3380en.pdf (Accessed: 12/08/2020).

Ouoba, J. B., Traore, K. A., Rouamba, H., Setondji, K. V.-M., Minoungou, G. L., Ouoba, B. L., Ouedraogo, A., Moctar, S., M'Bengue, A. K. and Kakou, S. N. (2019) 'Prevalence of anti-hepatitis E virus antibodies in domestic animal from three representative provinces of Burkina Faso', *Veterinary and Animal Science,* 7, pp. 100059.

Owolodun, O. A., Giménez-Lirola, L. G., Gerber, P. F., Sanford, B. J., Feagins, A. R., Meng, X.-J., Halbur, P. G. and Opriessnig, T. (2013) 'Development of a fluorescent microbead-based immunoassay for the detection of hepatitis E virus IgG antibodies in pigs and comparison to an enzyme-linked immunoassay', *Journal of virological methods,* 193(2), pp. 278-283.

O'Hara, Z., Crossan, C., Craft, J. and Scobie, L. (2018) 'First Report of the Presence of Hepatitis E Virus in Scottish-Harvested Shellfish Purchased at Retail Level', *Food and environmental virology*, pp. 1-5.

Pas, S. D., Rob, A., Mulders, C., Balk, A. H., van Hal, P. T., Weimar, W., Koopmans, M. P., Osterhaus, A. D. and van der Eijk, A. A. (2012) 'Hepatitis E virus infection among solid organ transplant recipients, the Netherlands', *Emerging infectious diseases,* 18(5), pp. 869.

Pathak, R. and Barde, P. V. (2017) 'Detection of Genotype 1a and 1f of Hepatitis E Virus in Patients Treated at Tertiary Care Hospitals in Central India', *Intervirology,* 60(5), pp. 201-206.

Pavio, N., Merbah, T. and Thebault, A. (2014) 'Frequent hepatitis E virus contamination in food containing raw pork liver, France', *Emerging infectious diseases,* 20(11), pp. 1925.

Peron, J., Bureau, C., Poirson, H., Mansuy, J., Alric, L., Selves, J., Dupuis, E., Izopet, J. and Vinel, J. (2007) 'Fulminant liver failure from acute autochthonous hepatitis E in France: description of seven patients with acute hepatitis E and encephalopathy', *Journal of viral hepatitis,* 14(5), pp. 298-303.

Pina, S., Buti, M., Cotrina, M., Piella, J. and Girones, R. (2000) 'HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain', *Journal of Hepatology,* 33(5), pp. 826-833.

Prevention, C. f. D. C. a. (2020) *Hepatitis E Questions and Answers for Health Professionals*. Available at: https://www.cdc.gov/hepatitis/hev/hevfaq.htm (Accessed: 15 December 2020).

Pujols, J., Rodríguez, C., Navarro, N., Pina-Pedrero, S., Campbell, J. M., Crenshaw, J. and Polo, J. (2014) 'No transmission of hepatitis E virus in pigs fed diets containing commercial spray-dried porcine plasma: a retrospective study of samples from several swine trials', *Virology journal,* 11(1), pp. 232.

Pérez-Gracia, M. T., García, M., Suay, B. and Mateos-Lindemann, M. L. (2015) 'Current knowledge on hepatitis E', *Journal of clinical and translational hepatology,* 3(2), pp. 117.

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T. F. and Beutler, N. A. (2017) 'Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples', *nature protocols,* 12(6), pp. 1261.

Rein, D. B., Stevens, G. A., Theaker, J., Wittenborn, J. S. and Wiersma, S. T. (2012) 'The global burden of hepatitis E virus genotypes 1 and 2 in 2005', *Hepatology,* 55(4), pp. 988-997.

Reuter, G., Fodor, D., Forgách, P., Kátai, A. and Szűcs, G. (2009) 'Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary', *Journal of Clinical Virology,* 44(4), pp. 277-281.

Rivadulla, E., Varela, M. F., Mesquita, J. R., Nascimento, M. S. and Romalde, J. L. (2019) 'Detection of hepatitis E virus in shellfish harvesting areas from Galicia (Northwestern Spain)', *Viruses,* 11(7), pp. 618.

Rivero-Juarez, A., Frias, M., Martinez-Peinado, A., Risalde, M., Rodriguez-Cano, D., Camacho, A., García-Bocanegra, I., Cuenca-Lopez, F., Gomez-Villamandos, J. and Rivero, A. (2017) 'Familial hepatitis E outbreak linked to wild boar meat consumption', *Zoonoses and public health*.

Rodriguez-Manzano, J., Miagostovich, M., Hundesa, A., Clemente-Casares, P., Carratala, A., Buti, M., Jardi, R. and Girones, R. (2010) 'Analysis of the evolution in the circulation of HAV and HEV in eastern Spain by testing urban sewage samples', *Journal of water and health,* 8(2), pp. 346-354.

Rose, N., Lunazzi, A., Dorenlor, V., Merbah, T., Eono, F., Eloit, M., Madec, F. and Pavio, N. (2011) 'High prevalence of Hepatitis E virus in French domestic pigs', *Comparative immunology, microbiology and infectious diseases,* 34(5), pp. 419-427.

Roth, A., Lin, J., Magnius, L., Karlsson, M., Belák, S., Widén, F. and Norder, H. (2016) 'Markers for ongoing or previous hepatitis E virus infection are as common in wild ungulates as in humans in Sweden', *Viruses,* 8(9), pp. 259.

Rutjes, S., Bouwknegt, M., Van Der Giessen, J., de Roda Husman, A. and Reusken, C. (2014) 'Seroprevalence of hepatitis E virus in pigs from different farming systems in The Netherlands', *Journal of food protection,* 77(4), pp. 640-642.

Rutjes, S., Lodder-Verschoor, F., Lodder, W., Van der Giessen, J., Reesink, H., Bouwknegt, M. and de Roda Husman, A. (2010) 'Seroprevalence and molecular detection of hepatitis E virus in wild boar and red deer in The Netherlands', *Journal of virological methods,* 168(1-2), pp. 197-206.

Ryll, R., Eiden, M., Heuser, E., Weinhardt, M., Ziege, M., Höper, D., Groschup, M. H., Heckel, G., Johne, R. and Ulrich, R. G. (2018) 'Hepatitis E virus in feral rabbits along a rural-urban transect in Central Germany', *Infection, genetics and evolution,* 61, pp. 155-159.

Said, B., Ijaz, S., Chand, M., Kafatos, G., Tedder, R. and Morgan, D. (2014) 'Hepatitis E virus in England and Wales: indigenous infection is associated with the consumption of processed pork products', *Epidemiology & Infection,* 142(7), pp. 1467-1475.

Said, B., Ijaz, S., Kafatos, G., Booth, L., Thomas, H. L., Walsh, A., Ramsay, M. and Morgan, D. (2009) 'Hepatitis E outbreak on cruise ship', *Emerging infectious diseases,* 15(11), pp. 1738.

Sanford, B., Emerson, S., Purcell, R., Engle, R., Dryman, B., Cecere, T., Buechner-Maxwell, V., Sponenberg, D. and Meng, X. (2013) 'Serological Evidence for a Hepatitis E Virus-Related Agent in Goats in the U nited S tates', *Transboundary and emerging diseases,* 60(6), pp. 538-545.

Sanford, B. J., Dryman, B. A., Huang, Y.-W., Feagins, A. R., LeRoith, T. and Meng, X.-J. (2011) 'Prior infection of pigs with a genotype 3 swine hepatitis E virus (HEV) protects against subsequent challenges with homologous and heterologous genotypes 3 and 4 human HEV', *Virus research,* 159(1), pp. 17-22.

Schemmerer, M., Apelt, S., Trojnar, E., Ulrich, R. G., Wenzel, J. J. and Johne, R. (2016) 'Enhanced replication of hepatitis E virus strain 47832c in an A549-derived subclonal cell line', *Viruses,* 8(10), pp. 267.

Schielke, A., Filter, M., Appel, B. and Johne, R. (2011) 'Thermal stability of hepatitis E virus assessed by a molecular biological approach', *Virology journal,* 8(1), pp. 487.

Slot, E., Zaaijer, H. L., Molier, M., Van den Hurk, K., Prinsze, F. and Hogema, B. M. (2017) 'Meat consumption is a major risk factor for hepatitis E virus infection', *PloS one,* 12(4), pp. e0176414.

Smith, D. B., Paddy, J. O. and Simmonds, P. (2016) 'The use of human sewage screening for community surveillance of hepatitis E virus in the UK', *Journal of medical virology,* 88(5), pp. 915-918.

Society, M. C. (2011) *Combined Sewer Overflows Pollution Policy and Position Statement*: Marine Conservation Society. Available at: https://www.mcsuk.org/downloads/pollution/CSO%20policy.pdf.

Sonoda, H., Abe, M., Sugimoto, T., Sato, Y., Bando, M., Fukui, E., Mizuo, H., Takahashi, M., Nishizawa, T. and Okamoto, H. (2004) 'Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan', *Journal of Clinical Microbiology,* 42(11), pp. 5371-5374.

Sooryanarain, H., Heffron, C. L., Hill, D. E., Fredericks, J., Rosenthal, B. M., Werre, S. R., Opriessnig, T. and Meng, X.-J. (2020) 'Hepatitis E virus in pigs from slaughterhouses, United States, 2017–2019', *Emerging infectious diseases,* 26(2), pp. 354.

Spancerniene, U., Grigas, J., Buitkuviene, J., Zymantiene, J., Juozaitiene, V., Stankeviciute, M., Razukevicius, D., Zienius, D. and Stankevicius, A. (2018) 'Prevalence and phylogenetic analysis of hepatitis E virus in pigs, wild boars, roe deer, red deer and moose in Lithuania', *Acta veterinaria scandinavica,* 60(1), pp. 13.

Spina, A., Lenglet, A., Beversluis, D., de Jong, M., Vernier, L., Spencer, C., Andayi, F., Kamau, C., Vollmer, S. and Hogema, B. (2017) 'A large outbreak of Hepatitis E virus genotype 1 infection in an urban setting in Chad likely linked to household level transmission factors, 2016-2017', *PLoS One,* 12(11), pp. e0188240.

Szabo, K., Trojnar, E., Anheyer-Behmenburg, H., Binder, A., Schotte, U., Ellerbroek, L., Klein, G. and Johne, R. (2015) 'Detection of hepatitis E virus RNA in raw sausages and liver sausages from retail in Germany using an optimized method', *International journal of food microbiology,* 215, pp. 149-156.

Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F. and Okamoto, H. (2003) 'Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus', *Journal of General Virology,* 84(4), pp. 851-862.

Tanaka, T., Takahashi, M., Kusano, E. and Okamoto, H. (2007) 'Development and evaluation of an efficient cell-culture system for Hepatitis E virus', *Journal of General Virology,* 88(3), pp. 903-911.

Tei, S., Kitajima, N., Takahashi, K. and Mishiro, S. (2003) 'Zoonotic transmission of hepatitis E virus from deer to human beings', *The Lancet,* 362(9381), pp. 371-373.

Terio, V., Bottaro, M., Pavoni, E., Losio, M., Serraino, A., Giacometti, F., Martella, V., Mottola, A., Di Pinto, A. and Tantillo, G. (2017) 'Occurrence of hepatitis A and E and norovirus GI and GII in ready-to-eat vegetables in Italy', *International journal of food microbiology,* 249, pp. 61-65.

Tomiyama, D., Inoue, E., Osawa, Y. and Okazaki, K. (2009) 'Serological evidence of infection with hepatitis E virus among wild Yezo-deer, Cervus nippon yesoensis, in Hokkaido, Japan', *Journal of viral hepatitis,* 16(7), pp. 524-528.

Vercouter, A.-S., Sayed, I. M., Lipkens, Z., De Bleecker, K., De Vliegher, S., Colman, R., Koppelman, M., Supré, K. and Meuleman, P. (2018) 'Absence of zoonotic hepatitis E virus infection in Flemish dairy cows', *International journal of food microbiology,* 281, pp. 54-59.

Villalba, M. C. M., Martínez, D. C., Ahmad, I., Lay, L. A. R., Corredor, M. B., March, C. G., Martínez, L. S., Martínez-Campo, L. S. and Jameel, S. (2017) 'Hepatitis E virus in bottlenose dolphins Tursiops truncatus', *Diseases of aquatic organisms,* 123(1), pp. 13-18.

Vitral, C. L., Pinto, M. A., Lewis-Ximenez, L. L., Khudyakov, Y. E., dos Santos, D. R. and Gaspar, A. M. C. (2005) 'Serological evidence of hepatitis E virus infection in different animal species from the Southeast of Brazil', *Memorias do Instituto Oswaldo Cruz,* 100(2), pp. 117-122.

Wang, Y., Zhang, H., Li, Z., Gu, W., Lan, H., Hao, W., Ling, R., Li, H. and Harrison, T. J. (2001) 'Detection of sporadic cases of hepatitis E virus (HEV) infection in China using immunoassays based on recombinant open reading frame 2 and 3 polypeptides from HEV genotype 4', *Journal of clinical microbiology,* 39(12), pp. 4370-4379.

Weger, S., Elkin, B., Lindsay, R., Bollinger, T., Crichton, V. and Andonov, A. (2017) 'Hepatitis E Virus Seroprevalence in Free-Ranging Deer in Canada', *Transboundary and emerging diseases,* 64(3), pp. 1008-1011.

Wenzel, J. J., Preiß, J., Schemmerer, M., Huber, B., Plentz, A. and Jilg, W. (2011) 'Detection of hepatitis E virus (HEV) from porcine livers in Southeastern Germany and high sequence homology to human HEV isolates', *Journal of clinical virology,* 52(1), pp. 50-54.

Wilhelm, B., Leblanc, D., Houde, A., Brassard, J., Gagné, M.-J., Plante, D., Bellon-Gagnon, P., Jones, T. H., Muehlhauser, V. and Janecko, N. (2014) 'Survey of Canadian retail pork chops and pork livers for detection of hepatitis E virus, norovirus, and rotavirus using real time RT-PCR', *International journal of food microbiology,* 185, pp. 33-40.

World Health Organisation. 2019. Hepatitis E. *In:* World Health Organisation. (ed.).

Xia, J., Zeng, H., Liu, L., Zhang, Y., Liu, P., Geng, J., Wang, L., Wang, L. and Zhuang, H. (2015) 'Swine and rabbits are the main reservoirs of hepatitis E virus in China: detection of HEV RNA in feces of farmed and wild animals', *Archives of virology,* 160(11), pp. 2791-2798.

Xie, X., Bil, J., Shantz, E., Hammermueller, J., Nagy, E. and Turner, P. V. (2017) 'Prevalence of lapine rotavirus, astrovirus, and hepatitis E virus in Canadian domestic rabbit populations', *Veterinary Microbiology,* 208, pp. 146-149.

Yan, B., Zhang, L., Gong, L., Lv, J., Feng, Y., Liu, J., Song, L., Xu, Q., Jiang, M. and Xu, A. (2016) 'Hepatitis E virus in yellow cattle, Shandong, Eastern China', *Emerging infectious diseases,* 22(12), pp. 2211.

Yazaki, Y., Mizuo, H., Takahashi, M., Nishizawa, T., Sasaki, N., Gotanda, Y. and Okamoto, H. (2003) 'Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by

the presence of hepatitis E virus in pig liver as food', *Journal of general virology,* 84(9), pp. 2351-2357.

Yin, W., Han, Y., Xin, H., Liu, W., Song, Q., Li, Z., Gao, S., Jiang, F., Cao, J., Bi, S. and Liu, H. (2019) 'Hepatitis E Outbreak in a Mechanical Factory in Qingdao City, China', *Int J Infect Dis*.

Yoo, D., Willson, P., Pei, Y., Hayes, M. A., Deckert, A., Dewey, C. E., Friendship, R. M., Yoon, Y., Gottschalk, M. and Yason, C. (2001) 'Prevalence of hepatitis E virus antibodies in Canadian swine herds and identification of a novel variant of swine hepatitis E virus', *Clin. Diagn. Lab. Immunol.,* 8(6), pp. 1213-1219.

Yugo, D. M., Cossaboom, C. M., Heffron, C. L., Huang, Y. W., Kenney, S. P., Woolums, A. R., Hurley, D. J., Opriessnig, T., Li, L. and Delwart, E. (2019) 'Evidence for an unknown agent antigenically related to the hepatitis E virus in dairy cows in the United States', *Journal of medical virology,* 91(4), pp. 677-686.

Zemtsova, G. E., Montgomery, M. and Levin, M. L. (2015) 'Relative sensitivity of conventional and real-time PCR assays for detection of SFG Rickettsia in blood and tissue samples from laboratory animals', *PloS one,* 10(1).

Zhang, X.-X., Qin, S.-Y., Zhang, Y., Meng, Q.-F., Jiang, J., Yang, G.-L., Zhao, Q. and Zhu, X.-Q. (2015) 'First report of hepatitis E virus infection in sika deer in China', *BioMed research international,* 2015.

