Relative sensitivity of immunohistochemistry, multiple reaction monitoring mass spectrometry, *in situ* hybridization and PCR to detect Coxsackievirus B1 in A549 cells

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Abstract

Background Enteroviruses (EVs) have been linked to the pathogenesis of several diseases and there is a collective need to develop improved methods for the detection of these viruses in tissue samples.

Objectives This study evaluates the relative sensitivity of immunohistochemistry (IHC), proteomics, *in situ* hybridization (ISH) and RT-PCR to detect one common EV, Coxsackievirus B1 (CVB1), in acutely infected human A549 cells *in vitro*.

Study design A549 cells were infected with CVB1 and diluted with uninfected A549 cells to produce a limited dilution series in which the proportion of infected cells ranged from 10^{-1} to 10^{-8}. Analyses were carried out by several laboratories using IHC with different anti-EV antibodies, ISH with both ViewRNA and RNAScope systems, Liquid Chromatography Multiple Reaction Monitoring Mass Spectrometry (LC/MRM/MS/MS), and two modifications of RT-PCR.

Results RT-PCR was the most sensitive method for EV detection yielding positive signals in the most diluted sample (10^{-8}). LC/MRM/MS/MS detected viral peptides at dilutions as high as 10^{-7}. The sensitivity of IHC depended on the antibody used, and the most sensitive antibody (Dako clone 5D8/1) detected virus proteins at a dilution of 10^{-6}, while ISH detected the virus at dilutions of 10^{-4}.

Conclusions All methods were able to detect CVB1 in infected A549 cells. RT-PCR was most sensitive followed by LC/MRM/MS/MS and then IHC. The results from this *in vitro* survey suggest that all methods are suitable tools for EV detection but that their differential sensitivities need to be considered when interpreting the results from such studies.

47 **Keywords**

48 Sensitivity, Proteomics, *In situ* hybridization, Immunohistochemisrty, RT-PCR

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Background

Enterovirus[1] (EV) infections are common in all age groups. They are usually asymptomatic or cause only mild respiratory symptoms, but can also lead to more severe illness including hand, foot and mouth disease, myocarditis, meningitis, encephalitis, pancreatitis, systemic infection in newborns and paralysis. EV infections may also play a role in the pathogenesis of chronic diseases such as dilated cardiomyopathy (1), chronic fatigue syndrome (2) and type 1 diabetes[2] (T1D) (3-5).

Laboratory diagnosis of EV infection is based on virus detection in stools, nasal/throat swabs or cerebrospinal fluid, as well as on EV-specific antibody responses in serum. However, studies evaluating the pathogenesis of EV infections and their possible role in chronic diseases (where levels of viral infection may be low but persistent) require additional technologies and there is an increasing need for direct virus detection in tissue samples. Traditionally, EVs are detected in tissue samples either by RT-PCR or immunohistochemistry[3] (IHC). In addition to these, the new single molecule hybridization (6,7) as well as mass spectrometry/proteomics technologies offer new opportunities for viral detection. However, there are no previous reports in which the performance of these various technologies has been evaluated in relation to one another.

Objectives

The aim of the study was to evaluate the relative sensitivities of proteomics, ISH, IHC and RT-PCR methods to detect one common EV, Coxsackievirus B1[4] (CVB1), using human A549 cells diluted to contain differing ratios of uninfected to *in vitro* EV-infected cells.

[1] EV, enterovirus

[2] T1D, type 1 diabetes

[3] IHC, immunohistochemistry

[4] CVB, Coxsackievirus B

Study design

Preparation of EV-infected cell arrays

Human A549 alveolar basal epithelial cells were grown in monolayers in Nutrient Mixture F-12 Ham, N 6658 (Sigma-Aldrich®) medium in T175 bottles and infected with CVB1, ATCC strain (10-15 MOI). The infection was stopped at four different time points (1 h, 2 h, 4 h, and 6 h post infection) to obtain a series of infected cells representing different stages of viral replication cycle. The cells from different time points were mechanically detached, pooled and washed with the growth medium. The cells were then immediately diluted with uninfected A549 cells to produce a dilution series ranging from 10^{-1} to 10^{-8}, as well as an undiluted sample (positive control) and a sample of uninfected A549 cells (negative control). Each dilution aliquot was further divided into ten sub-aliquots, each containing about 1 million cells. These sub-aliquots were fixed or stored in an optimal way for each of the different methodologies employed. Some of the aliquots were fixed in 10 % neutral-buffered formalin for 24 h and paraffin-embedded using standard procedures for IHC and ISH analyses. The rest were quickly frozen in liquid nitrogen and stored at -70°C for RT-PCR and proteomics analyses. From the individual formalin-fixed paraffin-embedded[5] (FFPE) samples, a cell microarray was created using TMA Master (3D Histech Kft, Hungary) and 5 µm-thick sections were cut for histological stainings.

