Comparing the selective and co-selective effects of different antimicrobials in bacterial communities

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Abstract

Bacterial communities are exposed to a cocktail of antimicrobial agents, including antibiotics, heavy metals and biocidal antimicrobials such as quaternary ammonium compounds (QACs). The extent to which these compounds may select or co-select for antimicrobial resistance (AMR) is not fully understood. In this study, human associated, wastewater derived, bacterial communities were exposed to either benzalkonium chloride (BAC), ciprofloxacin or trimethoprim at sub-point of use concentrations for one week, in order to determine selective and co-selective potential. Metagenome analyses were performed to determine effects on bacterial community structure and prevalence of antibiotic resistance genes (ARGs) and metal or biocide resistance genes (MBRGS). Ciprofloxacin had the greatest co-selective potential, significantly enriching for resistance mechanisms to multiple antibiotic classes. Conversely, BAC exposure significantly reduced relative abundance of ARGs and MBRGS, including the well characterised *qac* efflux genes. However, BAC exposure significantly impacted bacterial community structure. This suggests BAC and potentially other QACs did not play as significant a role in co-selection for AMR relative to antibiotics such as ciprofloxacin at below point of use concentrations in this study. This approach can be used to identify priority compounds for further study, to better understand evolution of AMR in bacterial communities exposed to sub-point of use concentrations of antimicrobials.

Keywords: antibiotic; antimicrobial; biocide; quaternary ammonium compound; resistance; evolution; metagenomics

1. Introduction

Antimicrobial resistance (AMR) occurs naturally in a variety of environments [1]; but anthropogenic use, overuse and misuse of antibiotics and other antimicrobials has selected for

increased levels of resistance [2]. Direct selection for AMR can arise when bacteria are exposed to a single compound; for example, exposure to ciprofloxacin can result in increased numbers of bacteria harbouring a *gyrA* mutation which confers resistance to ciprofloxacin [3]. Conversely, co-selection is indirect selection that can occur via two mechanisms: cross-resistance or co-resistance [4]. Cross-resistance occurs when one resistance gene can confer resistance to many antimicrobials [4]. For example, the *qac* resistance genes encode multidrug efflux pumps with the ability to efflux many different quaternary ammonium compounds (QACs) [5]. Therefore, exposure to one of these compounds would result in selection for the efflux gene. Co-resistance is when a resistance gene will be maintained / selected if it is genetically linked to another gene (though not necessarily a resistance gene) which is under positive selection [4]. *Qac* genes may also be co-selected via co-resistance as they are often located on integrons, which in turn can carry a vast diversity of antibiotic resistance genes [6, 7].

There are two main types of antimicrobial agents. Antibiotics are used therapeutically and prophylactically in humans and animals; and as growth promoters in animal husbandry in some parts of the world. Antibiotics are not fully metabolised by humans and animals, and in some cases >90% of an antibiotic can be excreted in an active form [8]. Other compounds with antimicrobial effects include biocides such as QACs and heavy metals. QACs are used widely for equipment sterilisation, product preservation and surface decontamination in a variety of settings including in hospitals, farms and in the household [9]. Heavy metals, though required by most bacteria for growth, are toxic at high concentrations. Heavy metals are used in animal feed [10], antibacterial products such as wound dressings [11] and they can accumulate in the environment due to industrial contamination [12]. In theory, each antimicrobial has the potential to co-select for resistance to another.

Antibiotics concentration gradients exist within human, animal and environmental microbiomes from point of use until they are diluted to extinction. Several studies have indicated sub-inhibitory concentrations of antibiotics exhibit biological effects and can even select for AMR [3, 13-15], but few have experimentally looked at co-selective effects. The selective and co-selective effects of different antimicrobials at below point of use concentrations levels have not previously been compared within bacterial communities. In this study, we exposed a wastewater derived bacterial community (includes gut microbiome bacteria and the WHO critically important *Enterobacteriaceae* [16], from a large number of individuals) to either the QAC biocide benzalkonium chloride (BAC), ciprofloxacin or trimethoprim at below point of use concentrations in serial passage experiments for 7 days. BAC was chosen on the basis it would likely co-select for resistance via cross-resistance and co-resistance via the *qac* multidrug efflux genes. Ciprofloxacin was included in this study as it has been previously shown to be selective at sub-inhibitory concentrations [3]. Finally, trimethoprim was chosen as *dhfr* genes are of the most common antibiotic resistance genes associated with class 1 integrons [6], and so may co-select for integron-borne resistance via co-resistance.

