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Large-scale characterization of hospital wastewater system microbiomes and clinical isolates from infected patients: profiling of multi-drug-resistant microbial species

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SUMMARY

Background: Hospital-acquired infections (HAIs) and infectious agents exhibiting antimicrobial resistance (AMR) are challenges globally. Environmental patient-facing wastewater apparatus including handwashing sinks, showers and toilets are increasingly identified as sources of infectious agents and AMR genes.

Aim: To provide large-scale metagenomics analysis of wastewater systems in a large teaching hospital in the Republic of Ireland experiencing multi-drug-resistant HAI outbreaks.

Methods: Wastewater pipe sections (N=20) were removed immediately prior to refurbishment of a medical ward where HAIs had been endemic. These comprised toilet U-bends, and sink and shower drains. Following DNA extraction, each pipe section underwent metagenomic analysis.

Findings: Diverse taxonomic and resistome profiles were observed, with members of phyla Proteobacteria and Actinobacteria dominating (38.23 ± 5.68% and 15.78 ± 3.53%, respectively). Genomes of five clinical isolates were analysed. These AMR bacterial isolates were from patients >48 h post-admission to the ward. Genomic analysis determined that the isolates bore a high number of antimicrobial resistance genes (ARGs).

Conclusion: Comparison of resistome profiles of isolates and wastewater metagenomes revealed high degrees of similarity, with many identical ARGs shared, suggesting probable acquisition post-admission. The highest numbers of ARGs observed were those encoding resistance to clinically significant and commonly used antibiotic classes. Average nucleotide identity analysis confirmed the presence of highly similar or identical genomes in clinical isolates and wastewater pipes. These unique large-scale analyses reinforce the

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need for regular cleaning and decontamination of patient-facing hospital wastewater pipes and effective infection control policies to prevent transmission of nosocomial infection and emergence of AMR within potential wastewater reservoirs.

Introduction

Multi-drug-resistant infections are an increasingly deleterious problem for healthcare settings given the rise in antimicrobial resistance (AMR) [1,2]. The clinical environment has been noted as a source of infection-causing micro-organisms and antimicrobial resistance genes (ARGs) contained therein, highlighting the need for effective infection control policies to reduce the rate of nosocomial transmission [3—5]. The clinical setting has a number of ecological niches, including patient-facing wastewater (WW) apparatus, which have the potential to act as reservoirs of ARGs and pathogenic bacteria due to their physical design and human activities associated with their use [6—8].

Hospital WW is known to contain many ARGs and, indeed, residual antibiotic compounds [9—11]. Healthcare facility effluent has been demonstrated to impact downstream municipal WW, although this effect remains poorly understood [12,13]. Specifically, multiple studies have reported that although hospital WW may comprise ARGs, this may not influence downstream municipal WW [14—16], and associated AMR emergence may be specific to certain species of bacteria [17]. Included in these species are clinically relevant bacteria that have been detected on clinical surfaces and in patient-facing sections of the hospital WW system. The isolates have been shown to bear ARGs, such as New Delhi metallo beta-lactamase, which confer resistance to carbapenem antimicrobials which have been referred to as 'antibiotics of last resort' [18]. The challenges of gene transfer, and associated multi-drug-resistant nosocomial infection, have been elucidated clearly using comparative genomics of clinical and environmental bacterial isolates, implicating the clinical environment as a reservoir of infectious agents [19].