RT-PCR

RT-PCR was performed in two different laboratories (Tampere and Uppsala), each analyzing similar aliquots of the dilution series. In Tampere, RNA was extracted from 140 µl cell sample using the Viral RNA Kit (Qiagen, Hilden, Germany), and real-time RT-PCR was performed as previously described (8). In Uppsala, viral RNA was extracted from 100 µl using RNeasy Mini kit

[5] FFPE, formalin-fixed paraffin-embedded

92 (Qiagen). 50 ng total RNA/sample were primed with virus specific primers and reverse transcribed
93 to cDNA with SuperScriptII™ RT (Invitrogen) according to the manufacturer's instructions. A
94 semi-nested EV PCR was performed as described previously (3).

95 Proteomics

96 The dilution series samples were solubilized using 50% Trifluoroethanol in 50 mM ammonium
97 bicarbonate as previously described (9). Protein concentration was determined by bicinchoninic
98 acid[6] (BCA) assay (Pierce, Rockford, Ill.). Concentration normalized samples from each of the
99 dilution steps were reduced and alkylated as previously described (10). Proteins were digested with
100 trypsin at a ratio of 1:50 at 37°C for 18 h to generate peptides. The peptides were purified using C18
101 columns, eluted using 80% acetonitrile in 0.1% formic acid and dried in a SpeedVac. Peptides were
102 reconstituted in 0.1% formic acid prior to Liquid chromatography multiple reaction monitoring
103 mass spectrometry[7] (LC/MRM/MS/MS). LC/MRM/MS/MS on a triple quadrupole (QqQ) mass
104 spectrometer[8] provides superior rapid, sensitive, and specific identification and quantitation of
105 targeted compounds in highly complex samples (11,12).

106 LC/MRM/MS analysis of the tryptic peptides from the 10 A549 dilution series cell samples was
107 performed on a 4000 QTRAP® mass spectrometer coupled to a Tempo NanoLC system (ABSciex,
108 Foster City, CA) (10). Skyline was used to generate and optimize tryptic peptides and tandem
109 MS/MS fragmentation data for developing MRM transitions pairs for CVB1 peptides (13). A CVB1
110 2C protein peptide SVATNLIGR was selected for subsequent analysis and quantitation based on its
111 abundance and high signal intensities for both the precursor ion (Q1 m/z) and fragment ions (Q3
112 m/z) and absence of signals in non-infected A549 cells. MRM Pilot ™ software (ABSciex) was

[6] BCA, bicinchoninic acid

[7] LC/MRM/MS/MS, liquid chromatography multiple reaction monitoring mass spectrometry

[8] QqQ, triple quadrupole mass spectrometer

used to optimize the assay conditions for the SVATNLIGR peptide with the following transition pairs of 465.7720/572.3515 and 465.7720/744.4363. The Q1 m/z (465.7720) for the MH^{2+} peptide parent mass and the Q3 m/zs correspond to y5 (572.3515) and y7 (744.4363) fragment ions. The final MRM assay conditions are detailed in Supplementary data.

The tryptic peptides corresponding to 1.6 µg of sample were injected and analyzed for each sample. The samples were sequentially analyzed starting with the non-infected samples and the low dilutions, to the non-diluted samples with multiple washing steps using blanks (buffer A) between each sample to avoid carry-over. Each sample was analyzed in duplicate experiments. Relative quantitation was achieved by comparing the area under the curve for the peptide transition pairs in the extracted ion chromatograms[9] (XIC) for each dilution step. The acquired data were processed and analyzed using Analyst 1.2 (ABSciex).