Metagenome analyses of communities exposed to these antimicrobials were performed to determine effects on bacterial community structure and prevalence of antibiotic resistance genes (ARGs) and metal or biocide resistance genes (MBRGs). Our findings indicate BAC was not a potent co-selective compound in this experimental system, unlike ciprofloxacin. We identified potentially important gene targets for tracking QAC resistance, in addition to the most well-studied *qac* efflux genes. Finally, results illustrated the potential for metagenome analyses to identify priority antimicrobial compounds for further study, on the basis of their selective and co-selective potential and corresponding threat to human health.

2. Materials and Methods

2.1. Evolution experiment

Untreated wastewater was collected from a sewage treatment plant (population equivalent of 43,000) in October 2015 and frozen at 50 % v/v in 40 % glycerol at 80°C until use. Frozen samples underwent two steps of centrifugation (3,500 *x g* for 10 minutes) and resuspension in equal volume 0.85 % sterile saline to minimise chemical and nutrient carry over.

There were 3 replicate microcosms for each antimicrobial. Compounds used were BAC (8 mg/L), ciprofloxacin (0.5 mg/L) and trimethoprim (2 mg/L) at half the clinical breakpoint concentrations [17] for *Enterobacteriaceae* (ciprofloxacin and trimethoprim) or half the MIC of the susceptible K12 *Escherichia coli* strain (BAC), as determined by the standard MIC plating method [18]. This was based on the assumption that a significant portion of the human derived waste water would include this family of bacteria, and based on a previous study in the same experimental model system where *E. coli* was the prominent detected species [19]. Antimicrobial-amended microcosms (n=3 per antimicrobial, with n=3 antimicrobial free control) comprising of 5ml Iso-sensitest broth (Oxoid) and 1 % v/v processed wastewater sample were incubated overnight at 37 °C, shaking at 180 rpm.

Each day, 1 % v/v of culture was inoculated into fresh, antimicrobial-amended media. This was repeated for a total of 6 days. On the 7th day, 1 ml culture was centrifuged (21,000 *x g*) for 2 minutes, resuspended in equal volume of 20 % glycerol, and stored at -80 °C.

2.2. DNA extraction, clean up and sequencing

Total bacterial DNA was extracted using the MoBio ultraclean kit, according to instructions but with the initial spin extended to 3 minutes. All DNA was stored at -20 °C until use.

DNA was cleaned and concentrated using Ampure™ beads, as previously described [20]. Nextera XT libraries were prepared and sequenced on the Illumina HiSeq 2500 platform by Exeter Sequencing Service (ESS), generating 300 bp paired end reads.

2.3. Metagenome analyses

Successful removal of adaptor sequences and low quality reads was performed with Skewer [21] and confirmed with MultiQC [22] before and after trimming. Number of reads for each sample after trimming are reported in the Supplementary Data (Table S1).

Extraction and analyses of 16S rRNA sequences were performed as described previously [20]. Briefly, reads were paired with FLASH version 2 [23] and 16S rRNA reads were extracted with MetaPhlan2 [24]. Community diversity visualisation was performed with HClust2 [25] using Bray Curtis distance measurements between samples and features (species). Biomarker species / genera were identified with LEfSe (linear discriminant analysis effect size) [26].

ARGs were identified with the ARGs-OAP pipeline, which identifies ARGs at the antibiotic class and within class level, and normalises these hits to both the length of the ARG itself and either parts per million, 16S rRNA copy number or cell number to derive a ARG relative abundance [27]. The default cut-off values for ARG assignment were used (25 amino acid, e-value of 1e-07 and 80% identity). MBRGs were identified through BacMet Scan against the experimentally confirmed BacMet database, using default search parameters and

cut off values [28]. All ARGs and MBRGs hits were normalised to hits per million reads. Heatmaps were generated using various python packages [29-31].

2.4. Statistical methods

Normally distributed data were analysed with parametric one-way ANOVA and Tukey post hoc tests. Non-normally distributed data unable to be transformed with log or square root functions into a normal distribution underwent non-parametric Kruskal Wallis and Dunn's tests. *P* values for the post-hoc Tukey test or Dunn's test are reported. Spearman's rank correlation between hits of ARGs and MBRGs per million reads determined whether a positive or negative correlation existed for all three tested antimicrobials.

3. Results

3.1. Effects on community structure

A wastewater bacterial community was exposed to sub-point of use concentrations of either BAC (8 mg/L), ciprofloxacin (0.5 mg/L) or trimethoprim (2 mg/L) equating to half the BAC MIC for susceptible *E. coli* and half the clinical breakpoint for the two antibiotics [17]. Metagenome analyses were performed on biological replicates for each of the antimicrobial treatments and from the cultured control. The top 25 detected species for each replicate are shown in Figure 1 (for all detected species, see Figure S1).