Numerous studies have correlated contaminated handwashing sinks and outbreaks in hospital settings, in particular with carbapenemase-producing Enterobacterales (CPE) [20—22]. A laboratory model system demonstrated direct linkage between dispersal of carbapenem-resistant Enterobacterales from sinks and drain position or drainage rates with contaminated splash water travelling up to 1 m from the sink. This work underlines the need for well-designed blockage-free WW apparatus in patient-facing clinical areas [23]. Complementary data implicate droplet-mediated transmission as the primary mechanism of bacterial spread from contaminated handwashing sink traps, rather than aerosols [24]. Such dispersal of potential pathogens from contaminated hospital WW systems has been associated definitively with outbreaks involving extended-spectrum β-lactamase (ESBL)-producing bacteria, such as ESBL-producing *Enterobacter cloacae* and a number of *Klebsiella* spp. [25—28]. Unsurprisingly, acknowledgement of risks attributable to plumbing systems in hospitals has resulted in research to develop optimal strategies and interventions for their mitigation or management. Exemplifying this, in one wide-ranging study involving two English hospitals, faulty sink, shower and toilet design was posited as a contributing factor to clinical area contamination by multi-drug-resistant *Pseudomonas aeruginosa*. Replacement with easier-to-clean models less prone to splashback, as well as reviews of cleaning and waste disposal protocols, were followed by a significant reduction in cases of infection [29,30]. Similarly, implementation of waterless patient care involving removal of sinks in patient rooms was successful in termination of a *P. aeruginosa* Verona integron-encoded metallo-beta-lactamase outbreak in the intensive care unit of a Swiss tertiary care hospital. In this case, whole-genome sequencing confirmed the epidemiological link between clinical and environmental *P. aeruginosa* strains and the monoclonal pattern of the outbreak [31]. Acetic acid has also been demonstrated as a simple method to decontaminate sink drains and prevent nosocomial transmission of metallo-β-lactamase-producing *P. aeruginosa* (Pae-MBL). Weekly washes of colonized sink drains using this approach resulted in negative cultures, and terminated the transmission of a Pae-MBL outbreak which had persisted for several years prior to the intervention [32].

In the context of evident relationships between WW and contribution to outbreaks, in addition to potential distribution of ARGs to the wider adjacent community, this study represents a unique large-scale metagenomic analysis of the WW system in a large teaching hospital, specifically in the Republic of Ireland, which is experiencing ongoing multi-drug-resistant organism outbreaks. In particular, emphasis was placed on the microbiome of WW pipe sections originating close to or directly at the interface of the WW system and human activity which were obtained for analysis following outbreaks. In parallel, the resistomes of clinical bacterial isolates known to have caused multi-drug-resistant infections in patients treated in the same rooms from which the WW pipes were removed were interrogated. Both the resistomes and overall nucleotide profiles of the clinical isolates were compared with environmental metagenomes to determine any relationships between infection-causing bacteria and the WW system.

Methods

Background and setting

University Hospital Limerick (UHL) is a tertiary care teaching hospital in the West of Ireland, comprising 533 inpatient beds. The ward involved is a multi-bedded facility encompassing seven single-occupancy rooms, two double-occupancy rooms, a four-bedded room, a five-bedded room and a 12-bedded open 'Nightingale' room. Each of the smaller rooms includes one en-suite facility with shower, toilet and handwash basin, with two such facilities in the 12- bedded room. Figure 1 provides a map of the rooms within the ward. UHL has a well-documented extensive history of detection of antimicrobial-resistant