Immunohistochemistry

IHC was performed in three laboratories (Tampere, Exeter and Uppsala). Primary analyses were done using a commercially available antibody raised against EV VP1[10] protein (clone 5D8/1; DAKO, Glostrup, Denmark). In Tampere and Exeter, IHC was performed as previously described (5,14,15). In Uppsala, the sections were counterstained with haematoxylin (DAKO) before addition of the primary antibody (diluted 1:2000) in autostainer Link 48 DAKO. Visualization was achieved with the DAKO Envision K8000. In addition, polyclonal antibodies produced in rabbits (see Supplementary) against each of the viral capsid proteins VP1, VP2, VP3 and VP4 of CVB4 Tuscany strain, were analyzed in the Tampere and Exeter laboratories. Antibodies from the first bleed (VP1A and VP3A) and from the last bleed (VP1B, VP2B, VP3B and VP4B) were used. In Tampere, staining was performed using the automated system and similar conditions to those for

[9] XIC, extracted ion chromatogram

[10] VP, viral protein

clone 5D8/1. In Exeter, following heat-induced epitope retrieval in 10mM citrate, pH6.0, the VP1-VP4 antibodies were incubated for 1h at room temperature. The DAKO Envision Detection System was used for antigen detection as per the manufacturer's instructions and sections were counterstained with haematoxylin. The concentrations of CVB4 VP1-VP4 antibodies used in both laboratories are detailed in Supplementary Table 1.

In situ hybridization

ISH assays were performed in two laboratories (Tampere and Gainesville). In Tampere, the QuantiGene® ViewRNA (Affymetrix, Santa Clara, California, USA) was used with two different EV-specific probe sets (EV AB and CVB1), according to the manufacturer's instructions and as previously described (6). In Gainesville, ISH was performed using the RNAscope 2.0 High Definition Assay (Advanced Cell Diagnostics, Hayward, California, USA) according to the manufacturer's instructions. Two EV-specific probes were tested to detect serotypes CVB1-6 and CVB3. Deparaffinized sections were hybridized to probes followed by amplification by serial application of amplifiers followed by peroxidase labels and detection with DAB.

Results

RT-PCR

Viral RNA was detected by RT-PCR in all samples although the semi-nested method was most sensitive. This yielded a positive signal from even the most dilute sample (10^{-8}) whereas the real-time RT-PCR method gave a positive signal in the second most dilute sample (10^{-7}). Ct values from real-time RT-PCR experiments with different dilutions of infected cells are shown in Supplementary Table 2.

Proteomics

MS-based targeted LC/MRM/MS/MS assay focused on the CVB1 2C protein peptide SVATNLIGR and the peptide signal was detectable at cell dilutions as high as 10^{-7}. Figure 1 shows the LC/MRM/MS/MS results for the relative abundance of the 2C protein peptide in undiluted, virus-infected cells. It also shows the detection of the MRM transition pairs signals, and the enhanced product ion scan[11] (EPI) showing the MS/MS fragmentation spectrum for the peptide.