There were 26 – 62 bacterial species detected in total across treatments (Figure 1 and Figure S1). These included mostly facultative anaerobes as well as some microaerophilic bacteria. Linear Discriminant Analysis Effect Size ('LEfSe') identifies 'features' (such as bacterial species or genera) which can be used to highlight differences between, for example, experimental treatments, different body sites or environments by combining statistical significance testing with tests which consider biological consistency and effect relevance.

169 [26] (Table S2). LEfSe was used to identify species significantly associated with different
170 treatments (species 'biomarkers'). LEfSe defined the control treatment as having the greatest
171 number of biomarker bacterial genera, with *Streptococcus, Staphylococcus, Acinetobacter,*
172 *Eggerthella, Enterobacter* and *Cronobacter* species all significantly associated with the
173 control treatment, indicating equal representation of Gram negative and Gram positive
174 biomarker genera (Table S2).

175 BAC had the greatest effect on community structure, resulting in complete loss of 18
176 species relative to the control (Figure 1). Only 5 of the original 28 bacterial genera persisted
177 in BAC treatments; only Gram negative genera including *Citrobacter, Escherichia,*
178 *Klebsiella, Morganella* and *Pseudomonas. P. aeruginosa, K. pneumoniae* and *M. morganii*
179 were determined as biomarkers in the BAC treatment (Figure S1). Interestingly, the
180 opportunistic pathogen *P. aeruginosa* was below the limit of detection in the control
181 treatment, but was enriched to a high abundance in the BAC treatment indicating strong
182 selection for this often intrinsically resistant organism. Only two bacterial genera were
183 biomarkers for the ciprofloxacin treatment, namely *Escherichia* and *Lactobacillus* (Table S2).
184 Trimethoprim had three biomarker genera, including *Veillonella, Bacteriodes* and
185 *Bifidobacterium* (Table S2). Therefore unlike the BAC treatment, some Gram positive
186 bacteria persisted following ciprofloxacin and trimethoprim exposure.

187 Generally, *E. coli* were the most abundant species in control and antibiotic treatments,
188 though ciprofloxacin and trimethoprim exposure resulted in slight decreases in *E. coli*
189 abundance compared to the control. In the BAC treatment, *E. coli* relative abundance was
190 much lower and only detected in a single treatment replicate (Figure 1).

191

192 **3.2. Co-selective potential of different antimicrobials for ARGs**

193 The ARGs-OAP pipeline [27] was used to identify ARGs within all treatment replicates.
194 ARG hits were normalised to number of hits per million reads and summed per antimicrobial
195 treatment. The total number of ARG hits was highest following ciprofloxacin exposure, with
196 replicate number 3 having >18,000 ARG hits per million reads (Table S3). Overall, total
197 number of ARG hits per million reads was significantly different to the control ($p = 0.02$,
198 Figure 2). However, the sum of ARGs in both BAC and trimethoprim treatments were not
199 significantly different to the control.

200 Multi-drug resistance mechanisms were the most abundant type of resistance
201 mechanism in all treatments (Figure S2), but there was little variability between treatments.
202 Ciprofloxacin was the most co-selective antimicrobial of the three tested, as significant
203 enrichment for aminoglycoside ($p = 0.011$), beta-lactam ($p = 0.016$), chloramphenicol ($p =$
204 0.019), macrolide-lincosamide-streptogramin ('MLS', $p = 0.035$), sulphonamide ($p = 0.033$),
205 trimethoprim ($p = 0.035$) and vancomycin ($p = 0.023$) compared to the control (Figure 3).
206 Conversely, no significant increases in any ARGs were observed following BAC treatment.
207 Rather, BAC treatment resulted in significant decreases in multidrug resistance genes ($p =$
208 0.029) and genes conferring resistance to other antibiotics ($p = 0.008$). Trimethoprim had
209 little effect on relative abundance of ARGs, with the only significance increase observed for
210 chloramphenicol resistance genes ($p = 0.046$) (Figure 3).
211 Surprisingly, ARGs conferring resistance to ciprofloxacin or trimethoprim were not
212 significantly enriched following exposure to either compound (e.g. significant enrichment of
213 quinolone ARGs was not observed following ciprofloxacin exposure).
214

215 **3.3. Co-selective potential of different antimicrobials for MBRGs**

216 Finally, all metagenomes were screened against the experimentally confirmed BacMet
217 database [28], which contains MBRGs. Again, total numbers of MBRG hits were normalised

per million reads. There was a statistically significant positive relationship between total numbers of ARG and MBRG hits ($r = 0.91$, df = 9, $p < 0.0001$). The only significant difference for the sum of MBRGs after antimicrobial treatment compared to the control was for BAC, where total numbers of MBRGs actually decreased significantly compared to the control ($p = 0.007$, Figure 2).

