Title

Comparative metagenomics reveals a diverse range of antimicrobial resistance genes in effluents entering a river catchment

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Abstract

The aquatic environment has been implicated as a reservoir for antimicrobial resistance genes. In order to identify sources that are contributing to these gene reservoirs, it is crucial to assess effluents that are entering the aquatic environment. Here we describe a metagenomic assessment for two types of effluent entering a river catchment. We investigated the diversity and abundance of resistance genes, mobile genetic elements and pathogenic bacteria. Findings were normalised to a background sample of river source water. Our results show that effluent contributed an array of genes to the river catchment, the most abundant being tetracycline resistance genes *tetC* and *tetW* from farm effluents and the sulfonamide resistance gene *sul2* from wastewater treatment plant effluents. In nine separate samples taken across three years we found 53 different genes conferring resistance to 7 classes of antimicrobial. Compared to the background sample taken up river from effluent entry, the average abundance of genes was three times greater in the farm effluent and two times greater in the wastewater treatment plant effluent. We conclude that effluents disperse antimicrobial resistance genes, mobile genetic elements and pathogenic bacteria within a river catchment, thereby contributing to environmental reservoirs of antimicrobial resistance genes.

Key words 3-6

Antimicrobial resistance, metagenomics, aquatic environment, resistome

Introduction

Antimicrobial resistance remains a significant and growing concern for both human and veterinary clinical practice (Levy and Marshall 2004, Davies and Davies 2010), with infections that were once readily treated now being resilient to antimicrobial therapy (WHO 2012). The use of antimicrobial compounds exerts selection pressures on bacteria, leading to the fixation of gene mutations, selection of resistant precursors and the up-regulation and lateral transfer of antimicrobial resistance genes (ARGs) within prokaryotic communities (Gillings 2013). The maintenance and transfer of ARGs is responsible in part for the rising threat of antimicrobial resistance (Laxminarayan et al. 2013).

The collective pool of ARGs in a given environment is termed the resistome (D'Costa et al. 2006, Wright 2007). Although a proportion of these ARGs are genes that have evolved to utilise antimicrobial compounds for functions other than defence, such as signalling molecules or constituents of metabolic pathways (Linares et al. 2006, Dantas et al. 2008), the resistome may also serve as a reservoir for ARGs that can be transferred to clinically significant pathogens (Forsberg et al. 2012, Wellington et al. 2013). Indeed, ARGs are commonly associated with Mobile Genetic Elements (MGEs) that facilitate the transfer of ARGs between bacteria and enable their entry into the accessory genome of pathogenic bacteria (William et al. 2013).

There is growing evidence showing that aquatic environments harbour ARGs, MGEs and pathogenic bacteria (Chen et al. 2013, Lu et al. 2015, Devarajan et al. 2015). It is also likely that these environments may host many uncharacterised and novel ARGs that may be selected for under sufficient selection pressures (Bengtsson-Palme et al. 2014). Effluents that feed into the aquatic environment have also been shown to contain ARGs, such as the effluents of urban residential areas and hospitals (Li et al. 2015), as well as other wastewater and faecal sources (Li et al. 2012, Pruden et al. 2006, Zhang et al. 2009) but the abundance and diversity of these genes relative to background samples needs to be clarified. It is therefore crucial to establish whether effluents entering the aquatic environment are carrying ARGs, along with MGEs and pathogenic bacteria, thus contributing to the reservoirs of resistance genes that may be utilised by pathogenic bacteria and subsequently re-enter human and animal populations (Berendonk et al. 2015).

Previous studies into the presence of ARGs within the aquatic environment have utilised techniques such as bacterial culture and polymerase chain reaction (Lu et al. 2015, Tao et al. 2010, Zhang and Zhang 2011). These techniques offer the ability to detect phenotypic resistance (culture), or a panel of ARGs, but they are limited by culturing bias or inadequate detection panels. Next generation sequencing techniques, such as metagenomics, offer the ability to circumvent these limitations and identify all known ARGs within a sample (if suitable reference sequences are available), providing a new approach for the environmental monitoring of antibiotic resistance (Port et al. 2014).