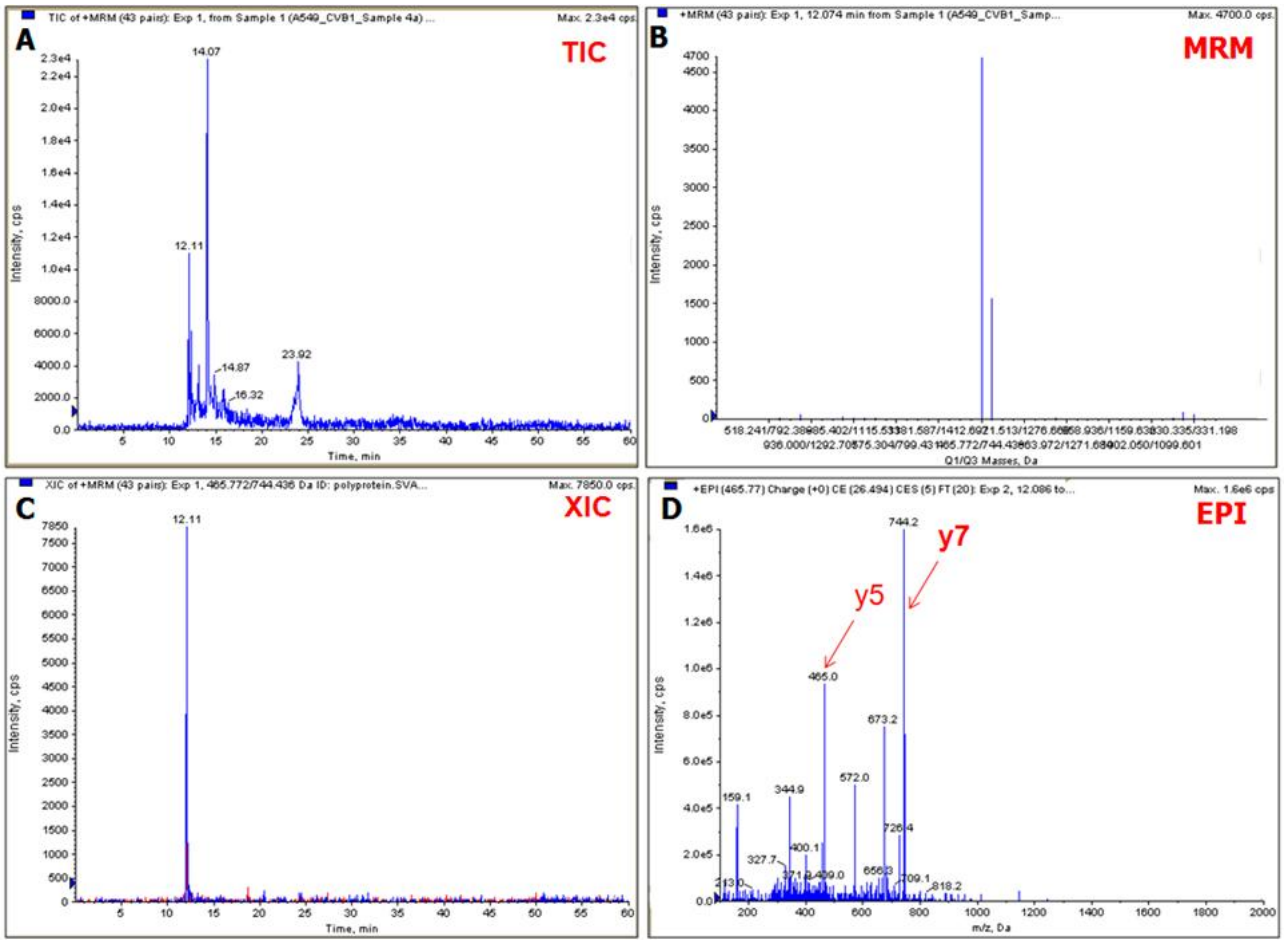
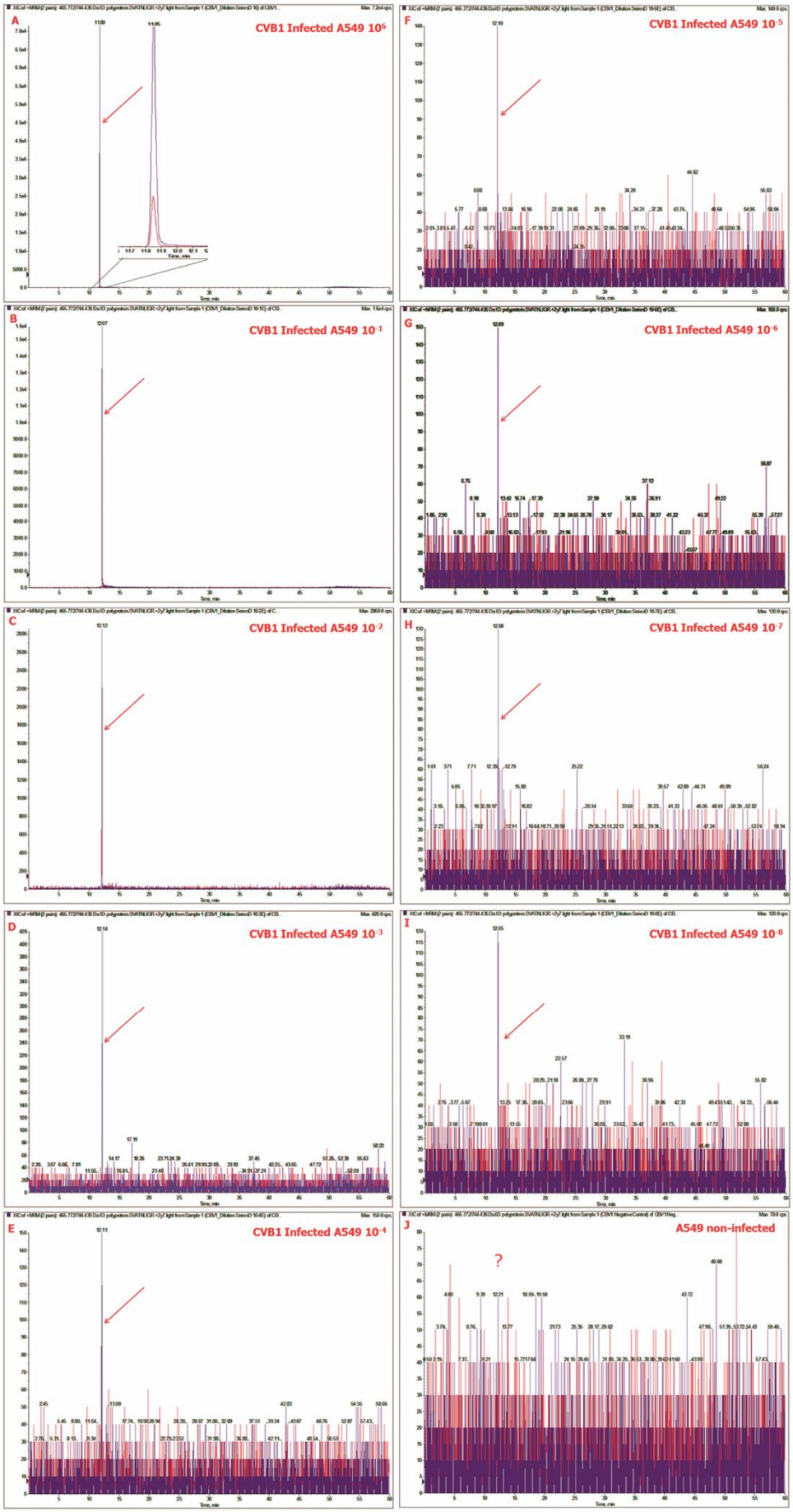