This was an interesting finding, combined with the lack of selection for ARGs. There are only three *qac* genes in the ARGs-OAP database [27], and so it was expected that there would be a greater number of hits for *qac* genes and other QAC resistance mechanisms when searched against the BacMet database [28]. Currently in the BacMet database, there are 64 experimentally confirmed BAC resistance mechanisms, 13 of which have been found on plasmids. Plasmid-encoded genes include *oqxA*, *oqxB*, all *qac* genes, and *sugE* (BacMet search, 7th March 2018). We compared the total number of *qac* genes, *oqxA/B* or *sugE* genes between treatments and found that ciprofloxacin significantly enriched for *qac* genes ($p = 0.034$). Total hits for *oqxA/B* genes were significantly lower following ciprofloxacin exposure compared to the control ($p = 0.016$). The only plasmid-borne BAC resistance genes which increased in relative abundance following BAC exposure were the *oqxA/B* genes, though this was not significant. Surprisingly, *qac* genes and *sugE* genes decreased in relative abundance following treatment with BAC; though again these differences were not significant. There were no significant differences in total number of hits for *qac*, *sugE* or *oqx* genes between the control and trimethoprim treatment.

Detected chromosomally-encoded BAC resistance genes and their total number of hits were also investigated (Table 1). Only 6 of a possible 51 chromosomally-encoded BAC resistance genes were detected in any treatment in this study. The *acrE/envC* and *acrF/envD* efflux systems were common across all treatments and formed the largest portion of chromosomal BAC hits. Also detected were the *abeS* and *adeT1* genes which encode efflux

pumps; these were found in the control treatment only and a single replicate of the BAC treatment, respectively. The *cpx* genes *cpxA* and *cpxR* were found in 2 BAC replicates and 1 trimethoprim replicate. When examining total hits for detected chromosomally-encoded BAC resistance genes, there was a significant decrease in hits in the BAC treatment compared to the control ($p = 0.033$).

4. Discussion

Bacterial communities are exposed to a variety of antimicrobial compounds. Previous observational studies have found correlative evidence which suggest QACs co-select for antimicrobial resistance in QAC polluted environments [32, 33]. More recent experimental studies have observed direct selection for QAC resistance in bioreactors of bacterial communities exposed to BAC [34], but did not investigate AMR co-selection. Currently, there are no studies which have examined the potential for biocides, such as QACs, to co-select for antibiotic resistance in bacterial communities and compared this to direct selection by antibiotic exposure.

Our findings agree with previous results, in that BAC exposure has significant effects on bacterial community structure, resulting in competitive exclusion of susceptible bacteria and clonal expansion of a few resistant bacterial species [34]. At the end of this study, all bacteria in BAC treatment replicates were Gram negative and comprised of only 8 detected species. *E. coli* were almost fully outcompeted and were detected in only a single replicate, even though the exposure concentration was half of the MIC for a susceptible *E. coli* lab strain. Though the starting inoculum metagenome was not sequenced in this study, the no antimicrobial control treatments control for potential effects on the community. Further studies should increase the sequencing frequency, so such dynamics can be better understood.

Previous work suggests QACs have a high predicted co-selective potential compared to other biocides and heavy metals, due to close genetic proximity of additional resistance mechanisms which could be co-selected by co-resistance [35]. We show for the first time through an experimental approach that the majority of ARGs and MBRGs are lost following BAC exposure at half the MIC for susceptible *E. coli* (relative to the control). There are two likely explanations for loss of ARGs / MBRGs, which are not mutually exclusive. The first is that the resistance gene sequences enriched by the BAC treatment are not currently deposited in the ARGs-OAP and BacMet databases. The second possibility is that BAC at 8 mg/L enriches for intrinsically resistant organisms, which outcompete susceptible organisms including those harbouring mobile resistance mechanisms (which may have increased fitness costs). The latter of these scenarios is supported not only by the enrichment of solely Gram negative bacteria, which generally have elevated levels of resistance to QACs compared to Gram positive bacteria; but by the analysis of mobile QAC resistance mechanisms (i.e. plasmid borne genes). There were no significant differences in the relative abundance of mobile QAC resistance genes between BAC and control treatments, including the well-characterised *qac* resistance genes. This finding indicates the need for continued efforts to identify potentially novel resistance genes which confer QAC resistance, as *qac* genes may not be as significant in QAC resistance as the literature suggests. In addition, it indicates the potential utility that bacterial community analyses combined with ARGs/MBRG mining can provide in determining the selective and co-selective potential of different antimicrobials. Intrinsically resistant organisms pose a considerably reduced risk to human health compared to bacteria which can readily transfer resistance, as their resistance mechanisms are not readily mobilisable. Therefore, a metagenome approach can be used to prioritise antimicrobials in terms of their potential exposure and therefore human health risk through a combination of: identifying compounds with strong selective potential, compounds which