In this study we have identified two distinct effluents that enter a single river catchment. Both effluents originate from faecal sources and were sampled several times, immediately prior to them entering the environment. Using a comparative metagenomic approach, we describe the ARG content of these effluents, characterise the MGEs and pathogenic bacteria present, and relate the abundance of these features to a background sample of the river source water, taken from upstream of the effluent entry points.

Methods

Sample collection and DNA sequencing

Water samples were collected from three sources within the River Cam Catchment, Cambridge, UK. A pilot collection was made on 21st June 2012 (Rowe et al. 2015). Further collections were made on the 2nd May 2013 and 4th August 2014. The effluent of the municipal wastewater treatment plant (WWTP) (latitude: 52.234469, longitude: 0.154614) was collected annually from the treated effluent discharge pipe that enters the River Cam. The effluent of the University of Cambridge dairy farm (latitude: 52.22259, longitude: 0.02603) was collected annually prior to it being applied to the surrounding fields as fertiliser, where it subsequently enters drainage ditches that drain into the River Cam. The river source water of the River Cam was collected at Ashwell Spring (latitude: 52.0421, longitude: 0.1497) once on the 4th August 2014. Samples were collected in 10L sterile polypropylene containers, transported at 4°C to the laboratory and processed within 2 hours.

Sample filtration, metagenomic DNA extraction and sequencing

Similarly as in Dancer et al. (Dancer et al. 2014), samples were filtered under pressure at approximately 2 bar using a pressure vessel system (10 L SM 1753, Sartorius). Samples were first pre-filtered through 3.0 µm membranes (Millipore) at 2 Bar to remove eukaryotic cells and debris. The filtrate was subsequently filtered through 0.22 µm membranes (Millipore) to capture the prokaryotic cells, metagenomic DNA was then extracted by washing and vortexing the membranes in phosphate buffered saline with Tween20 (2%) before enzymatic lysis (Meta-G-Nome DNA isolation kit; Epicentre). Assessment of DNA quality and concentration was made by TBE agarose (2%) gel electrophoresis and spectrophotometry (Nanodrop ND-1000; ThermoScientific). For each sample, 2 µg of DNA was used to generate Illumina paired-end libraries that were sequenced using an Illumina HiSeq2500. A full description of the metagenomic samples used in this study is available in the supplemental material (Table S1.)

Bioinformatic analyses

Identification of ARGs

ARGs were identified using the Search Engine for Antimicrobial Resistance (SEAR) (Rowe et al. 2015). In brief, the pipeline quality checks and filters metagenomic reads, clusters the filtered reads to the ARG-annot (Gupta et al. 2014) database of horizontally acquired ARGs and uses the resulting clusters to map the reads and generate a consensus sequence for each ARG in the query metagenome. Consensus sequences are then aligned to online databases (NCBI genbank, RAC, ARDB), annotated and given an abundance value based on the Reads Per Kilobase per Million (RPKM) value from the read-mapping stage. A full description of SEAR is available in supplemental methods.

Identification of mobile genetic elements

MGEs were identified by mapping metagenomic reads to a custom MGE database using BWA-mem (default options) (Li and Durbin 2009). The MGE database was built from the NCBI Refseq plasmid genomes dataset, combined with the representative sequences generated from clustering the Integrall dataset (Moura et al. 2009) at 97% identity using USEARCH (Edgar 2010). MGE mapping results with less than 90% coverage of the reference sequence were discarded from the analysis. Successfully mapped sequences where then binned into class I and class II integrons, transposons and mobilisable plasmids.

Abundance analysis

The ARG and MGE abundance data was normalised to the number of 16S rRNA sequences as in Bengtsson-Palme et al. (Bengtsson-Palme et al. 2014). In brief, bacterial 16S rRNA sequences were extracted from each metagenome using Metaxa 2.0 (Bengtsson-Palme et al. 2015) using default settings and then grafted to sequences from the SILVA RNA database using Megraft (Bengtsson et al. 2012) and subsequently clustered using USEARCH (Edgar 2010). ARG abundance values were normalised to 16S sequences by dividing the number of extracted 16S sequences by the length of the 16S gene (Bengtsson-Palme et al. 2014).