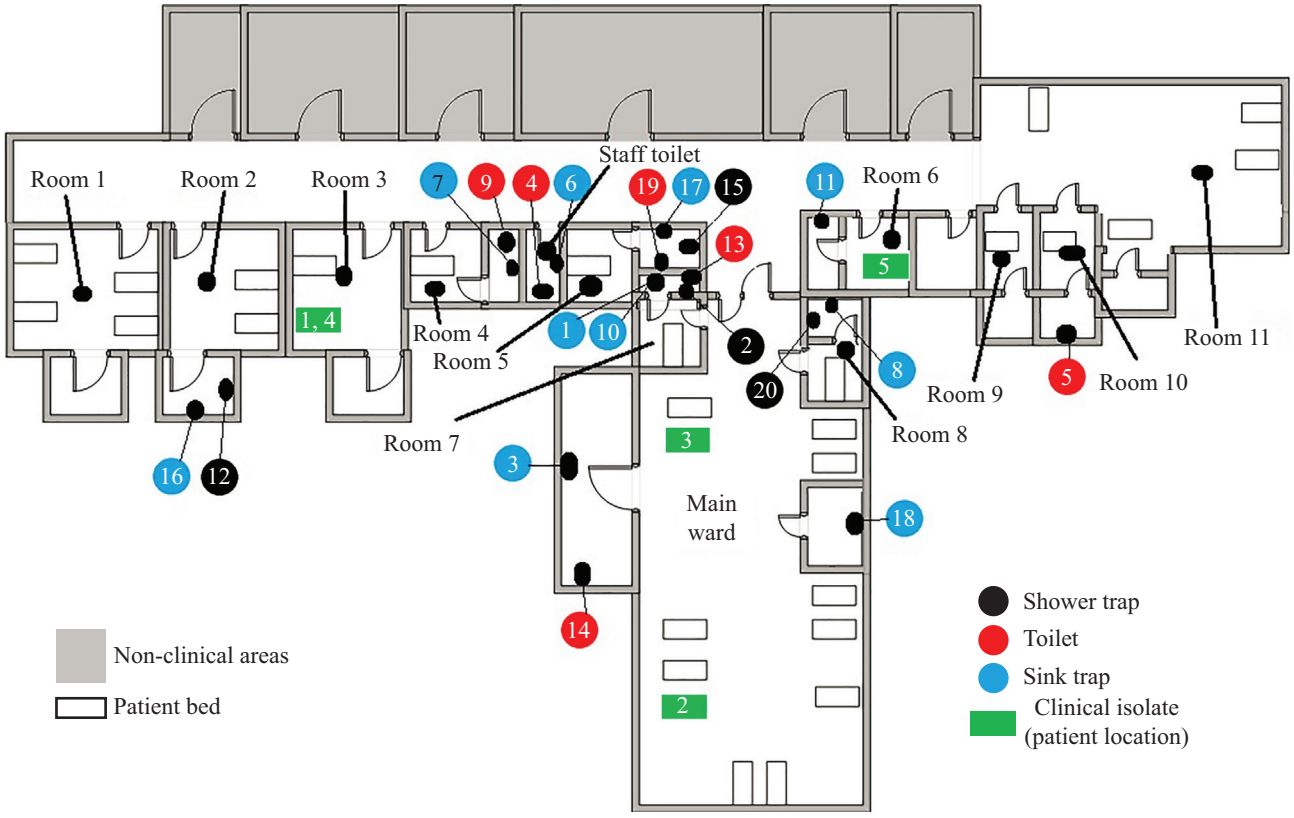

Figure 1. Layout of the ward showing the locations of the wastewater pipe sections used for metagenomic analysis, and the locations of the patients that acquired the clinical isolates investigated (not to scale). The numbers represent the sample numbers for the 20 pipe samples tested.

organisms, with reported outbreaks of linezolid-resistant [33] and ESBL-producing organisms [34]. CPE-producing bacteria are recognized as endemic at UHL [35—38]. Indeed, UHL was the site of the first CPE detection in Ireland [39]. Subsequently, the incidence of CPE at UHL has been amongst the highest in Ireland [i.e. the most recent Irish national data state that UHL contributes 8.6% of CPE cases [40], which is disproportionally high when correlated with only 4.6% of national hospital beds [41]]. Despite this, the level of carbapenem-resistant *Escherichia coli* at UHL (0.6%, 2020) is lower than the national level [9.5%, European Antimicrobial Resistance Surveillance Network (EARS-Net)] and the European Union (EU) level (2.7%, EARS-Net) [42]. Similarly, observed *Klebsiella pneumoniae* resistance is lower for both carbapenem (0.0% vs 0.3% nationally vs 1.1% median EU) and third-generation cephalosporin (3 GC) (14.3% vs 18.4% nationally vs 27.8% median EU). However, *E. coli* 3 GC resistance is high (i.e. 16.0% vs 11.3% nationally vs 12.3% median EU), as is *Enterococcus faecium* vancomycin resistance (50.0% vs 35.9% nationally vs 11.9% median EU). In that context, the study ward has been a facility associated consistently with the highest numbers of CPE cases (2018—2019), with the greatest number of nosocomial ESBL cases (2021) and vancomycin-resistant enterococci (VRE) cases (2018—2020).

Samples and DNA extraction

Immediately prior to refurbishment of the ward, toilet U-bends (N=6), sink drains (N=10) and shower drains (N=4) were removed and sealed in sterile plastic bags for transport to the laboratory for study. DNA was extracted using well-established phenol:chloroform extraction methods [43]. Briefly, the material adhering to the 20 parts was sampled using a sterile swab and sterile water, and added to a tube containing 210 μL 0.1 mm zirconium beads, 210 μL 20% SDS, 500 μL DNA extraction buffer (200 mM NaCl, 200 mM Tris, 20 mM EDTA, pH 8.0) and 500 μL phenol:chloroform:isoamyl alcohol. Cells were lysed by vortexing, followed by centrifugation and a further addition of 600 μL phenol:chloroform:isoamyl alcohol to the supernatant, which was gently mixed by inversion. Following a further round of centrifugation, DNA was precipitated from the supernatant using 1/10 volume 3 M sodium acetate and 1 volume isopropanol. Following overnight incubation at -20 °C, the solution was centrifuged and the supernatant was discarded. The pellet was washed with 100% ethanol, dissolved in TE buffer (pH 8.0) and treated with RNase. DNA was purified for sequencing using a GeneJet PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Sequencing and read analysis