Zhang, Y., Gong, W., Song, W. T., Fu, H., Wang, L., Li, M., Wang, L. and Zhuang, H. (2017) 'Different susceptibility and pathogenesis of rabbit genotype 3 hepatitis E virus (HEV-3) and human HEV-3 (JRC-HE3) in SPF rabbits', *Veterinary microbiology,* 207, pp. 1-6.

Zhao, C., Li, Z., Yan, B., Harrison, T. J., Guo, X., Zhang, F., Yin, J., Yan, Y. and Wang, Y. (2007) 'Comparison of real-time fluorescent RT-PCR and conventional RT-PCR for the detection of hepatitis E virus genotypes prevalent in China', *Journal of medical virology,* 79(12), pp. 1966-1973.

Bibliography

Abravanel, F., Lhomme, S., El Costa, H., Schvartz, B., Peron, J.-M., Kamar, N. and Izopet, J. (2017) 'Rabbit hepatitis E virus infections in humans, France', *Emerging infectious diseases,* 23(7), pp. 1191.

Abutaleb, A. and Kottilil, S. (2020) 'Hepatitis A: Epidemiology, natural history, unusual clinical manifestations, and prevention', *Gastroenterology Clinics,* 49(2), pp. 191-199.

Agrawal, S., Orschler, L. and Lackner, S. (2021) 'Long-term monitoring of SARS-CoV-2 RNA in wastewater of the Frankfurt metropolitan area in Southern Germany', *Scientific reports,* 11(1), pp. 5372-5372.

Ahn, H. S., Park, B. J., Han, S. H., Kim, Y. H., Kim, D. H., Kim, B. S., Lee, J. B., Park, S. Y., Song, C. S. and Lee, S. W. (2017) 'Prevalence and genetic features of rabbit hepatitis E virus in Korea', *Journal of medical virology,* 89(11), pp. 1995-2002.

Alfano-Sobsey, E., Sweat, D., Hall, A., Breedlove, F., Rodriguez, R., Greene, S., Pierce, A., Sobsey, M., Davies, M. and Ledford, S. L. (2012) 'Norovirus outbreak associated with undercooked oysters and secondary household transmission', *Epidemiol Infect,* 140(2), pp. 276-82.

Almand, E. A., Moore, M. D. and Jaykus, L.-A. (2017) 'Norovirus binding to ligands beyond histo-blood group antigens', *Frontiers in microbiology,* 8, pp. 2549.

Alsadey, S. and Mansour, O. (2020) 'Wastewater Treatment Plants in Libya: Challenges and Future Prospects', 6, pp. 76-80.Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) 'Basic local alignment search tool', *J Mol Biol,* 215(3), pp. 403-10.

Andraud, M., Dumarest, M., Cariolet, R., Aylaj, B., Barnaud, E., Eono, F., Pavio, N. and Rose, N. (2013) 'Direct contact and environmental contaminations are responsible for HEV transmission in pigs', *Veterinary research,* 44(1), pp. 102.

Anheyer-Behmenburg, H. E., Szabo, K., Schotte, U., Binder, A., Klein, G. and Johne, R. (2017) 'Hepatitis E virus in wild boars and spillover infection in red and roe deer, Germany, 2013–2015', *Emerging infectious diseases,* 23(1), pp. 130.

Aprea, G., Amoroso, M. G., Di Bartolo, I., D'Alessio, N., Di Sabatino, D., Boni, A., Cioffi, B., D'Angelantonio, D., Scattolini, S., De Sabato, L., Cotturone, G., Pomilio, F., Migliorati, G., Galiero, G. and Fusco, G. (2018) 'Molecular detection and phylogenetic analysis of hepatitis E virus strains circulating in wild boars in south-central Italy', *Transboundary and Emerging Diseases,* 65(1), pp. e25-e31.

Arankalle, V. A., Chadha, M. S. and Chobe, L. P. (1999) 'Long-term serological follow up and cross-challenge studies in rhesus monkeys experimentally infected with hepatitis E virus', *Journal of hepatology,* 30(2), pp. 199-204.

Arnold, B. F., Schiff, K. C., Griffith, J. F., Gruber, J. S., Yau, V., Wright, C. C.,

Wade, T. J., Burns, S., Hayes, J. M., McGee, C., Gold, M., Cao, Y., Weisberg, S. B. and Colford, J. M. J. (2013) 'Swimmer Illness Associated with Marine Water Exposure and Water Quality Indicators: Impact of Widely Used Assumptions', *Epidemiology,* 24(6), pp. 845-853.

Baechlein, C. and Becher, P. (2017) 'No evidence for zoonotic hepatitis E virus infection through dairy milk in Germany', *Hepatology,* 65(1), pp. 394-395.

Baechlein, C., Schielke, A., Johne, R., Ulrich, R. G., Baumgaertner, W. and Grummer, B. (2010) 'Prevalence of Hepatitis E virus-specific antibodies in sera of German domestic pigs estimated by using different assays', *Veterinary microbiology,* 144(1), pp. 187-191.

Baert, L., Uyttendaele, M., Stals, A., Van Coillie, E., Dierick, K., Debevere, J. and Botteldoorn, N. (2009) 'Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context', *Epidemiology & Infection,* 137(3), pp. 316-325.

Baez, P. A., Lopez, M. C., Duque-Jaramillo, A., Pelaez, D., Molina, F. and Navas, M.-C. (2017) 'First evidence of the Hepatitis E virus in environmental waters in Colombia', *PloS one,* 12(5), pp. e0177525.

Barnaud, E., Rogée, S., Garry, P., Rose, N. and Pavio, N. (2012) 'Thermal inactivation of infectious hepatitis E virus in experimentally contaminated food', *Applied and environmental microbiology,* 78(15), pp. 5153-5159.

Batista, F. M., Stapleton, T., Lowther, J. A., Fonseca, V. G., Shaw, R., Pond, C., Walker, D. I., van Aerle, R. and Martinez-Urtaza, J. (2020) 'Whole Genome Sequencing of Hepatitis A Virus Using a PCR-Free Single-Molecule Nanopore Sequencing Approach', *Front Microbiol,* 11, pp. 874.

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. and Sayers, E. W. (2013) 'GenBank', *Nucleic Acids Res,* 41(Database issue), pp. D36-42.

Bernard, H., Faber, M., Wilking , H., Haller, S., Höhle, M., Schielke, A., Ducomble, T., Siffczyk, C., Merbecks, S. S., Fricke, G., Hamouda, O., Stark, K., Werber, D. and Team, o. b. o. t. O. I. (2014) 'Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012', *Eurosurveillance,* 19(8), pp. 20719.

Berto, A., Martelli, F., Grierson, S. and Banks, M. (2012) 'Hepatitis E virus in pork food chain, United Kingdom, 2009–2010', *Emerging infectious diseases,* 18(8), pp. 1358.

Beyer, S., Szewzyk, R., Gnirss, R., Johne, R. and Selinka, H.-C. (2020) 'Detection and characterization of hepatitis E Virus genotype 3 in wastewater and urban surface waters in Germany', *Food and environmental virology*, pp. 1-11.

Bigoraj, E., Kozyra, I., Kwit, E. and Rzeżutka, A. (2020) 'Detection of hepatitis E virus (rabbit genotype) in farmed rabbits entering the food chain', *International Journal of Food Microbiology,* 319, pp. 108507.

Boadella, M., Casas, M., Martín, M., Vicente, J., Segalés, J., De la Fuente, J. and Gortázar, C. (2010) 'Increasing contact with hepatitis E virus in red deer, Spain', *Emerging infectious diseases,* 16(12), pp. 1994.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M. and Girones, R. (2006) 'Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices', *Appl. Environ. Microbiol.,* 72(12), pp. 7894-7896.

Bortagaray, V., Girardi, V., Pou, S., Lizasoain, A., Tort, L. F. L., Spilki, F. R., Colina, R. and Victoria, M. (2020) 'Detection, Quantification, and Microbial Risk Assessment of Group A Rotavirus in Rivers from Uruguay', *Food Environ Virol,* 12(2), pp. 89-98.

Bouwknegt, M., Lodder-Verschoor, F., Van Der Poel, W. H., Rutjes, S. A. and De Roda Husman, A. M. (2007) 'Hepatitis E virus RNA in commercial porcine livers in The Netherlands', *Journal of food protection,* 70(12), pp. 2889-2895.

Bouwknegt, M., Rutjes, S. A., Reusken, C. B., Stockhofe-Zurwieden, N., Frankena, K., de Jong, M. C., de Roda Husman, A. M. and van der Poel, W. H. (2009) 'The course of hepatitis E virus infection in pigs after contact-infection and intravenous inoculation', *BMC veterinary research,* 5(1), pp. 7.

Boxman, I. L., Jansen, C. C., Hägele, G., Zwartkruis-Nahuis, A., Cremer, J., Vennema, H. and Tijsma, A. S. (2017) 'Porcine blood used as ingredient in meat productions may serve as a vehicle for hepatitis E virus transmission', *International journal of food microbiology,* 257, pp. 225-231.

Boxman, I. L., Jansen, C. C., Hägele, G., Zwartkruis-Nahuis, A., Tijsma, A. S. and Vennema, H. (2019) 'Monitoring of pork liver and meat products on the Dutch market for the presence of HEV RNA', *International journal of food microbiology,* 296, pp. 58-64.

Boxman, I. L., Verhoef, L., Vennema, H., Ngui, S.-L., Friesema, I. H., Whiteside, C., Lees, D. and Koopmans, M. (2016) 'International linkage of two food-borne hepatitis A clusters through traceback of mussels, the Netherlands, 2012', *Eurosurveillance,* 21(3), pp. 30113.

Bruggink, L. D., Moselen, J. M. and Marshall, J. A. (2017) 'The molecular epidemiology of norovirus outbreaks in Victoria, 2014 to 2015', *Communicable diseases intelligence quarterly report,* 41(1), pp. E21-E32.

Bull, R. A., Adikari, T. N., Ferguson, J. M., Hammond, J. M., Stevanovski, I., Beukers, A. G., Naing, Z., Yeang, M., Verich, A., Gamaarachchi, H., Kim, K. W., Luciani, F., Stelzer-Braid, S., Eden, J.-S., Rawlinson, W. D., van Hal, S. J. and Deveson, I. W. (2020) 'Analytical validity of nanopore sequencing for rapid SARS-CoV-2 genome analysis', *Nature Communications,* 11(1), pp. 6272.

Burri, C., Vial, F., Ryser-Degiorgis, M. P., Schwermer, H., Darling, K., Reist, M., Wu, N., Beerli, O., Schöning, J. and Cavassini, M. (2014) 'Seroprevalence of hepatitis E virus in domestic pigs and wild boars in Switzerland', *Zoonoses and public health,* 61(8), pp. 537-544.

Burt, S. A., Veltman, J., Hakze-van der Honing, R., Schmitt, H. and van der

Poel, W. H. (2016) 'Hepatitis E virus in farmed rabbits, wild rabbits and petting farm rabbits in the Netherlands', *Food and environmental virology,* 8(3), pp. 227-229.

Campos, C. J., Avant, J., Gustar, N., Lowther, J., Powell, A., Stockley, L. and Lees, D. N. (2015) 'Fate of human noroviruses in shellfish and water impacted by frequent sewage pollution events', *Environmental science & technology,* 49(14), pp. 8377-8385.

Campos, C. J., Avant, J., Lowther, J., Till, D. and Lees, D. N. (2016) 'Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes', *Water research,* 103, pp. 224-232.

Campos, C. J., Kershaw, S. R. and Lee, R. J. (2013) 'Environmental influences on faecal indicator organisms in coastal waters and their accumulation in bivalve shellfish', *Estuaries and coasts,* 36(4), pp. 834-853.

Capelli, N., Marion, O., Dubois, M., Allart, S., Bertrand-Michel, J., Lhomme, S., Abravanel, F., Izopet, J. and Chapuy-Regaud, S. (2019) 'Vectorial Release of Hepatitis E Virus in Polarized Human Hepatocytes', *J Virol,* 93(4).

Casas, M., Pina, S., de Deus, N., Peralta, B., Martín, M. and Segalés, J. (2009) 'Pigs orally inoculated with swine hepatitis E virus are able to infect contact sentinels', *Vet Microbiol,* 138(1-2), pp. 78-84.

Centers for Disease Control and Prevention. (2020) *Hepatitis E Questions and Answers for Health Professionals*. Available at: https://www.cdc.gov/hepatitis/hev/hevfaq.htm (Accessed: 15 December 2020).

Chang, Y., Wang, L., Geng, J., Zhu, Y., Fu, H., Ren, F., Li, L., Wang, X. and Zhuang, H. (2009) 'Zoonotic risk of hepatitis E virus (HEV): A study of HEV infection in animals and humans in suburbs of Beijing', *Hepatology Research,* 39(12), pp. 1153-1158.

Chen, C. M., Chen, S. C., Yang, H. Y., Yang, S. T. and Wang, C. M. (2016) 'Hospitalization and mortality due to hepatitis A in Taiwan: a 15-year nationwide cohort study', *Journal of viral hepatitis,* 23(11), pp. 940-945.

Chhabra, P., de Graaf, M., Parra, G. I., Chan, M. C.-W., Green, K., Martella, V., Wang, Q., White, P. A., Katayama, K. and Vennema, H. (2019) 'Updated classification of norovirus genogroups and genotypes', *The Journal of general virology,* 100(10), pp. 1393.

Choi, C. and Kingsley, D. H. (2016) 'Temperature-dependent persistence of human norovirus within oysters (Crassostrea virginica)', *Food and environmental virology,* 8(2), pp. 141-147.

Clemente-Casares, P., Pina, S., Buti, M., Jardi, R., Martín, M., Bofill-Mas, S. and Girones, R. (2003) 'Hepatitis E virus epidemiology in industrialized countries', *Emerging infectious diseases,* 9(4), pp. 449.

Clemente-Casares, P., Rodriguez-Manzano, J. and Girones, R. (2009) 'Hepatitis E virus genotype 3 and sporadically also genotype 1 circulate in the population of Catalonia, Spain', *Journal of water and health,* 7(4), pp. 664-673.

Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal, V., Gallian, P., Heyries, L., Raoult, D. and Gerolami, R. (2010) 'Pig liver sausage as a source of hepatitis E virus transmission to humans', *The Journal of infectious diseases,* 202(6), pp. 825-834.

Comber, S. D. W., Gardner, M. J. and Ellor, B. (2020) 'Seasonal variation of contaminant concentrations in wastewater treatment works effluents and river waters', *Environmental Technology,* 41(21), pp. 2716-2730.

Corbett, S. J., Rubin, G. L., Curry, G. K. and Kleinbaum, D. G. (1993) 'The health effects of swimming at Sydney beaches. The Sydney Beach Users Study Advisory Group', *Am J Public Health,* 83(12), pp. 1701-6.

Corman, V. M., Hilgensloh, L., Voigt, U., Marklewitz, M., Siebert, U., Drosten, C. and Drexler, J. F. (2019) 'Hepatitis E Virus Infection in European Brown Hares, Germany, 2007–2014', *Emerging infectious diseases,* 25(6), pp. 1233.

Cossaboom, C. M., Córdoba, L., Dryman, B. A. and Meng, X.-J. (2011) 'Hepatitis E virus in rabbits, Virginia, USA', *Emerging infectious diseases,* 17(11), pp. 2047.

Costafreda, M. I., Bosch, A. and Pintó, R. M. (2006) 'Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples', *Appl. Environ. Microbiol.,* 72(6), pp. 3846-3855.

Costantini, V., Morantz, E. K., Browne, H., Ettayebi, K., Zeng, X.-L., Atmar, R. L., Estes, M. K. and Vinjé, J. (2018) 'Human norovirus replication in human intestinal enteroids as model to evaluate virus inactivation', *Emerging infectious diseases,* 24(8), pp. 1453.

Coutts, S. P., Sturge, K., Lalor, K., Marshall, J. A., Bruggink, L. D., Subasinghe, N. and Easton, M. (2017) 'An outbreak of foodborne norovirus gastroenteritis linked to a restaurant in Melbourne, Australia, 2014', *Western Pac Surveill Response J,* 8(2), pp. 12-16.

Crits-Christoph, A., Kantor, R. S., Olm, M. R., Whitney, O. N., Al-Shayeb, B., Lou, Y. C., Flamholz, A., Kennedy, L. C., Greenwald, H., Hinkle, A., Hetzel, J., Spitzer, S., Koble, J., Tan, A., Hyde, F., Schroth, G., Kuersten, S., Banfield, J. F. and Nelson, K. L. (2021) 'Genome Sequencing of Sewage Detects Regionally Prevalent SARS-CoV-2 Variants', *mBio,* 12(1), pp. e02703-20.

Cross, L. J. (2004) *The development and application of quantitative methods for norovirus detection in sewage effluents and seawater.* University of Surrey (United Kingdom).

Crossan, C., Baker, P. J., Craft, J., Takeuchi, Y., Dalton, H. R. and Scobie, L. (2012) 'Hepatitis E virus genotype 3 in shellfish, United Kingdom', *Emerging infectious diseases,* 18(12), pp. 2085.

da Silva, A. K., Le Saux, J.-C., Parnaudeau, S., Pommepuy, M., Elimelech, M. and Le Guyader, F. S. (2007) 'Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II', *Appl. Environ. Microbiol.,* 73(24), pp. 7891-

7897.