Figure 1. Multiple reaction monitoring assay for CVB1 virus peptides. The total ion chromatogram and MRM peaks are shown in panels A and B. The extracted ion chromatogram (XIC) for the Protein 2C peptide is shown in panel C and the enhanced product ion scan (EPI) spectrum with the Q3 y5 and y7 fragment ions are shown in panel D. These two ions are the most intense in the tandem mass spectrum and their primary sequences correspond to the following c-terminus fragments of the peptide. y5 NLIGR (m/z = 572.3515) and y7 ATNLIGR (m/z = 744.4363).

[11] EPI, enhanced product ion scan

Figure 2 shows extracted ion chromatograms of the two transition pairs 465.7720/572.3515 (red) and 465.7720/744.4363 (blue) for the non-diluted, infected A549 cells (Panel A), the dilution series of the infected cells (Panels B - J) and the non-infected A549 cells (Panel K). The relative intensity and the accompanying signal for the MRM assay decreases from that of the peptide.


A
CVB1 Infected A549 10-6
F
CVB1 Infected A549 10-5
B
CVB1 Infected A549 10-1
G
CVB1 Infected A549 10-6
C
CVB1 Infected A549 10-2
H
CVB1 Infected A549 10-7
D
CVB1 Infected A549 10-3
I
CVB1 Infected A549 10-8
E
CVB1 Infected A549 10-4
J
A549 non-infected
?

175

Figure 2. MRM Detection of CVB1 peptide in A549 cells LC/MRM/MS. Extracted ion chromatograms of the two transition pairs 465.7720/572.3515 (red) and 465.7720/744.4363 (purple) for the non-diluted CVB1 infected A549 cells panel (A), the dilution series of the infected cells (Panels B - I) and the non-infected A549 cells (Panel J). The MRM peaks in the samples are marked with a red arrow. Note the absence of signal (?) in Panel J. In panel A, the Zoom shows an expansion of the baseline to show the well resolved peaks of the extracted ion chromatograms of the two MRM transition pairs.

Immunohistochemistry

IHC also proved to be a sensitive method for detection of viral protein but was less sensitive than semi-nested RT-PCR, real-time RT-PCR and LC/MRM/MS/MS. IHC detected viral protein in virtually every cell in the undiluted sample, but the proportion of virus-positive cells decreased linearly as the dilution increased. Clone 5D8/1 was the most sensitive antibody tested detecting virus-positive cells at dilutions equal to or lower than 10^{-4} in Uppsala, 10^{-5} in Tampere and 10^{-6} in Exeter. At dilutions from 10^{-3} and beyond, the number of virus-positive cells was scarce, with only occasional cells stained positively (Fig. 3). In Tampere and Exeter, antibodies raised against CVB4 viral capsid proteins, stained efficiently the infected cells diluted over the range 10^{-2} to 10^{-4}. The intensity of the staining with these antibodies varied to some extent, with the VP1 (10^{-3}) and VP3 (10^{-4}) antibodies giving the highest sensitivities which were comparable in both Tampere and Exeter laboratories.