Extracted DNA underwent quality control (QC) on the AATI fragment analyser (Advanced Analytical Technologies Inc., Santa Clara, CA, USA), and library preparation was performed using the Illumina XT library prep kit (Illumina Inc., San Diego, CA, USA), followed by library QC. These libraries were normalized and pooled and checked for index balance, before running on the Illumina NovaSeq 6000 SP 300 flow cell (Illumina

Inc.). This produced 150-bp paired-end reads at 50M reads per sample. Sequencing reads were checked for quality using FASTQC v0.11.9 [44], and high-quality reads were confirmed across all samples. Fastq files were assembled into contigs using SPAdes v3.14.0 in –meta mode, using k-mer lengths 21, 33 and 55 [45]. Assembled contigs were processed for taxonomic classification using Kraken v2.0.8 [46].

Genome binning

Assembled contigs were built into a bowtie2 database file using the bowtie2-build command associated with Bowtie2 v2.4.2 [47]. Reads were aligned to this assembly using the bowtie2 command, and Samtools v1.3.17 [48] was used to index the resulting .bam file. Contig depths were summarized using JGI v2.15, and genomes were binned using the metabat2 command associated with Metabat2 v 2.2.15 [49]. Completeness and contamination of the resulting metagenomically-assembled genomes (MAGs) were checked using CheckM v1.1.3 [50], and those with >5% contamination or <50% completeness were discarded.

Taxonomic annotation and antimicrobial resistance gene analysis of bins

The phylophlan metagenomic command associated with PhyloPhlAn v3.0.60 was used to assign taxonomy to the selected MAGs. These were characterized to family, genus and species level. ARGs were identified via the Resistance Gene Identifier (RGI) using the Comprehensive Antibiotic Resistance Database (CARD) [51]. Gene counts were performed for each ARG identified across all samples.

Clinical isolates

Five biobanked clinical bacterial isolates were chosen based on the surveillance records of UHL. These were cultured from patients who acquired identified AMR pathogens during an admission to the ward, detected in the 12 months prior to the WW pipe sampling. The clinical isolates (N=5) were grown in LB broth at 37 °C, and genomic DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Merck, Darmstadt, Germany) in accordance with the manufacturer's protocol. Extracted whole-genome DNA underwent QC on the AATI fragment analyser, and library preparation was performed using the Illumina XT library preparation kit, followed by library QC as before. Libraries were normalized, pooled and run on the Illumina MiSeq V2 500. This produced 250-bp paired-end reads at 100 X coverage for each sample. Sequencing reads were checked for quality using FASTQC v0.11.9, and high-quality reads were confirmed across all samples. Fastq files were assembled into contigs using SPAdes v3.14.0, with k-mer lengths of 21, 33 and 55. Assembly quality metrics were performed using the Quality Assessment Tool (QUAST) associated with the Galaxy platform. ARG identification was performed as described above.

Comparison of clinical isolates to environmental DNA

Contigs assembled from the pipe metagenomes enabled generation of a local BLAST database using the makeblastdb command associated with NCBI+ BLAST v2.9.0 [52]. Prodigal v2.6.3 [53] was used to predict genes within the resultant database and translate them to amino acid sequences. Translated ARGs identified from the clinical isolate genomes were used as search terms to interrogate this database using the blastp function of NCBI+ BLAST v2.9.0. Average nucleotide identity similarity analysis between clinical isolate genomes and MAGs was performed using pyani v0.2.11 [54].

Visualization of results

Data were visualized using R v4.1.0, ggplot2 v3.3.6 and pheatmap v1.2.12.

Results

Sequencing metrics

DNA was extracted and sequenced from 20 pipe samples from patient rooms, including sinks (N=10), toilets (N=6) and showers (N=4). For environmental metagenomes, next-generation sequencing yielded 150-bp paired-end reads at 50M reads per sample. This amounted to an average data load of 18.1 Gb per sample, representing comprehensive sequencing coverage for each sample. FASTQC analysed a total of 906,956,118 sequences, with 0 of these flagged as being poor quality. The average GC content of sequences across all samples was 42%. For whole-genome sequencing of clinical isolates, next-generation sequencing similarly yielded 150-bp paired-end reads at 50M reads per sample. This yielded an average data load of 18.1 Gb per sample, again ensuring comprehensive sequencing of each genome. FASTQC analysis flagged 0 of these as being poor quality. The average GC content of sequences across all clinical isolates was 42%.