readily co-select for many types of different resistance mechanisms, and whether these resistance mechanisms are likely to be harboured by intrinsically resistant organisms (indicated by lack of community diversity).

While BAC exposure resulted in loss of ARGs and MBRGs, ciprofloxacin treatment enriched relative abundance of ARGs to 7 different antibiotic classes. Resistance genes detected in this study are not necessarily all being expressed, however this is not relevant for co-resistance (i.e. co-location) of genes on the chromosome or on mobile genetic elements. Our results combined with findings from other studies (that have shown selection can occur at very low concentrations of ciprofloxacin [3]) together demonstrate the high selective and co-selective potential of ciprofloxacin and suggest further research on this antibiotic is required.

Trimethoprim exposure resulted in significant enrichment of only chloramphenicol resistance genes. Interestingly, trimethoprim resistant species such as *Pseudomonas aeruginosa* were not selected for, indicating their relative fitness in these community was low compared to other resistant bacteria. Neither of the antibiotics directly selected for their own previously described resistance mechanisms (as in, ciprofloxacin exposure did not result in significant increases in relative abundance of quinolone ARGs, nor trimethoprim in relative abundance of trimethoprim ARGs). This indicates a high abundance of genes conferring cross-resistance to more than one compound; presence of intrinsically resistant organisms; and/or an incomplete database. Functional studies aiming to identify novel resistance genes and their complete antimicrobial susceptibility profiles are still critical for improving our understanding of selection and co-selection. New techniques such as emulsion, paired isolation and concatenation (EPIC) PCR [36] could be used to discern if resistance genes which are being selected for are present in only a few species (indicating selection of that

316 species), or if they are widespread throughout the bacterial population indicating potential for
317 horizontal gene transfer.
318
319
320 ## 5. Conclusions
321 In summary, this study compared selective and co-selective effects of different antimicrobials
322 at below point of use concentrations for the first time. Results indicate that QACs such as
323 BAC may exert relatively low selective pressure for AMR development in bacterial
324 communities, relative to antibiotics although this may be in part due to a high diversity of
325 uncharacterised resistance genes. Ciprofloxacin was shown to be the most selective
326 compound tested, and should be prioritised for further study to investigate the risk for
327 selection and co-selection occurring in a variety of settings. A metagenome approach to
328 quantify the risk of selection for AMR can be useful to identify additional priority
329 compounds based on their selective and co-selective potential, and whether this resistance is
330 likely to be readily mobilisable.
331
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340

7. References

Figure legends

Figure 1. Heatmap showing the 25 species with highest relative abundance for each biological replicate within each antimicrobial treatment, as determined with MetaPhlan2, using Bray-Curtis distance measurements for samples and features (species).

Figure 2. Total number of ARG/MBRG hits normalised per million reads (detected with ARGs-OAP and BacMetScan, respectively), averaged within treatment (n=3 for antimicrobial treatments, n=2) for the control. Significant differences relative to the control: + = significant increase in numbers of hits (p <0.05), * = significant decrease in numbers of hits (p <0.05).

Figure 3. Heatmap showing average relative abundance of ARG hits (antimicrobial treatments n=3, control n=2) detected for different antibiotic classes with the ARGs-OAP pipeline. Numbers of hits are normalised per million reads. 'MLS' = Macrolide-Lincosamide-Streptogramin resistance. Multi-drug resistance hits are excluded due to extremely high abundance (Figure S2).

Table legends

Table 1. Total number of experimentally confirmed, chromosomally encoded BAC resistance gene hits detected in this study with BacMetScan, normalised against per million reads. Hits are average within antimicrobial treatments (n = 3 for antimicrobials, n = 2 for control). * = significantly greater number of hits; ** = significantly reduced number of hits, relative to the control. ND = Not detected. [1]= detected in biological replicate 1 only.

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