Taxonomic profiling and pathogen detection

Taxonomic profiling of metagenomes was carried out by mapping sequencing reads to clade-specific marker genes using the Metaphlan package (Segata et al. 2012) (default parameters). Metaphlan output was then cross-referenced to the PATRIC database of pathogenic bacteria (Gillespie et al. 2011) to annotate potential human-specific bacterial pathogens. Biomarker discovery and identification of differentially abundant features between metagenomes from 2012, 2013 and 2014 was performed using LEfSe (Segata et al. 2011). Taxonomic profiling and pathogen data was then combined and presented using the Graphlan package (Segata 2014).

Results

Metagenome analysis

We generated 29.52 Giga base-pairs of data across all samples, with the number of reads produced from the total farm effluent samples being approximately double that produced from the total WWTP effluent samples (Table 1).

Table 1. Summary of the metagenomes generated in the present study.

Sample	Read pairs	Gbp	Total ARG reads	% ARGs	% 16S
Farm effluent 2012	44337147	4.4337	7715	0.0087	0.1199
Farm effluent 2013	33060321	3.3060	2317	0.0035	0.0396
Farm effluent 2014	92074704	9.2075	13094	0.0071	0.0775
WWTP effluent 2012	28696239	2.8696	4205	0.0073	0.1086
WWTP effluent 2013	32980301	3.2980	250	0.0004	0.0366
WWTP effluent 2014	36636758	3.6637	3767	0.0051	0.0862
River source water 2014	27399641	2.7400	181	0.0003	0.0201

Identification of antimicrobial resistance genes

In the effluent from the dairy farm we found an average of 7709 reads (0.007%) matching ARGs across the three samples. We found an average of 2740 reads (0.004%) matching ARGs across the three WWTP effluent samples. Only 181 reads (0.0003%) were found to match ARGs from the river source water. A significant diversity of ARGs was observed across the samples, with 53 different ARGs found in total, conferring resistance to seven antimicrobial classes (Figure 1, Table S2). There were 18 ARGs common between the farm and the WWTP effluent samples. The river source water contained the lowest diversity of ARGs (five ARGs, conferring resistance to two antimicrobial classes). When normalised to the number of 16S sequences in each sample, the most abundant ARG across all the samples was found to be *sul2* (sulfonamide resistance) in the WWTP effluent 2014 (0.097 copies per

16S sequence) and the least abundant ARG was *catB4* (phenicol resistance), found in the farm effluent 2014 (0.0001 copies per 16S sequence). When looking at the effluents individually, tetracycline resistance genes *tetC* (farm effluent 2012) and *tetW* (farm effluent 2013 and 2014) were the most abundant genes within the farm effluent samples. In comparison, the aminoglycoside resistance genes *strA*/*strB* (WWTP effluent 2012) and the sulfonamide resistance genes *sul1*/*sul2* (WWTP effluent 2013 and 2014) were the most abundant ARGs within the WWTP effluent samples. On average, the abundance of ARGs in the farm effluents was three times that of the river source water. Similarly, the average abundance of ARGs in the WWTP effluents was double that found in the river source water. In terms of the diversity of ARGs relative to the river source water, the farm effluent had an average of five different ARGs for each ARG found in the river source water, whereas the WWTP effluent had different 2 ARGs for each ARG present in the source water.

When comparing samples across the three years that the samples were taken, the abundance of ARGs was found to decrease year on year in the WWTP effluent for all but sulfonamide resistance genes, which were found to increase over time (11% average change in abundance of sulfonamide resistance genes over three years). The largest change over time for the farm effluent was the 10% increase in the abundance of aminoglycoside resistance genes observed between 2012-2013.

Figure 1. Abundance of ARGs found in each effluent sample, binned by antimicrobial class.
Abundance of antimicrobial resistance genes is normalised to the number of 16S sequences per sample. The MLS class of antimicrobial represents marcolides, lincosamides and streptogramins.

Identification of mobile genetic elements

In conjunction with determining the abundance and diversity of ARGs, the effluents were also interrogated for MGEs (Figure 2, Table S3). No MGEs were found to be present in the river source water. Mobilisable plasmids were the most abundant class of MGE found out of the combined metagenomic datasets, although no mobilisable plasmids were identified in the WWTP effluent 2012 or farm effluent 2014 samples. Class I and class II integrons, as well as transposon sequences, were found in all effluent samples. Class I integrons were more abundant in the collective farm effluent samples, compared to class II integrons that were more abundant in the collective WWTP effluent samples.