Dalton, H., Stableforth, W., Hazeldine, S., Thurairajah, P., Ramnarace, R., Warshow, U., Ijaz, S., Ellis, V. and Bendall, R. (2008) 'Autochthonous hepatitis E in Southwest England: a comparison with hepatitis A', *European Journal of Clinical Microbiology & Infectious Diseases,* 27(7), pp. 579-585.

Dalton, H., Thurairajah, P., Fellows, H., Hussaini, H., Mitchell, J., Bendall, R., Banks, M., Ijaz, S., Teo, C. G. and Levine, D. (2007) 'Autochthonous hepatitis E in southwest England', *Journal of viral hepatitis,* 14(5), pp. 304-309.

Dancho, B. A., Chen, H. and Kingsley, D. H. (2012) 'Discrimination between infectious and non-infectious human norovirus using porcine gastric mucin', *International Journal of Food Microbiology,* 155(3), pp. 222-226.

De Deus, N., Casas, M., Peralta, B., Nofrarías, M., Pina, S., Martín, M. and Segalés, J. (2008) 'Hepatitis E virus infection dynamics and organic distribution in naturally infected pigs in a farrow-to-finish farm', *Veterinary Microbiology,* 132(1), pp. 19-28.

de Oya, N. J., de Blas, I., Blázquez, A.-B., Martín-Acebes, M. A., Halaihel, N., Gironés, O., Saiz, J.-C. and Escribano-Romero, E. (2011) 'Widespread distribution of hepatitis E virus in Spanish pig herds', *BMC research notes,* 4(1), pp. 412.

De Sabato, L., Amoroso, M. G., Ianiro, G., Esposito, C., De Grossi, L., Fusco, G., Barone, A., Martini, E., Ostanello, F. and Di Bartolo, I. (2019) 'Detection of Hepatitis E Virus in Livers and Muscle Tissues of Wild Boars in Italy', *Food and environmental virology*, pp. 1-8.

Debode, F., Marien, A., Janssen, É., Bragard, C. and Berben, G. (2017) 'The influence of amplicon length on real-time PCR results', *Base*.

DEFRA (2002) Sewage Treatment in the UK: Department for Environment, Food and Rural Affairs. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/69582/pb6655-uk-sewage-treatment-020424.pdf (Accessed: 03 July 2022).

Degner, R. L. and Petrone, C. (1994) 'Consumer and restaurant manager reaction to depurated oysters and clams', *FAMRC industry report/(USA)*.

Demirci, M., Yiğin, A., Ünlü, Ö. and Kılıç, S. A. (2019) 'Detection of HEV RNA amounts and genotypes in raw milks obtained from different animals', *Mikrobiyoloji bulteni,* 53(1), pp. 43-52.

Desdouits, M., Wacrenier, C., Ollivier, J., Schaeffer, J. and Le Guyader, F. S. (2020) 'A Targeted Metagenomics Approach to Study the Diversity of Norovirus GII in Shellfish Implicated in Outbreaks', *Viruses,* 12(9), pp. 978.

Di Bartolo, I., Angeloni, G., Ponterio, E., Ostanello, F. and Ruggeri, F. M. (2015a) 'Detection of hepatitis E virus in pork liver sausages', *International journal of food microbiology,* 193, pp. 29-33.

Di Bartolo, I., De Sabato, L., Marata, A., Martinelli, N., Magistrali, C., Monini, M.,

Ponterio, E., Ostanello, F. and Ruggeri, F. (2016) 'Serological survey of hepatitis E virus infection in farmed and pet rabbits in Italy', *Archives of virology,* 161(5), pp. 1343-1346.

Di Bartolo, I., Diez-Valcarce, M., Vasickova, P., Kralik, P., Hernandez, M., Angeloni, G., Ostanello, F., Bouwknegt, M., Rodríguez-Lázaro, D. and Pavlik, I. (2012) 'Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010', *Emerging infectious diseases,* 18(8), pp. 1282.

Di Bartolo, I., Pavoni, E., Tofani, S., Consoli, M., Galuppini, E., Losio, M. N., Ruggeri, F. M. and Varisco, G. (2015b) 'Waterborne norovirus outbreak during a summer excursion in Northern Italy', *New Microbiol,* 38(1), pp. 109-12.

Di Bartolo, I., Ponterio, E., Angeloni, G., Morandi, F., Ostanello, F., Nicoloso, S. and Ruggeri, F. (2017) 'Presence of hepatitis E virus in a RED deer (Cervus elaphus) population in Central Italy', *Transboundary and emerging diseases,* 64(1), pp. 137-143.

Di Bartolo, I., Ponterio, E., Castellini, L., Ostanello, F. and Ruggeri, F. M. (2011) 'Viral and antibody HEV prevalence in swine at slaughterhouse in Italy', *Veterinary microbiology,* 149(3-4), pp. 330-338.

Di Martino, B., Di Profio, F., Melegari, I., Sarchese, V., Robetto, S., Marsilio, F. and Martella, V. (2016) 'Detection of hepatitis E virus (HEV) in goats', *Virus research,* 225, pp. 69-72.

Dorevitch, S., Panthi, S., Huang, Y., Li, H., Michalek, A. M., Pratap, P., Wroblewski, M., Liu, L., Scheff, P. A. and Li, A. (2011) 'Water ingestion during water recreation', *Water research,* 45(5), pp. 2020-2028.

Dufour, A. P., Evans, O., Behymer, T. D. and Cantu, R. (2006) 'Water ingestion during swimming activities in a pool: A pilot study', *Journal of Water and Health,* 4(4), pp. 425-430.

Dähnert, L., Eiden, M., Schlosser, J., Fast, C., Schröder, C., Lange, E., Gröner, A., Schäfer, W. and Groschup, M. H. (2018) 'High sensitivity of domestic pigs to intravenous infection with HEV', *BMC veterinary research,* 14(1), pp. 381.

Eden, J.-S., Hewitt, J., Lim, K. L., Boni, M. F., Merif, J., Greening, G., Ratcliff, R. M., Holmes, E. C., Tanaka, M. M. and Rawlinson, W. D. (2014) 'The emergence and evolution of the novel epidemic norovirus GII. 4 variant Sydney 2012', *Virology,* 450, pp. 106-113.

EFTEC (2002) 'Valuation of benefits to England and Wales of a revised bathing water quality directive and other beach characteristics using the choice experiment methodology', *Final Rep.*

Eiden, M., Vina-Rodriguez, A., Schlosser, J., Schirrmeier, H. and Groschup, M. H. (2016) 'Detection of hepatitis E virus in archived rabbit serum samples, Germany 1989', *Food and environmental virology,* 8(1), pp. 105-107.

El-Kafrawy, S. A., Hassan, A. M., El-Daly, M. M., Qadri, I., Tolah, A. M., Al-Subhi, T. L., Alzahrani, A. A., Alsaaidi, G. A., Al-Abdullah, N. and Kaki, R. M. (2020) 'Seroprevalence of Dromedary Camel HEV in Domestic and Imported

Camels from Saudi Arabia', *Viruses,* 12(5), pp. 553.

El-Senousy, W. M., Osman, G. A. and Melegy, A. A. (2014) 'Survival of adenovirus, rotavirus, hepatitis A virus, pathogenic bacteria and bacterial indicators in ground water', *World Applied Sciences Journal,* 29(3), pp. 337-348.

Emerson, S. U., Arankalle, V. A. and Purcell, R. H. (2005) 'Thermal stability of hepatitis E virus', *The Journal of infectious diseases,* 192(5), pp. 930-933.

Environment Agency. (2020a) 'Event Duration Monitoring - Storm Overflows - 2020' [Database of CSO use throughout UK in 2020]. Available at: https://environment.data.gov.uk/dataset/21e15f12-0df8-4bfc-b763-45226c16a8ac (Accessed 23 Jun 2021).

Environment Agency. (2020b) *Water and sewerage companies in England: environmental performance report for 2019*: Gov.UK. Available at: https://www.gov.uk/government/publications/water-and-sewerage-companies-in-england-environmental-performance-report-2019/water-and-sewerage-companies-in-england-environmental-performance-report-for-2019.

Environmental Protection Agency. *Agriculture Nutrient Management and Fertilizer*. USA. Available at: https://www.epa.gov/agriculture/agriculture-nutrient-management-and-fertilizer#Manure (Accessed: 08 September 2020).

European Commission. (2001) *Survey of wastes spread on land*: European Commission. Available at: https://ec.europa.eu/environment/waste/studies/compost/landspreading_4-6.pdf.

European Commission., European Union. (2007) *Portugal: Commission continues legal action over environmental and human health infringements.* Brussels: European Commission.

European Food Satety Authority. (2019) 'Analysis of the European baseline survey of norovirus in oysters', *EFSA Journal,* 17(7), pp. e05762.

Ettayebi, K., Crawford, S. E., Murakami, K., Broughman, J. R., Karandikar, U., Tenge, V. R., Neill, F. H., Blutt, S. E., Zeng, X. L., Qu, L., Kou, B., Opekun, A. R., Burrin, D., Graham, D. Y., Ramani, S., Atmar, R. L. and Estes, M. K. (2016) 'Replication of human noroviruses in stem cell-derived human enteroids', *Science,* 353(6306), pp. 1387-1393.

Farkas, K., Adriaenssens, E. M., Walker, D. I., McDonald, J. E., Malham, S. K. and Jones, D. L. (2019) 'Critical Evaluation of CrAssphage as a Molecular Marker for Human-Derived Wastewater Contamination in the Aquatic Environment', *Food and environmental virology,* 11(2), pp. 113-119.

Feagins, A., Opriessnig, T., Guenette, D., Halbur, P. and Meng, X. (2008) Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States', *International journal of food microbiology,* 123(1-2), pp. 32-37.

Feagins, A., Opriessnig, T., Guenette, D., Halbur, P. and Meng, X.-J. (2007) 'Detection and characterization of infectious Hepatitis E virus from commercial

pig livers sold in local grocery stores in the USA', *Journal of general virology,* 88(3), pp. 912-917.

Feurer, C., Le Roux, A., Rossel, R., Barnaud, E., Dumarest, M., Garry, P. and Pavio, N. (2018) 'High load of hepatitis E viral RNA in pork livers but absence in pork muscle at French slaughterhouses', *International journal of food microbiology,* 264, pp. 25-30.

Fisher, R. A. (1934) 'Statistical methods for research workers', *Statistical methods for research workers.,* (5th Ed).

Flannery J, Keaveney S, Rajko-Nenow P, O'Flaherty V, Doré W. 'Concentration of norovirus during wastewater treatment and its impact on oyster contamination'. Appl Environ Microbiol. 2012 May;78(9):3400-6. doi: 10.1128/AEM.07569-11. Epub 2012 Feb 24. PMID: 22367079; PMCID: PMC3346491.

Food and Agriculture Organisation. (2011) *Irrigation in Southern and Eastern Asia in figures*: Food and Agriculture Organisation. Available at: http://www.fao.org/3/i2809e/i2809e.pdf.

Food and Agriculture Organisation. (2016) *Irrigation in Eastern Europe in figures*: Food and Agriculture Organisation of the United Nations. Available at: http://www.fao.org/3/CA3380EN/ca3380en.pdf (Accessed: 12/08/2020).

Fong, T.-T. and Lipp, E. K. (2005) 'Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools', *Microbiology and molecular biology reviews : MMBR,* 69(2), pp. 357-371.

Formiga-Cruz, M., Tofino-Quesada, G., Bofill-Mas, S., Lees, D., Henshilwood, K., Allard, A., Conden-Hansson, A.-C., Hernroth, B., Vantarakis, A. and Tsibouxi, A. (2002) 'Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden, and the United Kingdom', *Applied and Environmental Microbiology,* 68(12), pp. 5990-5998.

Forni, D., Cagliani, R., Clerici, M. and Sironi, M. (2018) 'Origin and dispersal of hepatitis E virus', *Emerging microbes & infections,* 7(1), pp. 1-13.

Fraga, M., Doerig, C., Moulin, H., Bihl, F., Brunner, F., Müllhaupt, B., Ripellino, P., Semela, D., Stickel, F. and Terziroli Beretta-Piccoli, B. (2017) 'Hepatitis E virus as a cause of acute hepatitis acquired in Switzerland', *Liver International*.

Friedel, C. C., Dölken, L., Ruzsics, Z., Koszinowski, U. H. and Zimmer, R. (2009) 'Conserved principles of mammalian transcriptional regulation revealed by RNA half-life', *Nucleic acids research,* 37(17), pp. e115-e115.

Fusco, G., Anastasio, A., Kingsley, D. H., Amoroso, M. G., Pepe, T., Fratamico, P. M., Cioffi, B., Rossi, R., La Rosa, G. and Boccia, F. (2019) 'Detection of hepatitis A virus and other enteric viruses in shellfish collected in the Gulf of Naples, Italy', *International journal of environmental research and public health,* 16(14), pp. 2588.

Gao, J., Wu, H., Shi, X., Huo, Z., Zhang, J. and Liang, Z. (2016) 'Comparison of

Next-Generation Sequencing, Quantitative PCR, and Sanger Sequencing for Mutation Profiling of EGFR, KRAS, PIK3CA and BRAF in Clinical Lung Tumors', *Clin Lab,* 62(4), pp. 689-96.

Gao, S., Li, D., Zha, E., Zhou, T., Wang, S. and Yue, X. (2015) 'Surveillance of hepatitis E virus contamination in shellfish in China', *International journal of environmental research and public health,* 12(2), pp. 2026-2036.

Gao, Z., Liu, B., Yan, H., Li, W., Jia, L., Tian, Y., Chen, Y., Wang, Q. and Pang, X. (2019) 'Norovirus outbreaks in Beijing, China, from 2014 to 2017', *Journal of Infection,* 79(2), pp. 159-166.

García, M., Fernández-Barredo, S. and Pérez-Gracia, M. (2014) 'Detection of hepatitis E virus (HEV) through the different stages of pig manure composting plants', *Microbial biotechnology,* 7(1), pp. 26-31.

Gardinali, N. R., Barry, A. F., Otonel, R. A. A., Alfieri, A. F. and Alfieri, A. A. (2012) 'Hepatitis E virus in liver and bile samples from slaughtered pigs of Brazil', *Memorias do Instituto Oswaldo Cruz,* 107(7), pp. 935-939.

Garson, J. A., Ferns, R. B., Grant, P. R., Ijaz, S., Nastouli, E., Szypulska, R. and Tedder, R. S. (2012) 'Minor groove binder modification of widely used TaqMan probe for hepatitis E virus reduces risk of false negative real-time PCR results', *Journal of virological methods,* 186(1), pp. 157-160.

Geng, J., Wang, L., Wang, X., Fu, H., Bu, Q., Zhu, Y. and Zhuang, H. (2011a) 'Study on prevalence and genotype of hepatitis E virus isolated from Rex Rabbits in Beijing, China', *Journal of viral hepatitis,* 18(9), pp. 661-667.

Geng, Y., Zhao, C., Song, A., Wang, J., Zhang, X., Harrison, T. J., Zhou, Y., Wang, W. and Wang, Y. (2011b) 'The serological prevalence and genetic diversity of hepatitis E virus in farmed rabbits in China', *Infection, Genetics and Evolution,* 11(2), pp. 476-482.

Gherman, I., Holland, D., Clifford, R., Mahmoudzadeh, N., Uy, F., Adkin, A., Kainth, B., Cook, P., Day, A. and Wilson, A. (2020) 'Technical Report: Review of Quantitative Risk Assessment of foodborne norovirus transmission', *Science,* 1, pp. 1.

Giannini, P., Jermini, M., Leggeri, L., Nüesch-Inderbinen, M. and Stephan, R. (2017) 'Detection of hepatitis E virus RNA in raw cured sausages and raw cured sausages containing pig liver at retail stores in Switzerland', *Journal of food protection,* 81(1), pp. 43-45.

Gibson, K. E., Opryszko, M. C., Schissler, J. T., Guo, Y. and Schwab, K. J. (2011) 'Evaluation of human enteric viruses in surface water and drinking water resources in southern Ghana', *The American journal of tropical medicine and hygiene,* 84(1), pp. 20-29.

Givens, C. E., Kolpin, D. W., Borchardt, M. A., Duris, J. W., Moorman, T. B. and Spencer, S. K. (2016) 'Detection of hepatitis E virus and other livestock-related pathogens in Iowa streams', *Science of the Total Environment,* 566, pp. 1042-1051.

Graciaa, D. S., Cope, J. R., Roberts, V. A., Cikesh, B. L., Kahler, A. M., Vigar, M., Hilborn, E. D., Wade, T. J., Backer, L. C., Montgomery, S. P., Secor, W. E., Hill, V. R., Beach, M. J., Fullerton, K. E., Yoder, J. S. and Hlavsa, M. C. (2018) 'Outbreaks Associated with Untreated Recreational Water - United States, 2000-2014', *MMWR Morb Mortal Wkly Rep,* 67(25), pp. 701-706.