In situ hybridization

Both ISH methods (ViewRNA and RNAscope) demonstrated equal sensitivity, regardless of the probe used, detecting the virus at dilutions of 10^{-4} (Fig. 4). In the undiluted sample, almost all cells were EV-positive, and the number of positive cells decreased linearly as the ratio of CVB1-infected cells to uninfected cells was reduced.

Comparison of the sensitivity results between the methodologies is summarized in Table 1.

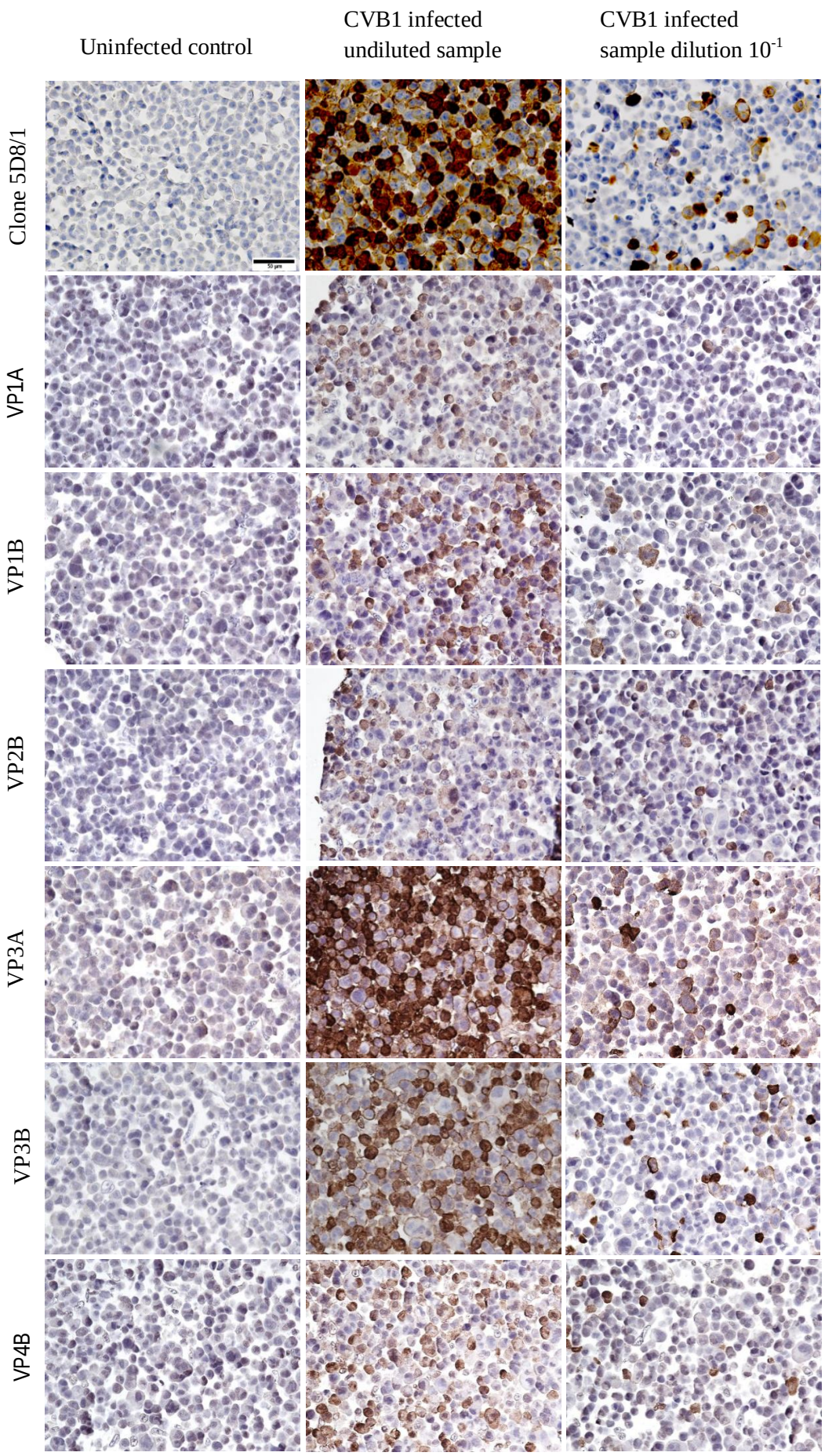

Uninfected control
CVB1 infected undiluted sample
CVB1 infected sample dilution 10^{-1}
Clone 5D8/1
VP1A
VP1B
VP2B
VP3A
VP3B
VP4B