Figure 2. Abundance of MGEs found in each effluent sample, binned by MGE type.
Plasmids were binned as mobilisation plasmids if they contained conjugation genes (tra, mob etc.) and integrons were binned as class I or II depending on the Integrall annotation. Relative abundance of MGEs is normalised to the number of 16S sequences per sample.

Taxonomic profiling and pathogen detection

Finally, the effluent metagenomes were subjected to taxonomic profiling. At genus level, the most abundant prokaryotes in the farm samples were *Pseudomonas* (farm effluent 2012) and *Butyrivibrio* (farm effluent 2013 and 2014). The most abundant prokaryotes at genus level in the WWTP samples were *Acinetobacter* (WWTP effluent 2012), *Thiomonas* (WWTP effluent 2013) and *Proteus* (WWTP effluent 2014). For the river source water, the most abundant prokaryotic genus was *Sphingobium*. After cross-referencing the identified species level, clade-specific marker genes for all the metagenomes to the PATRIC pathogen database, a total of 35 species of potential bacterial pathogens were identified (Figure 3, Table S4). The

most commonly identified species were *Escherichia coli*, *Arcobacter butzleri*, *Eubacterium rectale*, *Ruminococcus bromii* and *Salmonella enterica*. The WWTP effluent 2014 contained the greatest diversity of potential bacterial pathogens, whereas the river source water and the WWTP effluent 2012 were found to contain the lowest diversity.

Figure 3. Metagenomic phylogenetic analysis and annotation of potential bacterial pathogens.
The phylogenetic tree was built using Graphlan from the merged Metaphlan and LEfSe output for the effluent metagenomes. The PATRIC pathogens are highlighted as red stars and the external rings denote species prevalence in each metagenome.

Discussion

Through the use of a comparative metagenomic approach, we have shown that two types of effluent entering a shared river catchment contain ARGs and MGEs at higher average abundances than in a background sample of the river source water. This would suggest that effluents such as these are likely to serve as sources of ARGs and thus contribute to the environmental resistome of river catchments and other aquatic environments. It may be appropriate to routinely monitor such effluents as sources of ARGs, particularly when considering the current view of ARGs as environmental contaminants (Pruden et al. 2006) and the call for an environmental framework to tackle antimicrobial resistance (Berendonk et al. 2015).

One such reason for the high abundance of ARGs in effluents may be the presence of antimicrobial compounds that could consequently provide a selective pressure for the maintenance of ARGs. There have been several studies that document the presence of antimicrobial compounds, from both human and veterinary medicine, in the environment (Kemper 2008, Hu et al. 2010). Although these compounds are often present at relatively low concentrations, some studies have shown therapeutic concentrations of antimicrobials being discharged into the environment, such as the effluent from Indian drug manufacturers containing therapeutic concentrations of antimicrobial compounds (Larsson et al. 2007). Subsequent studies by Larsson et al. found a high abundance of ARGs downstream of the effluent discharge point relative to upstream of the manufacturers and when compared to a Swedish WWTP (Kristiansson et al. 2011). While the environmental release of antimicrobial compounds at therapeutic concentrations is largely prevented in the UK, Europe and US through proper wastewater management and controls, clinically important antimicrobials can be found in the environment at sub-inhibitory concentrations and it is possible that these very low antimicrobial concentrations could be enriching for resistant bacteria and promote increased persistence of ARGs (Gullberg et al. 2011). Thus, it may be pertinent to couple future environmental ARG monitoring studies and risk assessments with information on antimicrobial usage and the antimicrobial concentrations in the effluents being investigated.