Grierson, S., Heaney, J., Cheney, T., Morgan, D., Wyllie, S., Powell, L., Smith, D., Ijaz, S., Steinbach, F. and Choudhury, B. (2015) 'Prevalence of hepatitis E virus infection in pigs at the time of slaughter, United Kingdom, 2013', *Emerging infectious diseases,* 21(8), pp. 1396.

Grierson, S., Rabie, A., Lambert, M., Choudhury, B. and Smith, R. P. (2018) 'HEV infection not evident in rodents on English pig farms', *Veterinary Record,* 182(3), pp. 81-81.

Grodzki, M., Schaeffer, J., Piquet, J.-C., Le Saux, J.-C., Chevé, J., Ollivier, J., Le Pendu, J. and Le Guyader, F. S. (2014) 'Bioaccumulation efficiency, tissue distribution, and environmental occurrence of hepatitis E virus in bivalve shellfish from France', *Applied and environmental microbiology,* 80(14), pp. 4269-4276.

Guillois, Y., Abravanel, F., Miura, T., Pavio, N., Vaillant, V., Lhomme, S., Le Guyader, F. S., Rose, N., Le Saux, J.-C. and King, L. A. (2015) 'High proportion of asymptomatic infections in an outbreak of hepatitis E associated with a spit-roasted piglet, France, 2013', *Clinical Infectious Diseases,* 62(3), pp. 351-357.

Guillois-Bécel, Y., Couturier, E., Le Saux, J.-C., Roque-Afonso, A., Le Guyader, F., Le Goas, A., Pernes, J., Le Bechec, S., Briand, A. and Robert, C. (2009) 'An oyster-associated hepatitis A outbreak in France in 2007', *Eurosurveillance,* 14(10), pp. 19144.

Haas, C. N., Rose, J. B. and Gerba, C. P. (2014) *Quantitative microbial risk assessment.* John Wiley & Sons.

Halbur, P., Kasorndorkbua, C., Gilbert, C., Guenette, D., Potters, M., Purcell, R., Emerson, S., Toth, T. and Meng, X. (2001) 'Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human', *Journal of clinical microbiology,* 39(3), pp. 918-923.

Hall, V., Taye, A., Walsh, B., Maguire, H., Dave, J., Wright, A., Anderson, C. and Crook, P. (2017) 'A large outbreak of gastrointestinal illness at an open-water swimming event in the River Thames, London', *Epidemiology and Infection,* 145(6), pp. 1246-1255.

Hammerschmidt, F., Schwaiger, K., Dähnert, L., Vina-Rodriguez, A., Höper, D., Gareis, M., Groschup, M. and Eiden, M. (2017) 'Hepatitis E virus in wild rabbits and European brown hares in Germany', *Zoonoses and public health,* 64(8), pp. 612-622.

Hansman, G. S., Oka, T., Okamoto, R., Nishida, T., Toda, S., Noda, M., Sano, D., Ueki, Y., Imai, T. and Omura, T. (2007) 'Human sapovirus in clams, Japan', *Emerging infectious diseases,* 13(4), pp. 620.

Haramoto, E., Katayama, H., Oguma, K. and Ohgaki, S. (2005) 'Application of

cation-coated filter method to detection of noroviruses, enteroviruses, adenoviruses, and torque teno viruses in the Tamagawa River in Japan', *Applied and Environmental Microbiology,* 71(5), pp. 2403-2411.

Hardstaff, J., Clough, H., Harris, J., Lowther, J., Lees, D. and O'Brien, S. (2019) 'The use of capture-recapture methods to provide better estimates of the burden of norovirus outbreaks from seafood in England, 2004–2011', *Epidemiology & Infection,* 147.

Hassard, F., Sharp, J. H., Taft, H., LeVay, L., Harris, J. P., McDonald, J. E., Tuson, K., Wilson, J., Jones, D. L. and Malham, S. K. (2017) 'Critical review on the public health impact of norovirus contamination in shellfish and the environment: a UK perspective', *Food and environmental virology,* 9(2), pp. 123-141.

Hakze-van der Honing, R. W., van Coillie, E., Antonis, A. F. and van der Poel, W. H. (2011) 'First isolation of hepatitis E virus genotype 4 in Europe through swine surveillance in the Netherlands and Belgium', PLoS One, 6(8), pp. e22673.

He, X. and Huang, K. (2020) 'Chapter 7 - Assessment technologies for hazards/risks of wastewater', in Ren, H. and Zhang, X. (eds.) *High-Risk Pollutants in Wastewater*: Elsevier, pp. 141-167.

Hewitt, P. E., Ijaz, S., Brailsford, S. R., Brett, R., Dicks, S., Haywood, B., Kennedy, I. T., Kitchen, A., Patel, P. and Poh, J. (2014) 'Hepatitis E virus in blood components: a prevalence and transmission study in southeast England', *The Lancet,* 384(9956), pp. 1766-1773.

Hoehne, M. and Schreier, E. (2006) 'Detection of Norovirus genogroup I and II by multiplex real-time RT-PCR using a 3'-minor groove binder-DNA probe', *BMC infectious diseases,* 6(1), pp. 69.

Holland, D. and Mahmoudzadeh, N. (2020) *Foodborne Disease Estimates for the United Kingdom in 2018*.

Holm, S. (1979) 'A simple sequentially rejective multiple test procedure', *Scandinavian journal of statistics*, pp. 65-70.

Horvatits, T., Schulze zur Wiesch, J., Lütgehetmann, M., Lohse, A. W. and Pischke, S. (2019) 'The clinical perspective on hepatitis E', *Viruses,* 11(7), pp. 617.

Hu, G. and Ma, X. (2010) 'Detection and sequences analysis of bovine hepatitis E virus RNA in Xinjiang Autonomous Region', *Bing du xue bao= Chinese journal of virology,* 26(1), pp. 27-32.

Huang, F., Li, Y., Yu, W., Jing, S., Wang, J., Long, F., He, Z., Yang, C., Bi, Y. and Cao, W. (2016) 'Excretion of infectious hepatitis E virus into milk in cows imposes high risks of zoonosis', *Hepatology,* 64(2), pp. 350-359.

Huang, R., Li, D., Wei, S., Li, Q., Yuan, X., Geng, L., Li, X. and Liu, M. (1999) 'Cell culture of sporadic hepatitis E virus in China', *Clinical and diagnostic laboratory immunology,* 6(5), pp. 729-733.

Huang, W., Zhang, H., Harrison, T. J., Lang, S., Huang, G. and Wang, Y. (2008) 'Cross-protection of hepatitis E virus genotypes 1 and 4 in rhesus macaques', *Journal of medical virology,* 80(5), pp. 824-832.

Iaconelli, M., Bonanno Ferraro, G., Mancini, P., Suffredini, E., Veneri, C., Ciccaglione, A. R., Bruni, R., Della Libera, S., Bignami, F. and Brambilla, M. (2020) 'Nine-year nationwide environmental surveillance of hepatitis E virus in urban wastewaters in Italy (2011–2019)', *International journal of environmental research and public health,* 17(6), pp. 2059.

Iaconelli, M., Purpari, G., Della Libera, S., Petricca, S., Guercio, A., Ciccaglione, A., Bruni, R., Taffon, S., Equestre, M. and Fratini, M. (2015) 'Hepatitis A and E viruses in wastewaters, in river waters, and in Bivalve Molluscs in Italy', *Food and environmental virology,* 7(4), pp. 316-324.

Idolo, A., Serio, F., Lugoli, F., Grassi, T., Bagordo, F., Guido, M., Privitera, G., Lobreglio, G. and De Donno, A. (2013) 'Identification of HEV in symptom-free migrants and environmental samples in Italy', *Journal of viral hepatitis,* 20(6), pp. 438-443.

Iizuka, S., Oka, T., Tabara, K., Omura, T., Katayama, K., Takeda, N. and Noda, M. (2010) 'Detection of sapoviruses and noroviruses in an outbreak of gastroenteritis linked genetically to shellfish', *Journal of medical virology,* 82(7), pp. 1247-1254.

Ijaz, S., Said, B., Boxall, E., Smit, E., Morgan, D. and Tedder, R. S. (2013) 'Indigenous hepatitis E in England and Wales from 2003 to 2012: evidence of an emerging novel phylotype of viruses', *The Journal of infectious diseases,* 209(8), pp. 1212-1218.

Imagawa, T., Sugiyama, R., Shiota, T., Li, T.-C., Yoshizaki, S., Wakita, T. and Ishii, K. (2018) 'Evaluation of heating conditions for inactivation of hepatitis e virus genotypes 3 and 4', *Journal of food protection,* 81(6), pp. 947-952.

International Organization for Standardization. (2017)*: ISO 15216-1: 2017: Microbiology of food and animal feed—Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR.* Geneva.

Iwai, M., Hasegawa, S., Obara, M., Nakamura, K., Horimoto, E., Takizawa, T.,Kurata, T., Sogen, S.-i. and Shiraki, K. (2009) 'Continuous presence of noroviruses and sapoviruses in raw sewage reflects infections among inhabitants of Toyama, Japan (2006 to 2008)', *Applied and environmental microbiology,* 75(5), pp. 1264-1270.

Izopet, J., Dubois, M., Bertagnoli, S., Lhomme, S., Marchandeau, S., Boucher, S., Kamar, N., Abravanel, F. and Guérin, J.-L. (2012) 'Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France', *Emerging infectious diseases,* 18(8), pp. 1274.

Jefferson, T. A., Webber, M. A., Pitman, R. L. and Jarrett, B. (2011) *Marine Mammals of the World: A Comprehensive Guide to Their Identification.* Elsevier Science.

Jerrold, Z. (2005) 'Spearman rank correlation', *Encyclopedia of Biostatistics,* 7.

Jin, H., Zhao, Y., Zhang, X., Wang, B. and Liu, P. (2016) 'Case-fatality risk of pregnant women with acute viral hepatitis type E: a systematic review and meta-analysis', *Epidemiology & Infection,* 144(10), pp. 2098-2106.

Johne, R., Reetz, J., Ulrich, R., Machnowska, P., Sachsenröder, J., Nickel, P. and Hofmann, J. (2014) 'An ORF 1-rearranged hepatitis E virus derived from a chronically infected patient efficiently replicates in cell culture', *Journal of viral hepatitis,* 21(6), pp. 447-456.

Johne, R., Trojnar, E., Filter, M. and Hofmann, J. (2016) 'Thermal stability of hepatitis E virus as estimated by a cell culture method', *Applied and environmental microbiology,* 82(14), pp. 4225-4231.

Jothikumar, N., Cromeans, T. L., Robertson, B. H., Meng, X. and Hill, V. R. (2006) 'A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus', *Journal of virological methods,* 131(1), pp. 65-71.

Jung, Y. M., Park, S. J., Kim, J. S., Jang, J. H., Lee, S. H., Kim, J. W., Park, Y. M., Hwang, S. G., Rim, K. S. and Kang, S. K. (2010) 'Atypical manifestations of hepatitis A infection: a prospective, multicenter study in Korea', *Journal of medical virology,* 82(8), pp. 1318-1326.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., Takeda, N. and Katayama, K. (2003) 'Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR', *Journal of clinical microbiology,* 41(4), pp. 1548-1557.

Kamar, N., Dalton, H. R., Abravanel, F. and Izopet, J. (2014) 'Hepatitis E virus infection', *Clinical microbiology reviews,* 27(1), pp. 116-138.

Kanai, Y., Miyasaka, S., Uyama, S., Kawami, S., Kato-Mori, Y., Tsujikawa, M., Yunoki, M., Nishiyama, S., Ikuta, K. and Hagiwara, K. (2012) 'Hepatitis E virus in Norway rats (Rattus norvegicus) captured around a pig farm', BMC Res Notes, 5, pp. 4.

Kasorndorkbua, C., Guenette, D., Huang, F., Thomas, P., Meng, X.-J. and Halbur, P. (2004) 'Routes of transmission of swine hepatitis E virus in pigs', *Journal of clinical microbiology,* 42(11), pp. 5047-5052.

Kasorndorkbua, C., Opriessnig, T., Huang, F., Guenette, D., Thomas, P., Meng, X.-J. and Halbur, P. (2005) 'Infectious swine hepatitis E virus is present in pig manure storage facilities on United States farms, but evidence of water contamination is lacking', *Applied and environmental microbiology,* 71(12), pp. 7831-7837.

Kauppinen, A., Al-Hello, H., Zacheus, O., Kilponen, J., Maunula, L., Huusko, S., Lappalainen, M., Miettinen, I., Blomqvist, S. and Rimhanen-Finne, R. (2017) 'Increase in outbreaks of gastroenteritis linked to bathing water in Finland in summer 2014', *Eurosurveillance,* 22(8), pp. 30470.

Kauppinen, A., Pitkänen, T., Al-Hello, H., Maunula, L., Hokajärvi, A. M., Rimhanen-Finne, R. and Miettinen, I. T. (2019) 'Two Drinking Water Outbreaks Caused by Wastewater Intrusion Including Sapovirus in Finland', *Int J Environ*

Res Public Health, 16(22).

Kay, D., Jones, F., Wyer, M. D., Fleisher, J. M., Salmon, R. L., Godfree, A. F., Zelenauch-Jacquotte, A. and Shore, R. (1994) 'Predicting likelihood of gastroenteritis from sea bathing: results from randomised exposure', *The Lancet,* 344(8927), pp. 905-909.

Kenney, S. P. (2019) 'The current host range of hepatitis E viruses', *Viruses,* 11(5), pp. 452.

Kingsley, D. H., Fay, J. P., Calci, K., Pouillot, R., Woods, J., Chen, H., Niemira, B. A. and Van Doren, J. M. (2017) 'Evaluation of Chlorine Treatment Levels for Inactivation of Human Norovirus and MS2 Bacteriophage during Sewage Treatment', *Applied and environmental microbiology,* 83(23), pp. e01270-17.

Kinoti, W. M., Constable, F. E., Nancarrow, N., Plummer, K. M. and Rodoni, B. (2017) 'Generic amplicon deep sequencing to determine Ilarvirus species diversity in Australian Prunus', *Frontiers in microbiology,* 8, pp. 1219.

Kitajima, M., Iker, B. C., Pepper, I. L. and Gerba, C. P. (2014) 'Relative abundance and treatment reduction of viruses during wastewater treatment processes — Identification of potential viral indicators', *Science of The Total Environment,* 488-489, pp. 290-296.

Kitaura, S., Wakabayashi, Y., Okazaki, A., Okada, Y., Okamoto, K., Ikeda, M., Okugawa, S. and Moriya, K. (2020) 'The First Case Report of Acute Symptomatic HEV Genotype 4 Infection in an HIV-positive Patient in Japan', Intern Med, 59(13), pp. 1655-1658.

Kojima, S., Kageyama, T., Fukushi, S., Hoshino, F. B., Shinohara, M., Uchida, K., Natori, K., Takeda, N. and Katayama, K. (2002) 'Genogroup-specific PCR primers for detection of Norwalk-like viruses', *J Virol Methods,* 100(1-2), pp. 107-14.

Kokkinos, P., Kozyra, I., Lazic, S., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A. M., Kaupke, A. and Legaki, E. (2012) 'Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries', *Food and environmental virology,* 4(4), pp. 179-191.

Kono, N. and Arakawa, K. (2019) 'Nanopore sequencing: review of potential applications in functional genomics', *Development, growth & differentiation,* 61(5), pp. 316-326.

Krog, J. S., Larsen, L. E. and Schultz, A. C. (2014) 'Enteric porcine viruses in farmed shellfish in Denmark', *International journal of food microbiology,* 186, pp. 105-109.

Kroneman, A., de Sousa, R., Verhoef, L., Koopmans, M. P. and Vennema, H. (2018) 'Usability of the international HAVNet hepatitis A virus database for geographical annotation, backtracing and outbreak detection', *Eurosurveillance,* 23(37), pp. 1700802.

Kroneman, A., Vennema, H., Deforche, K., v d Avoort, H., Peñaranda, S.,

Oberste, M. S., Vinjé, J. and Koopmans, M. (2011) 'An automated genotyping tool for enteroviruses and noroviruses', *J Clin Virol,* 51(2), pp. 121-5.

Kruskal, W. H. and Wallis, W. A. (1952) 'Use of ranks in one-criterion variance analysis', *Journal of the American statistical Association,* 47(260), pp. 583-621.

Kukielka, D., Rodriguez-Prieto, V., Vicente, J. and Sánchez-Vizcaíno, J. (2016) 'Constant hepatitis E virus (HEV) circulation in wild boar and red deer in Spain: an increasing concern source of HEV zoonotic transmission', *Transboundary and emerging diseases,* 63(5), pp. e360-e368.

Kumar, N., Das, V., Agarwal, A., Pandey, A. and Agrawal, S. (2017) 'Fetomaternal outcomes in pregnant women with hepatitis E infection; still an important fetomaternal killer with an unresolved mystery of increased virulence in pregnancy', *Turkish Journal of Obstetrics and Gynecology,* 14(2), pp. 106.