Figure 3. Detection of CVB1 in infected A549 and uninfected A549 cells (FFPE) with different antibodies; commercial DAKO clone 5D8/1 and in-house anti-CVB4 antibodies VP1A, VP1B, VP2B, VP3A, VP3B and VP4B. Example micrographs of uninfected control, CVB1 infected undiluted sample and CVB1 infected dilution 10^{-1} are shown. 40X magnification. Scale bar = 50µm.

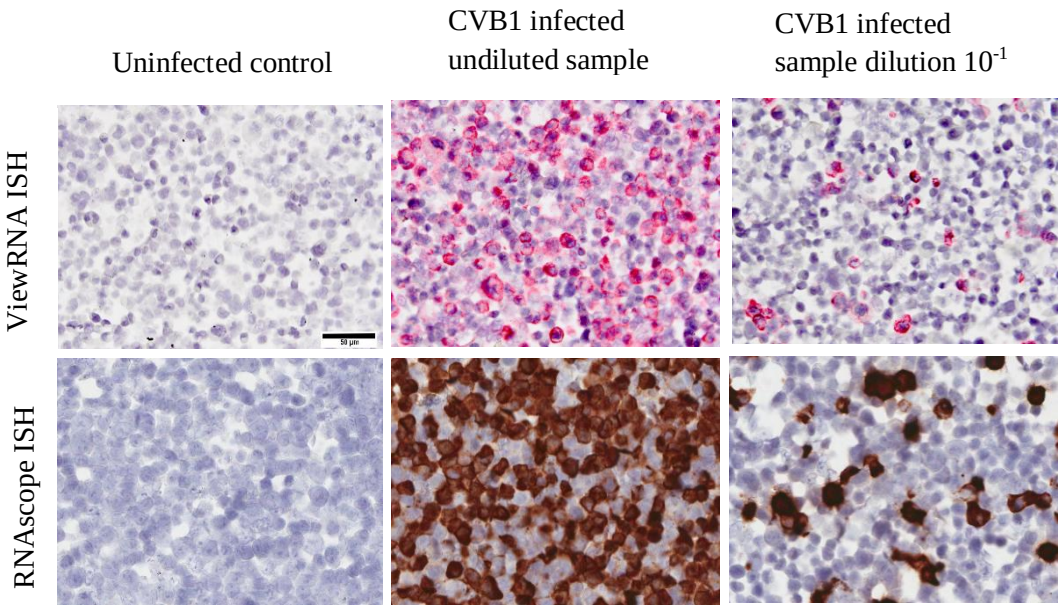


Figure 4. Detection of CVB1 in infected A549 and in uninfected A549 cells (FFPE) using two different commercially available ISH (ViewRNA and RNAscope) methods. Example micrographs of uninfected control, CVB1 infected undiluted sample and CVB1 infected dilution 10^{-1} are shown. 40X magnification. Scale bar = 50µm.

Table 1. Comparison of the sensitivity of different methodologies to detect CVB1 in A549 cells.

Method	Sensitivity (dilution)
RT-PCR (frozen cells)	
semi-nested (Uppsala)	10^{-8}
real-time (Tampere)	10^{-7}
Proteomics (frozen cells)	
LC/MRM/MS/MS	10^{-7}
MRM	10^{-7}
IHC (FFPE cells)	
anti-EV VP1: Clone 5D8/1	10^{-4} - 10^{-6}
anti-CVB4 VP1, -VP2, -VP3, -VP4	10^{-2} – 10^{-4}
ISH (FFPE cells)	
probes: EV AB[1], CVB1[2] (Affymetrix)	10^{-4}
probe: CVB1-6, CVB3 (RNAscope)	10^{-4}

[1] targets members of EV species A and B; [2] serotype-targeted probe

Discussion

The present study provides important information to guide the selection of assays capable of optimally detect EVs in infected cells. Although the conditions prevailing in mammalian cells infected *in vitro* do not completely resemble those in clinical tissue samples, the results provide a firm indication of the sensitivity and specificity of each method.