Interestingly, the average abundance of ARGs was found to be greater in the farm effluents than in the WWTP effluents (Figure 1). Although these two effluents are from differently treated faecal sources, one being a treated effluent (sedimentation treatment) from a municipal WWTP (i.e. predominantly human faecal source) and the other being an untreated effluent from a farm (predominantly bovine faecal source), this finding does offer some insight into the debate surrounding the relative impact of human and animal contributions to the development of antimicrobial resistance (Phillips et al. 2004, Mather et al. 2013). The fact that WWTP effluent had undergone a form of water treatment prior to being released into the river catchment, whereas the farm effluent did not, may suggest that some form of water treatment could reduce the abundance or diversity of ARGs. A comparison of WWTP crude

influent to the effluent could elaborate on the effectiveness of sedimentation treatment on the abundance of ARGs. Studies have shown that wastewater treatment processes do not completely remove ARGs (Wang et al. 2015) and that some WWTP processing can result in an increase in the proportion of antimicrobial resistant bacteria in WWTP effluents (Harris et al. 2012). Considering that effluents may also disseminate antimicrobial compounds, it raises the question as to whether the combination of ARGs and antimicrobial compounds within effluents is resulting in the expression of ARGs and the occurrence of phenotypic antimicrobial resistance. This should be addressed in future studies that aim to assess the risk of ARGs entering the environment.

In terms of the mobility of genes within the effluents, an array of mobilisable plasmids, integrons and transposons were present in the metagenomes (Figure 2) and many of the ARGs identified aligned to the Repository of Antibiotic resistance Cassettes (RAC) (Tsafnat et al. 2011). This raises the possibility that the ARGs within the effluents could be readily mobilised into other bacteria, including both directly into pathogens also discharged into the environment and environmental bacteria. These environmental bacteria in turn could pose a risk as potential bacterial intermediaries, harbouring these ARGs in the environment prior to transferring them into other pathogens.

Based on the observations in this study, it is recommended that future Risk Assessments should incorporate direct MGE and pathogen detection with metagenomic assessments of effluents entering river catchments, especially considering the absence of MGEs and the lower diversity of pathogens found in the river source water. This study also showed that a large amount of variation can occur between samples from the same sampling site, possibly as a result of seasonal variation or other environmental factor related to sample collection. It would be benifical to future environmental risk assessments if the impact of seasonal variation on ARG abundance could be determined.

We did however find five resistance genes in the river source water conferring resistance to two classes of antimicrobials. When normalised to 16S sequences the river source water was found to be accountable for the most abundant phenicol resistance gene and the third most abundant aminoglycoside resistance genes out of all the metagenome libraries examined. However, when using the raw SEAR abundance metric, that does not include normalisation to the 16S sequences within the sample, the relative abundance of ARGs from the river source water are reduced relative to the other effluent samples. This raises the question as to whether 16S normalisation is the most appropriate approach to metagenomic abundance estimates as factors such as variation in 16S copy number can skew the data generated as well as interpretation (Case et al. 2007). An alternative could be to use the RPKM value generated as part of the SEAR analysis and featured in table S2.

The metagenomic approach used was relatively less sensitive than more direct-targeted measures of known ARG abundance (e.g. qPCR-based detections (LaPara et al. 2011)). The lack of sample replication at each time point also made comparisons between effluents less certain. However the approach had the advantages that it was relatively unbiased and semi-quantitative, giving a good estimation of relative key ARG and MGE abundance and diversity across bacterial populations. It was also potentially able to detect novel ARGs that would otherwise not be found using these more targeted approaches.

Conclusion

We have presented a detailed metagenomic analysis of effluents entering a river catchment. Effluents were found to contain an array of ARGs, MGEs and pathogenic bacteria that, when compared to a background sample of the river source water, were found to be more diverse and abundant than in the river source water. This study has shown that the discharge of

effluents into river catchments contributes to the dissemination of ARGs, MGEs and pathogenic bacteria, and may play an important role in the propagation of environmental reservoirs of ARGs.

List of abbreviations

ARG	-	Antimicrobial Resistance Gene
MGE	-	Mobile Genetic Element
WWTP	-	Wastewater treatment plant
SEAR	-	Search Engine for Antimicrobial Resistance
RPMK	-	Reads Per Kilobase per Million
BWA	-	Burrows-Wheeler Aligner
RAC	-	Repository of Antibiotic resistance Cassettes

Acknowledgements

This research was funded by the Centre for Environment, Fisheries and Aquaculture Science, GlaxoSmithKline and the Biotechnology and Biological Sciences Research Council. Metagenomic sequencing was performed by the Sequencing Service and Wellcome Trust Biomedical Informatics Hub, Exeter (UK).

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