La Bella, G., Martella, V., Basanisi, M., Nobili, G., Terio, V. and La Salandra, G. (2017) 'Food-borne viruses in shellfish: investigation on norovirus and HAV presence in Apulia (SE Italy)', *Food and environmental virology,* 9(2), pp. 179-186.

La Rosa, G., Della Libera, S., Brambilla, M., Bisaglia, C., Pisani, G., Ciccaglione, A., Bruni, R., Taffon, S., Equestre, M. and Iaconelli, M. (2017a) 'Hepatitis E virus (genotype 3) in slurry samples from swine farming activities in Italy', *Food and environmental virology,* 9(2), pp. 219-229.

La Rosa, G., Della Libera, S., Iaconelli, M., Proroga, Y., De Medici, D., Martella, V. and Suffredini, E. (2017b) 'Detection of norovirus GII. 17 Kawasaki 2014 in shellfish, marine water and underwater sewage discharges in Italy', *Food and environmental virology,* 9(3), pp. 326-333.

La Rosa, G., Mancini, P., Bonanno Ferraro, G., Iaconelli, M., Veneri, C., Paradiso, R., De Medici, D., Vicenza, T., Proroga, Y. T. R. and Di Maro, O. (2021) 'Hepatitis A Virus Strains Circulating in the Campania Region (2015–2018) Assessed through Bivalve Biomonitoring and Environmental Surveillance', *Viruses,* 13(1), pp. 16.

La Rosa, G., Pourshaban, M., Iaconelli, M., Vennarucci, V. S. and Muscillo, M. (2010) 'Molecular detection of hepatitis E virus in sewage samples', *Applied and environmental microbiology,* 76(17), pp. 5870-5873.

La Rosa, G., Proroga, Y., De Medici, D., Capuano, F., Iaconelli, M., Della Libera, S. and Suffredini, E. (2018) 'First detection of hepatitis E virus in shellfish and in seawater from production areas in Southern Italy', *Food and environmental virology,* 10(1), pp. 127-131.

Lack, J. B., Volk, K. and Van Den Bussche, R. A. (2012) 'Hepatitis E virus genotype 3 in wild rats, United States', Emerg Infect Dis, 18(8), pp. 1268-73.

Lapp, R. T. and Rochling, F. (2013) 'Acute cholestatic hepatitis a virus infection presenting with hemolytic anemia and renal failure: a case report', *Case reports in hepatology,* 2013.

Le Guyader, F., Haugarreau, L., Miossec, L., Dubois, E. and Pommepuy, M.

(2000) 'Three-year study to assess human enteric viruses in shellfish', *Applied and environmental microbiology,* 66(8), pp. 3241-3248.

Ledesma, J., Williams, D., Stanford, F. A., Hewitt, P. E., Zuckerman, M., Bansal, S., Dhawan, A., Mbisa, J. L., Tedder, R. and Ijaz, S. (2019) 'Resolution by deep sequencing of a dual hepatitis E virus infection transmitted via blood components', *Journal of General Virology,* 100(11), pp. 1491-1500.

Lee, G.-H., Tan, B.-H., Teo, E. C.-Y., Lim, S.-G., Dan, Y.-Y., Wee, A., Aw, P. P. K., Zhu, Y., Hibberd, M. L. and Tan, C.-K. (2016) 'Chronic infection with camelid hepatitis E virus in a liver transplant recipient who regularly consumes camel meat and milk', *Gastroenterology,* 150(2), pp. 355-357. e3.

Lee, L. E., Cebelinski, E. A., Fuller, C., Keene, W. E., Smith, K., Vinjé, J. and Besser, J. M. (2012) 'Sapovirus outbreaks in long-term care facilities, Oregon and Minnesota, USA, 2002-2009', *Emerg Infect Dis,* 18(5), pp. 873-6.

Lee, S. D., Wang, Y. J., Lu, R. H., Chan, C. Y., Lo, K. J. and Moeckli, R. (1994) 'Seroprevalence of antibody to hepatitis E virus among Chinese subjects in Taiwan', *Hepatology,* 19(4), pp. 866-870.

Lees, D. (2000) 'Viruses and bivalve shellfish', *International journal of food microbiology,* 59(1-2), pp. 81-116.

Leonard, A. F., Zhang, L., Balfour, A. J., Garside, R. and Gaze, W. H. (2015) 'Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters', *Environment international,* 82, pp. 92-100.

Leonard, A. F. C., Singer, A., Ukoumunne, O. C., Gaze, W. H. and Garside, R. (2018) 'Is it safe to go back into the water? A systematic review and meta-analysis of the risk of acquiring infections from recreational exposure to seawater', *International journal of epidemiology,* 47(2), pp. 572-586.

Lhomme, S., Dubois, M., Abravanel, F., Top, S., Bertagnoli, S., Guerin, J.-L. and Izopet, J. (2013) 'Risk of zoonotic transmission of HEV from rabbits', *Journal of Clinical Virology,* 58(2), pp. 357-362.

Li, D., Butot, S., Zuber, S. and Uyttendaele, M. (2018) 'Monitoring of foodborne viruses in berries and considerations on the use of RT-PCR methods in surveillance', *Food Control,* 89, pp. 235-240.

Li, J. Y., Mai, W., Tan, H. Q., Jian, M. T., Deng, H., Chen, Z. P. and Chen, H. H. (2020a) '[An outbreak of acute gastroenteritis caused by Sapovirus in a community of Guangdong province]', *Zhonghua Liu Xing Bing Xue Za Zhi,* 41(2), pp. 226-230.

Li, M., Zhang, H., Wang, L., Li, Z., Wang, J., Xu, B., Hao, R., Liu, C., Fu, H. and Rao, H. (2020b) 'The investigation of hepatitis A virus and hepatitis E virus co-infection in humans and animals in China', *Acta Virologica,* 64(1), pp. 20-27.

Li, S., Li, M., He, Q., Liang, Z., Shu, J., Wang, L. and Wang, L. (2020c) 'Characterization of hepatitis E virus natural infection in farmed rabbits', *Journal of Viral Hepatitis*.

Li, S., Liu, M., Cong, J., Zhou, Y. and Miao, Z. (2017) 'Detection and

characterization of hepatitis E virus in goats at slaughterhouse in Tai'an region, China', *BioMed research international,* 2017.

Li, T.-C., Miyamura, T. and Takeda, N. (2007) 'Detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (Corbicula japonica) in Japan', *The American journal of tropical medicine and hygiene,* 76(1), pp. 170-172.

Li, T.-C., Yang, T., Shiota, T., Yoshizaki, S., Yoshida, H., Saito, M., Imagawa, T., Malbas, F. F., Lupisan, S. P. and Oshitani, H. (2014) 'Molecular detection of hepatitis E virus in rivers in the Philippines', *The American journal of tropical medicine and hygiene,* 90(4), pp. 764-766.

Lodder, W. J. and de Roda Husman, A. M. (2005) 'Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands', *Applied and environmental microbiology,* 71(3), pp. 1453-1461.

Loisy, F., Atmar, R., Guillon, P., Le Cann, P., Pommepuy, M. and Le Guyader,F. (2005) 'Real-time RT-PCR for norovirus screening in shellfish', *Journal of virological methods,* 123(1), pp. 1-7.

Loisy-Hamon, F. and Leturnier, G. (2015) 'Autochthonous cases of hepatitis E: where does the virus come from? Impact of pig slurry treatment on reduction of the viral load and prevalence of the virus in food substrates', *EuroReference,* 18, pp. 13-18.

Long, F., Yu, W., Yang, C., Wang, J., Li, Y., Li, Y. and Huang, F. (2017) 'High prevalence of hepatitis E virus infection in goats', *Journal of medical virology,* 89(11), pp. 1981-1987.

Lowther, J. (2011) 'Investigation into the prevalence, distribution and levels of norovirus titre in oyster harvesting areas in the UK', *Weymouth: CEFAS Weymouth laboratory*.

Lowther, J., Gustar, N., Powell, A., O'Brien, S. and Lees, D. (2018) 'A one-year survey of norovirus in UK oysters collected at the point of sale', *Food and environmental virology,* 10(3), pp. 278-287.

Lowther, J. A., Avant, J. M., Gizynski, K., Rangdale, R. E. and Lees, D. N. (2010) 'Comparison between Quantitative Real-Time Reverse Transcription PCR Results for Norovirus in Oysters and Self-Reported Gastroenteric Illness in Restaurant Customers', *Journal of Food Protection,* 73(2), pp. 305-311.

Lowther, J. A., Cross, L., Stapleton, T., Gustar, N. E., Walker, D. I., Sills, M., Treagus, S., Pollington, V. and Lees, D. N. (2019) 'Use of F-Specific RNA Bacteriophage to Estimate Infectious Norovirus Levels in Oysters', *Food and Environmental Virology,* 11(3), pp. 247-258.

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E. and Lees, D. N. (2012) 'Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom', *Applied and environmental microbiology,* 78(16), pp. 5812-5817.

Lu, J., Fang, L., Zheng, H., Lao, J., Yang, F., Sun, L., Xiao, J., Lin, J., Song, T., Ni, T., Raghwani, J., Ke, C., Faria, N. R., Bowden, T. A., Pybus, O. G. and Li, H.

(2016) 'The Evolution and Transmission of Epidemic GII.17 Noroviruses', *The Journal of Infectious Diseases,* 214(4), pp. 556-564.

Ma, L. P., Zhao, F., Yao, L., Li, X. G., Zhou, D. Q. and Zhang, R. L. (2013) 'The presence of genogroup II norovirus in retail shellfish from seven coastal cities in China', Food Environ Virol, 5(2), pp. 81-6.

Maalouf, H., Zakhour, M., Le Pendu, J., Le Saux, J.-C., Atmar, R. and Le Guyader, F. (2010) 'Distribution in Tissue and Seasonal Variation of Norovirus Genogroup I and II Ligands in Oysters', *Applied and Environmental Microbiology*, 76(16), pp. 5621-5630, *Applied and Environmental Microbiology* Available: American Society for Microbiology.

Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A. R., Potter, S. C. and Finn, R. D. (2019) 'The EMBL-EBI search and sequence analysis tools APIs in 2019', *Nucleic acids research,* 47(W1), pp. W636-W641.

Maguire, A. J., Green, J., Brown, D. W., Desselberger, U. and Gray, J. J. (1999) 'Molecular epidemiology of outbreaks of gastroenteritis associated with small round-structured viruses in East Anglia, United Kingdom, during the 1996–1997 season', *Journal of Clinical Microbiology,* 37(1), pp. 81-89.

Mallet, V., Bruneau, J., Zuber, J., Alanio, C., Leclerc-Mercier, S., Roque-Afonso, A.-M., Kraft, A. R., Couronné, L., Roulot, D. and Wedemeyer, H. (2017) 'Hepatitis E virus-induced primary cutaneous CD30 (+) T cell lymphoproliferative disorder', *Journal of Hepatology*.

Malmberg, M. M., Spangenberg, G. C., Daetwyler, H. D. and Cogan, N. O. I. (2019) 'Assessment of low-coverage nanopore long read sequencing for SNP genotyping in doubled haploid canola (Brassica napus L.)', *Sci Rep,* 9(1), pp. 8688.

Mancini, P., Bonanno Ferraro, G., Iaconelli, M., Suffredini, E., Valdazo-González, B., Della Libera, S., Divizia, M. and La Rosa, G. (2019) 'Molecular characterization of human Sapovirus in untreated sewage in Italy by amplicon-based Sanger and next-generation sequencing', *Journal of applied microbiology,* 126(1), pp. 324-331.

Manso, C., Polo, D., Vilariño, M. and Romalde, J. (2010) 'Genotyping of hepatitis A virus detected in bivalve shellfish in Galicia (NW Spain)', *Water Science and Technology,* 61(1), pp. 15-24.

Marcheggiani, S., D'Ugo, E., Puccinelli, C., Giuseppetti, R., D'Angelo, A. M., Gualerzi, C. O., Spurio, R., Medlin, L. K., Guillebault, D. and Weigel, W. (2015) 'Detection of emerging and re-emerging pathogens in surface waters close to an urban area', *International journal of environmental research and public health,* 12(5), pp. 5505-5527.

Marine Conservation Society. (2011) *Combined Sewer Overflows Pollution Policy and Position Statement*: Marine Conservation Society. Available at: https://www.mcsuk.org/downloads/pollution/CSO%20policy.pdf.

Martin, J., Klapsa, D., Wilton, T., Zambon, M., Bentley, E., Bujaki, E., Fritzsche,

M., Mate, R. and Majumdar, M. (2020) 'Tracking SARS-CoV-2 in Sewage: Evidence of Changes in Virus Variant Predominance during COVID-19 Pandemic', *Viruses,* 12(10), pp. 1144.

Masclaux, F. G., Hotz, P., Friedli, D., Savova-Bianchi, D. and Oppliger, A. (2013) 'High occurrence of hepatitis E virus in samples from wastewater treatment plants in Switzerland and comparison with other enteric viruses', *Water research,* 47(14), pp. 5101-5109.

Matos, A., Mesquita, J. R., Gonçalves, D., Abreu-Silva, J., Luxo, C. and Nascimento, M. S. (2018) 'First detection and molecular characterization of hepatitis E virus in water from wastewater treatment plants in Portugal', *Annals of Agricultural and Environmental Medicine,* 25(2), pp. 364-367.

Matsuura, Y., Suzuki, M., Yoshimatsu, K., Arikawa, J., Takashima, I., Yokoyama, M., Igota, H., Yamauchi, K., Ishida, S. and Fukui, D. (2007) 'Prevalence of antibody to hepatitis E virus among wild sika deer, Cervus nippon, in Japan', *Archives of virology,* 152(7), pp. 1375-1381.

Maunula, L., Kaupke, A., Vasickova, P., Söderberg, K., Kozyra, I., Lazic, S., van der Poel, W. H., Bouwknegt, M., Rutjes, S. and Willems, K. A. (2013) 'Tracing enteric viruses in the European berry fruit supply chain', *International journal of food microbiology,* 167(2), pp. 177-185.

McBride, G. B., Stott, R., Miller, W., Bambic, D. and Wuertz, S. (2013) 'Discharge-based QMRA for estimation of public health risks from exposure to stormwater-borne pathogens in recreational waters in the United States', *Water research,* 47(14), pp. 5282-5297.

McCaustland, K. A., Krawczynski, K., Ebert, J. W., Balayan, M. S., Andjaparidze, A. G., Spelbring, J. E., Cook, E. H., Humphrey, C., Yarbough, P. O., Favorov, M. O., Carson, D., Bradley, D. W. and Robertson, B. H. (2000) 'Hepatitis E virus infection in chimpanzees: a retrospective analysis', *Archives of Virology,* 145(9), pp. 1909-1918.

Mcleod, C., Hay, B., Grant, C., Greening, G. and Day, D. (2009) 'Localization of norovirus and poliovirus in Pacific oysters', *Journal of Applied Microbiology,* 106(4), pp. 1220-1230.

McNaughton, A. L., Roberts, H. E., Bonsall, D., de Cesare, M., Mokaya, J., Lumley, S. F., Golubchik, T., Piazza, P., Martin, J. B., de Lara, C., Brown, A., Ansari, M. A., Bowden, R., Barnes, E. and Matthews, P. C. (2019) 'Illumina and Nanopore methods for whole genome sequencing of hepatitis B virus (HBV)', *Sci Rep,* 9(1), pp. 7081.

Medema, G., Been, F., Heijnen, L. and Petterson, S. (2020a) 'Implementation of environmental surveillance for SARS-CoV-2 virus to support public health decisions: Opportunities and challenges', *Current Opinion in Environmental Science & Health*.Medema, G., Heijnen, L., Elsinga, G., Italiaander, R. and Brouwer, A. (2020b) 'Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands', *Environmental Science & Technology Letters,* 7(7), pp. 511-516.

Medrano, C., Boadella, M., Barrios, H., Cantu, A., Garcia, Z., de la Fuente, J. and Gortazar, C. (2012) 'Zoonotic pathogens among white-tailed deer, northern Mexico, 2004–2009', *Emerging Infectious Diseases,* 18(8), pp. 1372.

Meghnath, K., Hasselback, P., McCormick, R., Prystajecky, N., Taylor, M., McIntyre, L., Man, S., Whitfield, Y., Warshawsky, B., McKinley, M., Bitzikos, O., Hexemer, A. and Galanis, E. (2019) 'Outbreaks of Norovirus and Acute Gastroenteritis Associated with British Columbia Oysters, 2016-2017', *Food Environ Virol,* 11(2), pp. 138-148.

Meng, X.-J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., Purcell, R. H. and Emerson, S. U. (1998) 'Genetic and experimental evidence for cross-species infection by swine hepatitis E virus', *Journal of virology,* 72(12), pp. 9714-9721.

Meng, X.-J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. and Emerson, S. U. (1997) 'A novel virus in swine is closely related to the human hepatitis E virus', *Proceedings of the National Academy of Sciences,* 94(18), pp. 9860-9865.