All methods tested were able to detect CVB1 with good sensitivity. However, depending on the method used, the detection limit varied and RT-PCR was found to be the most sensitive one.

The new LC/MRM/MS/MS technology demonstrated also high sensitivity, while its sensitivity might be still further improved by use of higher capacity columns that allow the loading of larger amounts of peptides. This technology has the particular advantage that identification and validation experiments are performed at the same time; the overlapping extracted ion chromatograms for the MRM transition pairs provide confirmation that the signals are derived from the same peptide and the MS/MS spectrum data can be used for protein identification in database searches. These data highlight the potential utility of using modern sensitive MS approaches to identify viral sequences with a relatively high sensitivity, suggesting that its applicability for virus detection in human samples should be evaluated in detail. The differences in sensitivities observed among the laboratories using IHC approaches with the same antibody, and also the laboratories employing ISH, could be explained by the low number of virus-positive cells present in the more diluted samples. Once these dilutions are reached, the actual number of virus-positive cells is very low (1-2 cells per field); thus, positivity may vary from one section to another when cells are plated for analysis. Importantly, the different antisera tested exhibited broadly similar profiles among the different laboratories with the 5D8/1 clone consistently demonstrating the highest sensitivity. ISH sensitivity depends on a number of variables including the affinity with which the relevant probe sets bind to the CVB1 genome. Therefore, we cannot conclude that IHC and ISH data have yielded absolute sensitivities in each laboratory, but rather they provide an indication of the sensitivity range of each method.

Each of the tested methods clearly has its own advantages. The proteomics-based LC/MRM/MS/MS provides important molecular information about the detected viruses based on

peptide sequences. We have also previously used mass spectrometry imaging[12] (MSI) to identify insulin and other proteins in pancreas tissue since the technology is useful for the identification and determination of the spatial distribution of molecules in tissues (16). The preservation of tissue morphology is a clear advantage of IHC and ISH, thereby making it possible to localize the virus in individual cells. ISH appeared to be less sensitive than IHC, but this varied according to the type of antibodies used in IHC. The main advantage of RT-PCR is its high sensitivity and the possibility to derive sequence information from the viral genome.

This study also has certain limitations. Firstly, it was based on infected cells and not ex-vivo tissue samples, and therefore the relative sensitivity of each assay might be different when tissues are examined. Secondly, a single EV strain was used and, theoretically, the binding of antibodies, probes and primers to different EV strains may differ. However, the used antibodies bind to several different EV serotypes and strains, the used PCR primers amplify practically all EVs, the used ISH techniques allow specific probes to be designed, enabling the detection of the virus of interest (6), and proteomics analyses are not dependent on the EV type, suggesting that other EV strains should give comparable results. Third, it is difficult to exclude the possibility that in spite of the repeated washes of the infected cells during the preparation of infected cell arrays, remnants of extracellular viral peptides and RNA may have remained in the samples. This could have led to overestimation of the PCR and proteomics sensitivity, which can detect both intracellular and extracellular viruses compared to IHC and ISH, which mainly detect intracellular viruses. In addition, one needs to consider the fact that the sensitivity of RT-PCR and proteomics may depend on the sample volume, while ISH and IHC methods detect the virus on a thin (5 µm) tissue section. Thus, the results should be put into the context of these limitations and the use of more than one of these assays is recommended to reach optimal sensitivity and specificity.

[12] MSI, mass spectrometry imaging

In conclusion, all methods proved suitable for the detection of EV in FFPE or frozen samples. The new proteomics technologies offer one of the most attractive alternatives for frozen tissues, being relatively sensitive and providing sequence information about the detected virus. On the other hand, the new non-radioactive ISH methods work well in FFPE samples. Even if IHC and proteomics were relatively sensitive, RT-PCR remains one of the most sensitive methods when frozen or fresh samples are available. Importantly, this effort was launched as part of the collaborative efforts of the JDRF nPOD Working Group, and these results are guiding virus analyses of pancreas specimens collected from T1D patients.

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Conflict of Interest: HH is a minor (<5%) shareholder and a member of the Board in Vactech Ltd., which develops vaccines and diagnostic assays for picornavirus infections.

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