Mesquita, J. R., Oliveira, D., Rivadulla, E., Abreu-Silva, J., Varela, M. F., Romalde, J. L. and Nascimento, M. S. (2016) 'Hepatitis E virus genotype 3 in mussels (Mytilus galloprovinciallis), Spain', *Food microbiology,* 58, pp. 13-15.

Messner, M. J., Berger, P. and Nappier, S. P. (2014) 'Fractional Poisson—A Simple Dose-Response Model for Human Norovirus', *Risk Analysis,* 34(10), pp. 1820-1829.

Mizuo, H., Yazaki, Y., Sugawara, K., Tsuda, F., Takahashi, M., Nishizawa, T. and Okamoto, H. (2005) 'Possible risk factors for the transmission of hepatitis E virus and for the severe form of hepatitis E acquired locally in Hokkaido, Japan', *Journal of medical virology,* 76(3), pp. 341-349.

Mizuochi, M. (2008). ‘Small-Scale Domestic Wastewater Treatment Technology in Japan , and the Possibility of Technological Transfer’.

Moor, D., Liniger, M., Baumgartner, A. and Felleisen, R. (2018) 'Screening of ready-to-eat meat products for hepatitis E virus in Switzerland', *Food and environmental virology,* 10(3), pp. 263-271.

Mughini-Gras, L., Angeloni, G., Salata, C., Vonesch, N., D'amico, W., Campagna, G., Natale, A., Zuliani, F., Ceglie, L. and Monne, I. (2017) 'Hepatitis E virus infection in North Italy: high seroprevalence in swine herds and increased risk for swine workers', *Epidemiology & Infection,* 145(16), pp. 3375-3384.

Mulder, A. C., Kroneman, A., Franz, E., Vennema, H., Tulen, A. D., Takkinen, J., Hofhuis, A. and Adlhoch, C. (2019) 'HEVnet: a One Health, collaborative, interdisciplinary network and sequence data repository for enhanced hepatitis E virus molecular typing, characterisation and epidemiological investigations', *Euro Surveill,* 24(10).

Mykytczuk, O., Harlow, J., Bidawid, S., Corneau, N. and Nasheri, N. (2017) 'Prevalence and molecular characterization of the hepatitis E virus in retail pork

products marketed in Canada', *Food and environmental virology,* 9(2), pp. 208-218.

Müller, A., Collineau, L., Stephan, R., Müller, A. and Stärk, K. D. (2017) 'Assessment of the risk of foodborne transmission and burden of hepatitis E in Switzerland', *International journal of food microbiology,* 242, pp. 107-115.

Nasheri, N., Vester, A. and Petronella, N. (2019) 'Foodborne viral outbreaks associated with frozen produce', *Epidemiology & Infection,* 147.

National Office for Statistics. (2020) *Population estimates for the UK, England and Wales, Scotland and Northern Ireland: mid-2019*: Population estimates for the UK, England and Wales, Scotland and Northern Ireland: mid-2019. Available at: https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/bulletins/annualmidyearpopulationestimates/mid2019estimates.

Neumann, S., Hackl, S. S., Piepenschneider, M., Vina-Rodriguez, A., Dremsek, P., Ulrich, R. G., Groschup, M. H. and Eiden, M. (2016) 'Serologic and molecular survey of hepatitis E virus in German deer populations', *Journal of wildlife diseases,* 52(1), pp. 106-113.

Ngazoa, E., Fliss, I. and Jean, J. (2008) 'Quantitative study of persistence of human norovirus genome in water using TaqMan real-time RT-PCR', *Journal of Applied Microbiology,* 104(3), pp. 707-715.

Nguyen, L. T., Schmidt, H. A., von Haeseler, A. and Minh, B. Q. (2015) 'IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies', *Mol Biol Evol,* 32(1), pp. 268-74.

Nijskens, C. M., Pas, S. D., Cornelissen, J., Caliskan, K., Hoek, R. A., Hesselink, D. A., van der Eijk, A. A. and Robert, A. (2016) 'Hepatitis E virus genotype 3 infection in a tertiary referral center in the Netherlands: Clinical relevance and impact on patient morbidity', *Journal of Clinical Virology,* 74, pp. 82-87.

Nordgren, J., Kindberg, E., Lindgren, P.-E., Matussek, A. and Svensson, L. (2010) 'Norovirus gastroenteritis outbreak with a secretor-independent susceptibility pattern, Sweden', *Emerging infectious diseases,* 16(1), pp. 81.

Novel, C. E., Durupt, S., de Laforcade, L., Lecoq, M., Durieu, I. and Reynaud, Q. (2017) 'Acute kidney injury, a rare complication of acute hepatitis E infection', *Medecine et maladies infectieuses,* 47(7), pp. 502.

Oeser, C., Vaughan, A., Said, B., Ijaz, S., Tedder, R., Haywood, B., Warburton, F., Charlett, A., Elson, R. and Morgan, D. (2019) 'Epidemiology of Hepatitis E in England and Wales: A 10-Year Retrospective Surveillance Study, 2008–2017', *The Journal of Infectious Diseases,* 220(5), pp. 802-810.

O'Hara, Z., Crossan, C., Craft, J. and Scobie, L. (2018) 'First Report of the Presence of Hepatitis E Virus in Scottish-Harvested Shellfish Purchased at Retail Level', *Food and environmental virology*, pp. 1-5.

Oka, T., Katayama, K., Hansman, G. S., Kageyama, T., Ogawa, S., Wu, F. T., White, P. A. and Takeda, N. (2006) 'Detection of human sapovirus by real-time reverse transcription-polymerase chain reaction', *Journal of medical virology,* 78(10), pp. 1347-1353.

Oka, T., Wang, Q., Katayama, K. and Saif, L. J. (2015) 'Comprehensive review of human sapoviruses', *Clinical microbiology reviews,* 28(1), pp. 32-53.

Okonechnikov, K., Golosova, O., Fursov, M. and team, t. U. (2012) 'Unipro UGENE: a unified bioinformatics toolkit', *Bioinformatics,* 28(8), pp. 1166-1167.

Ouoba, J. B., Traore, K. A., Rouamba, H., Setondji, K. V.-M., Minoungou, G. L., Ouoba, B. L., Ouedraogo, A., Moctar, S., M'Bengue, A. K. and Kakou, S. N. (2019) 'Prevalence of anti-hepatitis E virus antibodies in domestic animal from three representative provinces of Burkina Faso', *Veterinary and Animal Science,* 7, pp. 100059.

Owolodun, O. A., Giménez-Lirola, L. G., Gerber, P. F., Sanford, B. J., Feagins, A. R., Meng, X.-J., Halbur, P. G. and Opriessnig, T. (2013) 'Development of a fluorescent microbead-based immunoassay for the detection of hepatitis E virus IgG antibodies in pigs and comparison to an enzyme-linked immunoassay', *Journal of virological methods,* 193(2), pp. 278-283.

Oxford Nanopore Technologies. (2021) *Guppy v5.0.7 release note*. Oxford Nanopore Technologies Community Posts. Available at: https://community.nanoporetech.com/posts/guppy-v5-0-7-release-note (Accessed: 02 September 2021 2021).

Pallerla, S. R., Schembecker, S., Meyer, C. G., Linh, L. T. K., Johne, R., Wedemeyer, H., Bock, C. T., Kremsner, P. G. and Velavan, T. P. (2021) 'Hepatitis E virus genome detection in commercial pork livers and pork meat products in Germany', *Journal of Viral Hepatitis,* 28(1), pp. 196-204.

Pas, S. D., Rob, A., Mulders, C., Balk, A. H., van Hal, P. T., Weimar, W., Koopmans, M. P., Osterhaus, A. D. and van der Eijk, A. A. (2012) 'Hepatitis E virus infection among solid organ transplant recipients, the Netherlands', *Emerging infectious diseases,* 18(5), pp. 869.

Pathak, R. and Barde, P. V. (2017) 'Detection of Genotype 1a and 1f of Hepatitis E Virus in Patients Treated at Tertiary Care Hospitals in Central India', *Intervirology,* 60(5), pp. 201-206.

Pavio, N., Merbah, T. and Thebault, A. (2014) 'Frequent hepatitis E virus contamination in food containing raw pork liver, France', *Emerging infectious diseases,* 20(11), pp. 1925.

Pellerin, M., Hirchaud, E., Blanchard, Y., Pavio, N. and Doceul, V. (2021) 'Characterization of a Cell Culture System of Persistent Hepatitis E Virus Infection in the Human HepaRG Hepatic Cell Line', Viruses, 13(3).

Peron, J., Bureau, C., Poirson, H., Mansuy, J., Alric, L., Selves, J., Dupuis, E., Izopet, J. and Vinel, J. (2007) 'Fulminant liver failure from acute autochthonous hepatitis E in France: description of seven patients with acute hepatitis E and encephalopathy', *Journal of viral hepatitis,* 14(5), pp. 298-303.

Petersen, L. M., Martin, I. W., Moschetti, W. E., Kershaw, C. M. and Tsongalis, G. J. (2019) 'Third-Generation Sequencing in the Clinical Laboratory: Exploring the Advantages and Challenges of Nanopore Sequencing', *Journal of clinical microbiology,* 58(1), pp. e01315-19.

Phillips, G., Tam, C. C., Rodrigues, L. and Lopman, B. (2010) 'Prevalence and characteristics of asymptomatic norovirus infection in the community in England', *Epidemiology & Infection,* 138(10), pp. 1454-1458.

Pina, S., Buti, M., Cotrina, M., Piella, J. and Girones, R. (2000) 'HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain', *Journal of Hepatology,* 33(5), pp. 826-833.

Pintó, R. M., Costafreda, M. I. and Bosch, A. (2009) 'Risk assessment in shellfish-borne outbreaks of hepatitis A', *Appl. Environ. Microbiol.,* 75(23), pp. 7350-7355.

Pires, S. M., Fischer-Walker, C. L., Lanata, C. F., Devleesschauwer, B., Hall, A. J., Kirk, M. D., Duarte, A. S., Black, R. E. and Angulo, F. J. (2015) 'Aetiology-specific estimates of the global and regional incidence and mortality of diarrhoeal diseases commonly transmitted through food', *PloS one,* 10(12), pp. e0142927.

Polo, D., Schaeffer, J., Fournet, N., Le Saux, J.-C., Parnaudeau, S., McLeod, C. and Le Guyader, F. S. (2016) 'Digital PCR for quantifying norovirus in oysters implicated in outbreaks, France', *Emerging infectious diseases,* 22(12), pp. 2189.

Potasman, I., Paz, A. and Odeh, M. (2002) 'Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective', *Clinical Infectious Diseases,* 35(8), pp. 921-928.

Pouillot, R., Smith, M., Van Doren, J. M., Catford, A., Holtzman, J., Calci, K. R., Edwards, R., Goblick, G., Roberts, C., Stobo, J., White, J., Woods, J., DePaola, A., Buenaventura, E. and Burkhardt, W. (2021) 'Risk Assessment of Norovirus Illness from Consumption of Raw Oysters in the United States and in Canada', *Risk Anal*.

Prato, R., Lopalco, P. L., Chironna, M., Barbuti, G., Germinario, C. and Quarto, M. (2004) 'Norovirus gastroenteritis general outbreak associated with raw shellfish consumption in south Italy', *BMC infectious diseases,* 4, pp. 37-37.

Primadharsini, P. P., Nagashima, S. and Okamoto, H. (2021) 'Mechanism of Cross-Species Transmission, Adaptive Evolution and Pathogenesis of Hepatitis E Virus', Viruses, 13(5).

Prüss, A. (1998) 'Review of epidemiological studies on health effects from exposure to recreational water', *International Journal of Epidemiology,* 27(1), pp. 1-9.

Public Health England . (2018a) *PHE National norovirus and rotavirus Report*: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/724329/Norovirus_update_2018_weeks_22_to_26.pdf.

Public Health England. (2018b) *PHE National norovirus and rotavirus Report*: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/724329/Norovirus_update_2018_weeks_22_to_26.pdf.

Public Health England. (2019) *Hepatitis E: symptoms, transmission, treatment and prevention*. gov.uk: Public Health England. Available at: https://www.gov.uk/government/publications/hepatitis-e-symptoms-transmission-prevention-treatment/hepatitis-e-symptoms-transmission-treatment-and-prevention (Accessed: 27 August 2019).

Public Health England. (2020) *PHE National norovirus and rotavirus Report*, GOV.UK: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/871753/Norovirus_update_2020_weeks_08_to_09.pdf.

Public Health England. (2021a) *2018 to 2019 Season Norovirus Report National norovirus laboratory and outbreak data in England*, gov.uk: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/966775/2021-01-29-Norovirus_annual_report_2018_to_2019.pdf.

Public Health England. (2021b) *Laboratory reports of hepatitis A infections in England and Wales, 2019*, UK: Public Health England. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/982962/hpr0715_HAV19_v2.pdf.

Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G. and Girones, R. (1994) 'Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification', *Appl. Environ. Microbiol.,* 60(8), pp. 2963-2970.

Pujols, J., Rodríguez, C., Navarro, N., Pina-Pedrero, S., Campbell, J. M., Crenshaw, J. and Polo, J. (2014) 'No transmission of hepatitis E virus in pigs fed diets containing commercial spray-dried porcine plasma: a retrospective study of samples from several swine trials', *Virology journal,* 11(1), pp. 232.

Purcell, R. H., Engle, R. E., Rood, M. P., Kabrane-Lazizi, Y., Nguyen, H. T., Govindarajan, S., St Claire, M. and Emerson, S. U. (2011) 'Hepatitis E virus in rats, Los Angeles, California, USA', Emerg Infect Dis, 17(12), pp. 2216-22.

Pérez-Gracia, M. T., García, M., Suay, B. and Mateos-Lindemann, M. L. (2015) 'Current knowledge on hepatitis E', *Journal of clinical and translational hepatology,* 3(2), pp. 117.

Qi, R., Huang, Y.-t., Liu, J.-w., Sun, Y., Sun, X.-f., Han, H.-J., Qin, X.-R., Zhao, M., Wang, L.-j. and Li, W. (2018) 'Global prevalence of asymptomatic norovirus infection: a meta-analysis', *EClinicalMedicine,* 2, pp. 50-58.

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T. F. and Beutler, A. (2017) 'Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples', *nature protocols,* 12(6), pp. 1261.

Razafimahefa, R. M., Ludwig-Begall, L. F. and Thiry, E. (2020) 'Cockles and mussels, alive, alive, oh—The role of bivalve molluscs as transmission vehicles for human norovirus infections', *Transboundary and emerging diseases,* 67, pp. 9-25.

R Core Team. (2018) *R: A Language and Environment for Statistical Computing* (Version 3.4.3). Available at: https://www.R-project.org/.

Rein, D. B., Stevens, G. A., Theaker, J., Wittenborn, J. S. and Wiersma, S. T. (2012) 'The global burden of hepatitis E virus genotypes 1 and 2 in 2005', *Hepatology,* 55(4), pp. 988-997.

Reuter, G., Fodor, D., Forgách, P., Kátai, A. and Szűcs, G. (2009) 'Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary', *Journal of Clinical Virology,* 44(4), pp. 277-281.

Riera-Montes, M., Brus Sjölander, K., Allestam, G., Hallin, E., Hedlund, K. O. and Löfdahl, M. (2011) 'Waterborne norovirus outbreak in a municipal drinking-water supply in Sweden', *Epidemiol Infect,* 139(12), pp. 1928-35.

Rivadulla, E., Varela, M. F., Mesquita, J. R., Nascimento, M. S. and Romalde, J. (2019) 'Detection of hepatitis E virus in shellfish harvesting areas from Galicia (Northwestern Spain)', *Viruses,* 11(7), pp. 618.

Rivero-Juarez, A., Frias, M., Martinez-Peinado, A., Risalde, M., Rodriguez-Cano, D., Camacho, A., García-Bocanegra, I., Cuenca-Lopez, F., Gomez-Villamandos, J. and Rivero, A. (2017) 'Familial hepatitis E outbreak linked to wild boar meat consumption', *Zoonoses and public health*.

Robilotti, E., Deresinski, S. and Pinsky, B. A. (2015) 'Norovirus', *Clin Microbiol Rev,* 28(1), pp. 134-64.

Rodriguez-Manzano, J., Miagostovich, M., Hundesa, A., Clemente-Casares, P., Carratala, A., Buti, M., Jardi, R. and Girones, R. (2010) 'Analysis of the evolution in the circulation of HAV and HEV in eastern Spain by testing urban sewage samples', *Journal of water and health,* 8(2), pp. 346-354.

Rose, N., Lunazzi, A., Dorenlor, V., Merbah, T., Eono, F., Eloit, M., Madec, F. and Pavio, N. (2011) 'High prevalence of Hepatitis E virus in French domestic pigs', *Comparative immunology, microbiology and infectious diseases,* 34(5), pp. 419-427.

Roth, A., Lin, J., Magnius, L., Karlsson, M., Belák, S., Widén, F. and Norder, H. (2016) 'Markers for ongoing or previous hepatitis E virus infection are as common in wild ungulates as in humans in Sweden', *Viruses,* 8(9), pp. 259.

Ruchusatsawat, K., Nuengjamnong, C., Tawatsin, A., Thiemsing, L., Kawidam, C., Somboonna, N. and Nuanualsuwan, S. (2021) 'Quantitative Risk Assessments of Hepatitis A Virus and Hepatitis E Virus from Raw Oyster Consumption', *Risk Analysis*.

Rutjes, S., Bouwknegt, M., Van Der Giessen, J., de Roda Husman, A. and Reusken, C. (2014) 'Seroprevalence of hepatitis E virus in pigs from different

farming systems in The Netherlands', *Journal of food protection,* 77(4), pp. 640-642.

Rutjes, S., Lodder-Verschoor, F., Lodder, W., Van der Giessen, J., Reesink, H., Bouwknegt, M. and de Roda Husman, A. (2010) 'Seroprevalence and molecular detection of hepatitis E virus in wild boar and red deer in The Netherlands', *Journal of virological methods,* 168(1-2), pp. 197-206.

Rutjes, S. A., Lodder, W. J., Lodder-Verschoor, F., van den Berg, H. H. J. L., Vennema, H., Duizer, E., Koopmans, M. and de Roda Husman, A. M. (2009) 'Sources of hepatitis E virus genotype 3 in The Netherlands', *Emerging infectious diseases,* 15(3), pp. 381-387.

Ryll, R., Eiden, M., Heuser, E., Weinhardt, M., Ziege, M., Höper, D., Groschup, H., Heckel, G., Johne, R. and Ulrich, R. G. (2018) 'Hepatitis E virus in feral rabbits along a rural-urban transect in Central Germany', *Infection, genetics and evolution,* 61, pp. 155-159.

Said, B., Ijaz, S., Chand, M., Kafatos, G., Tedder, R. and Morgan, D. (2014) 'Hepatitis E virus in England and Wales: indigenous infection is associated with the consumption of processed pork products', *Epidemiology & Infection,* 142(7), pp. 1467-1475.

Said, B., Ijaz, S., Kafatos, G., Booth, L., Thomas, H. L., Walsh, A., Ramsay, M. and Morgan, D. (2009) 'Hepatitis E outbreak on cruise ship', *Emerging infectious diseases,* 15(11), pp. 1738.

Sakon, N., Sadamasu, K., Shinkai, T., Hamajima, Y., Yoshitomi, H., Matsushima, Y., Takada, R., Terasoma, F., Nakamura, A., Komano, J., Nagasawa, K., Shimizu, H., Katayama, K. and Kimura, H. (2018) 'Foodborne Outbreaks Caused by Human Norovirus GII.P17-GII.17–Contaminated Nori, Japan, 2017', *Emerging Infectious Disease journal,* 24(5), pp. 920.

Salvador, D., Neto, C., Benoliel, M. J. and Caeiro, M. F. (2020) 'Assessment of the Presence of Hepatitis E virus in Surface Water and Drinking Water in Portugal', *Microorganisms,* 8(5), pp. 761.

Sanchez-Galan, J. E., Ureña, G., Escovar, L. F., Fabrega-Duque, J. R., Coles, A. and Kurt, Z. (2021) 'Challenges to Detect SARS-CoV-2 on Environmental Media, the Need and Strategies to Implement the Detection Methodologies in Wastewaters', *Journal of Environmental Chemical Engineering*, pp. 105881.

Sanford, B., Emerson, S., Purcell, R., Engle, R., Dryman, B., Cecere, T., Buechner-Maxwell, V., Sponenberg, D. and Meng, X. (2013) 'Serological Evidence for a Hepatitis E Virus-Related Agent in Goats in the United States', *Transboundary and emerging diseases,* 60(6), pp. 538-545.

Sanford, B. J., Dryman, B. A., Huang, Y.-W., Feagins, A. R., LeRoith, T. and Meng, X.-J. (2011) 'Prior infection of pigs with a genotype 3 swine hepatitis E virus (HEV) protects against subsequent challenges with homologous and heterologous genotypes 3 and 4 human HEV', *Virus research,* 159(1), pp. 17-22.

Sano, D., Pérez-Sautu, U., Guix, S., Pintó, R. M., Miura, T., Okabe, S. and

Bosch, A. (2011) 'Quantification and genotyping of human sapoviruses in the Llobregat river catchment, Spain', *Applied and environmental microbiology,* 77(3), pp. 1111-1114.

Santiana, M., Ghosh, S., Ho, B. A., Rajasekaran, V., Du, W. L., Mutsafi, Y., De Jesus-Diaz, D. A., Sosnovtsev, S. V., Levenson, E. A., Parra, G. I., Takvorian, P. M., Cali, A., Bleck, C., Vlasova, A. N., Saif, L. J., Patton, J. T., Lopalco, P., Corcelli, A., Green, K. Y. and Altan-Bonnet, N. (2018) 'Vesicle-Cloaked Virus Clusters Are Optimal Units for Inter-organismal Viral Transmission', Cell Host Microbe, 24(2), pp. 208-220.e8.

Santiso-Bellón, C., Randazzo, W., Pérez-Cataluña, A., Vila-Vicent, S., Gozalbo-Rovira, R., Muñoz, C., Buesa, J., Sanchez, G. and Rodríguez Díaz, J. (2020) 'Epidemiological surveillance of norovirus and rotavirus in sewage (2016–2017) in Valencia (Spain)', *Microorganisms,* 8(3), pp. 458.

Santos, L., Mesquita, J., Pereira, N. R., Lima-Alves, C., Serrão, R., Figueiredo, P., Reis, J., Simões, J., Nascimento, M. and Sarmento, A. (2013) 'Acute hepatitis E complicated by Guillain-Barre syndrome in Portugal, December 2012–a case report', *Platelets (x 109/L),* 328(308), pp. 286.

Schaeffer, J., Le Saux, J.-C., Lora, M., Atmar, R. L. and Le Guyader, F. S. (2013) 'Norovirus contamination on French marketed oysters', *International journal of food microbiology,* 166(2), pp. 244-248.

Schemmerer, M., Apelt, S., Trojnar, E., Ulrich, R. G., Wenzel, J. J. and Johne, R. (2016) 'Enhanced replication of hepatitis E virus strain 47832c in an A549-derived subclonal cell line', *Viruses,* 8(10), pp. 267.

Schets FM, van den Berg HHJL, Vennema H, Pelgrim MTM, Collé C, Rutjes SA, Lodder WJ. ‘Norovirus Outbreak Associated with Swimming in a Recreational Lake Not Influenced by External Human Fecal Sources in The Netherlands, August 2012’. Int J Environ Res Public Health. 2018 Nov 14;15(11):2550. doi: 10.3390/ijerph15112550. PMID: 30441782; PMCID: PMC6267268.

Schlosser, J., Dahnert, L., Dremsek, P., Tauscher, K., Fast, C., Ziegler, U., Groner, A., Ulrich, R. G., Groschup, M. H. and Eiden, M. (2018) 'Different Outcomes of Experimental Hepatitis E Virus Infection in Diverse Mouse Strains, Wistar Rats, and Rabbits', Viruses, 11(1).

Schielke, A., Filter, M., Appel, B. and Johne, R. (2011) 'Thermal stability of hepatitis E virus assessed by a molecular biological approach', *Virology journal,* 8(1), pp. 487.

Schijven, J. and de Roda Husman, A. M. (2006) 'A survey of diving behavior and accidental water ingestion among Dutch occupational and sport divers to assess the risk of infection with waterborne pathogenic microorganisms', *Environmental Health Perspectives,* 114(5), pp. 712-717.

Sedji, M. I., Varbanov, M., Meo, M., Colin, M., Mathieu, L. and Bertrand, I. (2018) 'Quantification of human adenovirus and norovirus in river water in the north-east of France', *Environmental science and pollution research,* 25(30), pp. 30497-30507.

Severn Trent Water. (None) *Making it clear: Sewage treatment*. Available at: https://www.stwater.co.uk/content/dam/stw/my-severn-trent/documents/Sewage-treatment-poster.pdf (Accessed: 26 June 2022)

Shapiro, S. S. and Wilk, M. B. (1965) 'An analysis of variance test for normality (complete samples)', *Biometrika,* 52(3/4), pp. 591-611.

Sharp, J. H., Clements, K., Diggens, M., McDonald, J. E., Malham, S. K. and Jones, D. L. (2021) 'E. coli Is a Poor End-Product Criterion for Assessing the General Microbial Risk Posed From Consuming Norovirus Contaminated Shellfish', *Front Microbiol,* 12, pp. 608888.

Silva, A. J., Yang, Z., Wolfe, J., Hirneisen, K. A., Ruelle, S. B., Torres, A., Williams-Hill, D., Kulka, M. and Hellberg, R. S. (2021) 'Application of whole-genome sequencing for norovirus outbreak tracking and surveillance efforts in Orange County, CA', Food Microbiology, 98, pp. 103796.

Sima, L. C., Schaeffer, J., Le Saux, J.-C., Parnaudeau, S., Elimelech, M. and Le Guyader, F. S. (2011) 'Calicivirus removal in a membrane bioreactor wastewater treatment plant', *Applied and environmental microbiology,* 77(15), pp. 5170-5177.

Slot, E., Zaaijer, H. L., Molier, M., Van den Hurk, K., Prinsze, F. and Hogema, B. M. (2017) 'Meat consumption is a major risk factor for hepatitis E virus infection', *PloS one,* 12(4), pp. e0176414.

Smith, A. J., McCarthy, N., Saldana, L., Ihekweazu, C., McPhedran, K., Adak, G. K., Iturriza-Gómara, M., Bickler, G. and O'Moore, É. (2012) 'A large foodborne outbreak of norovirus in diners at a restaurant in England between January and February 2009', *Epidemiol Infect,* 140(9), pp. 1695-701.

Smith, C., Halse, T. A., Shea, J., Modestil, H., Fowler, R. C., Musser, K. A., Escuyer, V. and Lapierre, P. (2020a) 'Assessing Nanopore Sequencing for Clinical Diagnostics: a Comparison of Next-Generation Sequencing (NGS) Methods for Mycobacterium tuberculosis', *J Clin Microbiol,* 59(1).

Smith, D. B., Izopet, J., Nicot, F., Simmonds, P., Jameel, S., Meng, X.-J., Norder, H., Okamoto, H., van der Poel, W. H. and Reuter, G. (2020b) 'Update: proposed reference sequences for subtypes of hepatitis E virus (species Orthohepevirus A)', *Journal of General Virology*, pp. jgv001435.

Smith, D. B., Paddy, J. O. and Simmonds, P. (2016) 'The use of human sewage screening for community surveillance of hepatitis E virus in the UK', *Journal of medical virology,* 88(5), pp. 915-918.

Smith, D. B., Simmonds, P., Izopet, J., Oliveira-Filho, E. F., Ulrich, R. G., Johne, R., Koenig, M., Jameel, S., Harrison, T. J. and Meng, X.-J. (2016) 'Proposed reference sequences for hepatitis E virus subtypes', *Journal of General Virology,* 97(3), pp. 537-542.

Smith, I., Said, B., Vaughan, A., Haywood, B., Ijaz, S., Reynolds, C., Brailsford, S., Russell, K. and Morgan, D. (2021) 'Case–Control Study of Risk Factors for Acquired Hepatitis E Virus Infections in Blood Donors, United Kingdom, 2018–

2019', *Emerging Infectious Diseases,* 27(6), pp. 1654-1661.

Soller, J. A., Schoen, M. E., Bartrand, T., Ravenscroft, J. E. and Ashbolt, N. J. (2010) 'Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination', *Water research,* 44(16), pp. 4674-4691.

Sonoda, H., Abe, M., Sugimoto, T., Sato, Y., Bando, M., Fukui, E., Mizuo, H., Takahashi, M., Nishizawa, T. and Okamoto, H. (2004) 'Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan', *Journal of Clinical Microbiology,* 42(11), pp. 5371-5374.

Sooryanarain, H., Heffron, C. L., Hill, D. E., Fredericks, J., Rosenthal, B. M., Werre, S. R., Opriessnig, T. and Meng, X.-J. (2020) 'Hepatitis E virus in pigs from slaughterhouses, United States, 2017–2019', *Emerging infectious diseases,* 26(2), pp. 354.

Spancerniene, U., Grigas, J., Buitkuviene, J., Zymantiene, J., Juozaitiene, V., Stankeviciute, M., Razukevicius, D., Zienius, D. and Stankevicius, A. (2018) 'Prevalence and phylogenetic analysis of hepatitis E virus in pigs, wild boars, roe deer, red deer and moose in Lithuania', *Acta veterinaria scandinavica,* 60(1), pp. 13.

Spina, A., Lenglet, A., Beversluis, D., de Jong, M., Vernier, L., Spencer, C., Andayi, F., Kamau, C., Vollmer, S. and Hogema, B. (2017) 'A large outbreak of Hepatitis E virus genotype 1 infection in an urban setting in Chad likely linked to household level transmission factors, 2016-2017', *PLoS One,* 12(11), pp. e0188240.

Stefan, C. P., Hall, A. T., Graham, A. S. and Minogue, T. D. (2022) 'Comparison of Illumina and Oxford Nanopore Sequencing Technologies for Pathogen Detection from Clinical Matrices Using Molecular Inversion Probes', The Journal of Molecular Diagnostics, 24(4), pp. 395-405.

Stone, D. L., Harding, A. K., Hope, B. K. and Slaughter-Mason, S. (2008) 'Exposure Assessment and Risk of Gastrointestinal Illness Among Surfers', *Journal of Toxicology and Environmental Health, Part A,* 71(24), pp. 1603-1615.

Sumner, J. and Ross, T. (2002) 'A semi-quantitative seafood safety risk assessment', *International Journal of Food Microbiology,* 77(1), pp. 55-59.

Sunaga, F., Masuda, T., Aoki, H., Ito, M., Sano, K., Naoi, Y., Katayama, Y., Omatsu, T., Oba, M. and Furuya, T. (2019) 'Complete genome sequencing and genetic characterization of porcine sapovirus genogroup (G) X and GXI: GVI, GVII, GX, and GXI sapoviruses share common genomic features and form a unique porcine SaV clade', *Infection, Genetics and Evolution,* 75, pp. 103959.

Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B. and Koopmans, M. (2007) 'Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005', *Journal of clinical microbiology,* 45(5), pp. 1389-1394.

Svraka, S., Vennema, H., van der Veer, B., Hedlund, K.-O., Thorhagen, M.,

Siebenga, J., Duizer, E. and Koopmans, M. (2010) 'Epidemiology and genotype analysis of emerging sapovirus-associated infections across Europe', *Journal of clinical microbiology,* 48(6), pp. 2191-2198.

Szabo, K., Trojnar, E., Anheyer-Behmenburg, H., Binder, A., Schotte, U., Ellerbroek, L., Klein, G. and Johne, R. (2015) 'Detection of hepatitis E virus RNA in raw sausages and liver sausages from retail in Germany using an optimized method', *International journal of food microbiology,* 215, pp. 149-156.

Sánchez, G., Pintó, R. M., Vanaclocha, H. and Bosch, A. (2002) 'Molecular characterization of hepatitis A virus isolates from a transcontinental shellfish-borne outbreak', *Journal of Clinical Microbiology,* 40(11), pp. 4148-4155.

Takahashi, M., Tanaka, T., Takahashi, H., Hoshino, Y., Nagashima, S., Jirintai, Mizuo, H., Yazaki, Y., Takagi, T., Azuma, M., Kusano, E., Isoda, N., Sugano, K. and Okamoto, H. (2010) 'Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: characterization of HEV virions in blood circulation', J Clin Microbiol, 48(4), pp. 1112-25.

Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F. and Okamoto, H. (2003) 'Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus', *Journal of General Virology,* 84(4), pp. 851-862.

Takahashi, M., Yamada, K., Hoshino, Y., Takahashi, H., Ichiyama, K., Tanaka, T. and Okamoto, H. (2008) 'Monoclonal antibodies raised against the ORF3 protein of hepatitis E virus (HEV) can capture HEV particles in culture supernatant and serum but not those in feces', Arch Virol, 153(9), pp. 1703-13.

Tallon, L. A., Love, D. C., Moore, Z. S. and Sobsey, M. D. (2008) 'Recovery and sequence analysis of hepatitis a virus from springwater implicated in an outbreak of acute viral hepatitis', *Applied and environmental microbiology,* 74(19), pp. 6158-6160.

Tam, C., Viviani, L., Adak, B., Bolton, E., Dodds, J., Cowden, J., Evans, M., Gray, J., Hunter, P. and Jackson, K. (2012) 'The second study of infectious intestinal disease in the community (IID2 study)', *UK Food Standards Agency*.

Tamada, Y., Yano, K., Yatsuhashi, H., Inoue, O., Mawatari, F. and Ishibashi, H. (2004) 'Consumption of wild boar linked to cases of hepatitis E', *Journal of Hepatology,* 40(5), pp. 869-870.

Tanaka, T., Takahashi, M., Kusano, E. and Okamoto, H. (2007) 'Development and evaluation of an efficient cell-culture system for Hepatitis E virus', *Journal of General Virology,* 88(3), pp. 903-911.

Tang, Y., Wang, J., Xu, Z., Guo, Y., Qian, W. and Xu, J. (1991) 'A serologically confirmed, case-control study, of a large outbreak of hepatitis A in China, associated with consumption of clams', *Epidemiology & Infection,* 107(3), pp. 651-657.

Tei, S., Kitajima, N., Takahashi, K. and Mishiro, S. (2003) 'Zoonotic transmission of hepatitis E virus from deer to human beings', *The Lancet,*

362(9381), pp. 371-373.

Terio, V., Bottaro, M., Pavoni, E., Losio, M., Serraino, A., Giacometti, F., Martella, V., Mottola, A., Di Pinto, A. and Tantillo, G. (2017) 'Occurrence of hepatitis A and E and norovirus GI and GII in ready-to-eat vegetables in Italy', *International journal of food microbiology,* 249, pp. 61-65.

Teunis, P. F., Moe, C. L., Liu, P., E. Miller, S., Lindesmith, L., Baric, R. S., Le Pendu, J. and Calderon, R. L. (2008a) 'Norwalk virus: how infectious is it?', *Journal of medical virology,* 80(8), pp. 1468-1476.

Teunis, P. F., Moe, C. L., Liu, P., Miller, S. E., Lindesmith, L., Baric, R. S., Le Pendu, J. and Calderon, R. L. (2008b) 'Norwalk virus: how infectious is it?', *J Med Virol,* 80(8), pp. 1468-76.

Teunis, P. F. M. and Havelaar, A. H. (2000) 'The Beta Poisson Dose-Response Model Is Not a Single-Hit Model', *Risk Analysis,* 20(4), pp. 513-520.

Thebault, A., Teunis, P. F. M., Le Pendu, J., Le Guyader, F. S. and Denis, J.-B. (2013) 'Infectivity of GI and GII noroviruses established from oyster related outbreaks', *Epidemics,* 5(2), pp. 98-110.

Thongprachum, A., Fujimoto, T., Takanashi, S., Saito, H., Okitsu, S., Shimizu, H., Khamrin, P., Maneekarn, N., Hayakawa, S. and Ushijima, H. (2018) 'Detection of nineteen enteric viruses in raw sewage in Japan', *Infection, Genetics and Evolution,* 63, pp. 17-23.

Todt, D., Gisa, A., Radonic, A., Nitsche, A., Behrendt, P., Suneetha, P. V., Pischke, S., Bremer, B., Brown, R. J. and Manns, M. P. (2016) 'In vivo evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome', *Gut,* 65(10), pp. 1733-1743.

Tomiyama, D., Inoue, E., Osawa, Y. and Okazaki, K. (2009) 'Serological evidence of infection with hepatitis E virus among wild Yezo-deer, Cervus nippon yesoensis, in Hokkaido, Japan', *Journal of viral hepatitis,* 16(7), pp. 524- 528.

Treagus, S., Wright, C., Baker-Austin, C., Longdon, B. and Lowther, J. (2021) 'The Foodborne Transmission of Hepatitis E Virus to Humans', *Food and Environmental Virology*, pp. 1-19.

Tripathy, A. S., Sharma, M., Deoshatwar, A. R., Babar, P., Bharadwaj, R. and Bharti, O. K. (2019) 'Study of a hepatitis E virus outbreak involving drinking water and sewage contamination in Shimla, India, 2015-2016', *Trans R Soc Trop Med Hyg,* 113(12), pp. 789-796.

Tyson, J. R., James, P., Stoddart, D., Sparks, N., Wickenhagen, A., Hall, G., Choi, J. H., Lapointe, H., Kamelian, K., Smith, A. D., Prystajecky, N., Goodfellow, I., Wilson, S. J., Harrigan, R., Snutch, T. P., Loman, N. J. and Quick, J. (2020) 'Improvements to the ARTIC multiplex PCR method for SARS-CoV-2 genome sequencing using nanopore', *bioRxiv*, pp. 2020.09.04.283077.

Ueki, Y., Shoji, M., Suto, A., Tanabe, T., Okimura, Y., Kikuchi, Y., Saito, N., Sano, D. and Omura, T. (2007) 'Persistence of caliciviruses in artificially contaminated oysters during depuration', *Applied and environmental*

microbiology, 73(17), pp. 5698-5701.

UK Government. EUR-Lex. (2021) *Commission Implementing Regulation (EU) 2019/627 of 15 March 2019 laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) No 2074/2005 as regards official controls*: UK Government.

United States Government, U.S House of Representatives. (2018) *The United States Code.* USA: Office of the Law Revision Counsel of the U.S. House of Representatives.

Van Abel, N., Schoen, M. E., Kissel, J. C. and Meschke, J. S. (2017) 'Comparison of Risk Predicted by Multiple Norovirus Dose–Response Models and Implications for Quantitative Microbial Risk Assessment', *Risk Analysis,* 37(2), pp. 245-264.

van Alphen, L. B., Dorléans, F., Schultz, A. C., Fonager, J., Ethelberg, S., Dalgaard, C., Adelhardt, M., Engberg, J. H., Fischer, T. K. and Lassen, S. G. (2014) 'The application of new molecular methods in the investigation of a waterborne outbreak of norovirus in Denmark, 2012', *PloS one,* 9(9), pp. e105053-e105053.

Van Dijk, P., Lacey, R. and Pike, E. (1996) 'Health effects of sea bathing—further analysis of data from UK beach surveys', *Department of the Environment, WRc pic, Medmenham*.

Varela, M. F., Polo, D. and Romalde, J. L. (2016) 'Prevalence and genetic diversity of human sapoviruses in shellfish from commercial production areas in Galicia, Spain', *Applied and environmental microbiology,* 82(4), pp. 1167-1172.

Vercouter, A.-S., Sayed, I. M., Lipkens, Z., De Bleecker, K., De Vliegher, S., Colman, R., Koppelman, M., Supré, K. and Meuleman, P. (2018) 'Absence of zoonotic hepatitis E virus infection in Flemish dairy cows', *International journal of food microbiology,* 281, pp. 54-59.

Vergara, G. G. R. V., Rose, J. B. and Gin, K. Y. H. (2016) 'Risk assessment of noroviruses and human adenoviruses in recreational surface waters', *Water Research,* 103, pp. 276-282.

Verhoef, L., Kouyos, R. D., Vennema, H., Kroneman, A., Siebenga, J., van Pelt, W. and Koopmans, M. (2011) 'An integrated approach to identifying international foodborne norovirus outbreaks', *Emerg Infect Dis,* 17(3), pp. 412-8.

Viau, E. J., Lee, D. and Boehm, A. B. (2011) 'Swimmer risk of gastrointestinal illness from exposure to tropical coastal waters impacted by terrestrial dry-weather runoff', *Environmental science & technology,* 45(17), pp. 7158-7165.

Villalba, M. C. M., Martínez, D. C., Ahmad, I., Lay, L. A. R., Corredor, M. B., March, C. G., Martínez, L. S., Martínez-Campo, L. S. and Jameel, S. (2017) 'Hepatitis E virus in bottlenose dolphins Tursiops truncatus', *Diseases of aquatic organisms,* 123(1), pp. 13-18.

Vitral, C. L., Pinto, M. A., Lewis-Ximenez, L. L., Khudyakov, Y. E., dos Santos, D. R. and Gaspar, A. M. C. (2005) 'Serological evidence of hepatitis E virus infection in different animal species from the Southeast of Brazil', *Memorias do Instituto Oswaldo Cruz,* 100(2), pp. 117-122.

Vivek, R., Zachariah, U. G., Ramachandran, J., Eapen, C. E., Rajan, D. P. and Kang, G. (2013) 'Characterization of hepatitis E virus from sporadic hepatitis cases and sewage samples from Vellore, south India', *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 107(6), pp. 363-367.

Wachtman, L. and Mansfield, K. (2012) 'Viral diseases of nonhuman primates', *Nonhuman primates in biomedical research*, pp. 1.

Wade TJ, Augustine SAJ, Griffin SM, Sams EA, Oshima KH, Egorov AI, Simmons KJ, Eason TN, Dufour AP. 'Asymptomatic norovirus infection associated with swimming at a tropical beach: A prospective cohort study'. PLoS One. 2018 Mar 28;13(3):e0195056. doi: 10.1371/journal.pone.0195056. PMID: 29590196; PMCID: PMC5874074.

Wade, T. J., Pai, N., Eisenberg, J. N. S. and Colford, J. M. (2003) 'Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis', *Environmental Health Perspectives,* 111(8), pp. 1102-1109.

Wang, L., Teng, J. L., Lau, S. K., Sridhar, S., Fu, H., Gong, W., Li, M., Xu, Q., He, Y. and Zhuang, H. (2019) 'Transmission of a novel genotype of hepatitis E virus from Bactrian camels to cynomolgus macaques', *Journal of Virology,* 93(7), pp. e02014-18.

Wang, Y., Zhang, H., Li, Z., Gu, W., Lan, H., Hao, W., Ling, R., Li, H. and Harrison, T. J. (2001) 'Detection of sporadic cases of hepatitis E virus (HEV) infection in China using immunoassays based on recombinant open reading frame 2 and 3 polypeptides from HEV genotype 4', *Journal of clinical microbiology,* 39(12), pp. 4370-4379.

Ward, P., Müller, P., Letellier, A., Quessy, S., Simard, C., Trottier, Y. L., Houde, A. and Brassard, J. (2008) 'Molecular characterization of hepatitis E virus detected in swine farms in the province of Quebec', *Can J Vet Res,* 72(1), pp. 27-31.

Webster, C. L., Waldron, F. M., Robertson, S., Crowson, D., Ferrari, G., Quintana, J. F., Brouqui, J.-M., Bayne, E. H., Longdon, B. and Buck, A. H. (2015) 'The discovery, distribution, and evolution of viruses associated with Drosophila melanogaster', *PLoS Biol,* 13(7), pp. e1002210.

Weesendorp, E., Willems, E. M. and Loeffen, W. L. A. (2010) 'The effect of tissue degradation on detection of infectious virus and viral RNA to diagnose classical swine fever virus', *Veterinary Microbiology,* 141(3), pp. 275-281.

Weger, S., Elkin, B., Lindsay, R., Bollinger, T., Crichton, V. and Andonov, A. (2017) 'Hepatitis E Virus Seroprevalence in Free-Ranging Deer in Canada', *Transboundary and emerging diseases,* 64(3), pp. 1008-1011.

Wenzel, J. J., Preiß, J., Schemmerer, M., Huber, B., Plentz, A. and Jilg, W.

(2011) 'Detection of hepatitis E virus (HEV) from porcine livers in Southeastern Germany and high sequence homology to human HEV isolates', *Journal of clinical virology,* 52(1), pp. 50-54.Wilcoxon, F. (1992) 'Individual comparisons by ranking methods', *Breakthroughs in statistics*: Springer, pp. 196-202.

Wilhelm, B., Leblanc, D., Houde, A., Brassard, J., Gagné, M.-J., Plante, D., Bellon-Gagnon, P., Jones, T. H., Muehlhauser, V. and Janecko, N. (2014) 'Survey of Canadian retail pork chops and pork livers for detection of hepatitis E virus, norovirus, and rotavirus using real time RT-PCR', *International journal of food microbiology,* 185, pp. 33-40.

Williams, S. and O'Brien, S. J. (2019) *NoVAS: Assessing the contribution made by the food chain to the burden of UK acquired norovirus infection*, UK: Cefas, Fera, Leatherhead Food Research, Liverpool Clinical Laboratories (Royal Liverpool and Broadgreen University Hospital Trust), UK Health Security Agency, University of East Anglia and University of Liverpool (Accessed: 21 July 2021).

World Health Organization. (2019) *Hepatitis E*: World Health Organization. Available at: https://www.who.int/news-room/fact-sheets/detail/hepatitis-e (Accessed: 08 September 2021).

Wyer, M. D., Kay, D., Morgan, H., Naylor, S., Clark, S., Watkins, J., Davies, C. M., Francis, C., Osborn, H. and Bennett, S. (2018) 'Within-day variability in microbial concentrations at a UK designated bathing water: Implications for regulatory monitoring and the application of predictive modelling based on historical compliance data', *Water Res X,* 1, pp. 100006.

Wyn-Jones, A. P., Carducci, A., Cook, N., D'agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R. and Höller, C. (2011) 'Surveillance of adenoviruses and noroviruses in European recreational waters', *Water research,* 45(3), pp. 1025-1038.

Xia, J., Zeng, H., Liu, L., Zhang, Y., Liu, P., Geng, J., Wang, L., Wang, L. and Zhuang, H. (2015) 'Swine and rabbits are the main reservoirs of hepatitis E virus in China: detection of HEV RNA in feces of farmed and wild animals', *Archives of virology,* 160(11), pp. 2791-2798.

Xie, X., Bil, J., Shantz, E., Hammermueller, J., Nagy, E. and Turner, P. V. (2017) 'Prevalence of lapine rotavirus, astrovirus, and hepatitis E virus in Canadian domestic rabbit populations', *Veterinary Microbiology,* 208, pp. 146-149.

Yan, B., Zhang, L., Gong, L., Lv, J., Feng, Y., Liu, J., Song, L., Xu, Q., Jiang, M. and Xu, A. (2016) 'Hepatitis E virus in yellow cattle, Shandong, Eastern China', *Emerging infectious diseases,* 22(12), pp. 2211.

Yang, N., Qi, H., Wong, M. M. L., Wu, R. S. S. and Kong, R. Y. C. (2012) 'Prevalence and diversity of norovirus genogroups I and II in Hong Kong marine waters and detection by real-time PCR', Marine Pollution Bulletin, 64(1), pp. 164-168.

Yazaki, Y., Mizuo, H., Takahashi, M., Nishizawa, T., Sasaki, N., Gotanda, Y.

and Okamoto, H. (2003) 'Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food', *Journal of general virology,* 84(9), pp. 2351-2357.

Yin, X., Ambardekar, C., Lu, Y. and Feng, Z. (2016) 'Distinct Entry Mechanisms for Nonenveloped and Quasi-Enveloped Hepatitis E Viruses', *J Virol,* 90(8), pp. 4232-4242.

Yin, W., Han, Y., Xin, H., Liu, W., Song, Q., Li, Z., Gao, S., Jiang, F., Cao, J., Bi, S. and Liu, H. (2019) 'Hepatitis E Outbreak in a Mechanical Factory in Qingdao City, China', *Int J Infect Dis*.

Yoo, D., Willson, P., Pei, Y., Hayes, M. A., Deckert, A., Dewey, C. E., Friendship, R. M., Yoon, Y., Gottschalk, M. and Yason, C. (2001) 'Prevalence of hepatitis E virus antibodies in Canadian swine herds and identification of a novel variant of swine hepatitis E virus', *Clin. Diagn. Lab. Immunol.,* 8(6), pp. 1213-1219.

Yugo, D. M., Cossaboom, C. M., Heffron, C. L., Huang, Y. W., Kenney, S. P., Woolums, A. R., Hurley, D. J., Opriessnig, T., Li, L. and Delwart, E. (2019) 'Evidence for an unknown agent antigenically related to the hepatitis E virus in dairy cows in the United States', *Journal of medical virology,* 91(4), pp. 677-686.

Zemtsova, G. E., Montgomery, M. and Levin, M. L. (2015) 'Relative sensitivity of conventional and real-time PCR assays for detection of SFG Rickettsia in blood and tissue samples from laboratory animals', *PloS one,* 10(1).

Zhang, X.-X., Qin, S.-Y., Zhang, Y., Meng, Q.-F., Jiang, J., Yang, G.-L., Zhao, Q. and Zhu, X.-Q. (2015) 'First report of hepatitis E virus infection in sika deer in China', *BioMed research international,* 2015.

Zhang, Y., Gong, W., Song, W. T., Fu, H., Wang, L., Li, M., Wang, L. and Zhuang, H. (2017) 'Different susceptibility and pathogenesis of rabbit genotype 3 hepatitis E virus (HEV-3) and human HEV-3 (JRC-HE3) in SPF rabbits', *Veterinary microbiology,* 207, pp. 1-6.

Zhao, C., Li, Z., Yan, B., Harrison, T. J., Guo, X., Zhang, F., Yin, J., Yan, Y. and Wang, Y. (2007) 'Comparison of real-time fluorescent RT-PCR and conventional RT-PCR for the detection of hepatitis E virus genotypes prevalent in China', *Journal of medical virology,* 79(12), pp. 1966-1973.

Zlot A, Simckes M, Vines J, Reynolds L, Sullivan A, Scott AK, McLuckie JM, Kromer D, Hill VR, Yoder JS, Hlavsa MC; Centers for Disease Control and Prevention (CDC). ‘Norovirus outbreak associated with a natural lake used for recreation - Oregon, 2014’. MMWR Morb Mortal Wkly Rep. 2015 May 15;64(18):485-90. PMID: 25974632; PMCID: PMC4584822.