Taxonomic breakdown and population diversity of wastewater pipe metagenomes

At phylum level, Proteobacteria were the most abundant across all samples, constituting a mean abundance of 38.23 ± 5.68% of classified bacterial reads (Figure 2 and Supplementary Information SI1). This was followed by Actinobacteria (15.78 ± 3.53%) and Bacteroidetes (2.23 ± 0.74%), regardless of sample type. Alpha diversity analysis revealed the presence of greater microbial diversity in sinks (Shannon index 3.82), compared with toilets (3.59) and showers (3.73) (Supplementary Figure 1A). Both S1 (a trap unit complete with U-bend of a sink) and S10 (pipe connection immediately into trap) were taken from the same sink and shared the same ecological signature. A growing number of studies have highlighted contamination issues with hospital handwashing sinks, implicating these niches as reservoirs of infection-causing bacteria and ARGs [20,22,23,25–27]. Principal co-ordinate analysis (PCoA) of a Bray–Curtis dissimilarity matrix showed that sample types were loosely grouped based on relative abundance at species level (Supplementary Figure 1B), suggesting that varying microbial profiles were present in different pipe samples. These groupings are likely to be due to the human behaviours and activities associated with each location (e.g. sinks or toilet). Elements of similarity across sample types are also to be

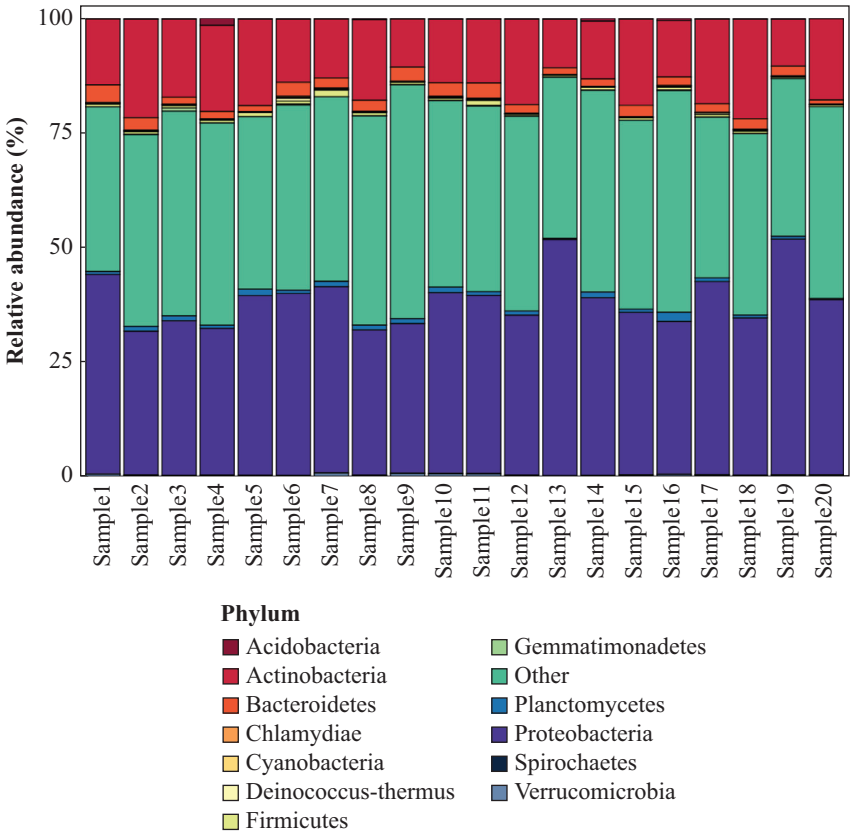

Figure 2. Metagenomic analysis of the clinical wastewater environment showing microbial taxonomic breakdown at phylum level across all 20 pipe sections examined. Taxonomic analysis was performed using Kraken2 v2.0.8 following assembly with SPAdes v3.14.0.

expected given their proximity and the interconnectedness of the WW pipe system within the hospital.

Antimicrobial resistome analysis of metagenomically-assembled genomes

MAGs were generated from reads of pipe sample metagenomes using Metabat2. In total, 469 MAGs were generated across the 20 metagenomes which met the set criteria for contamination and completeness. The number of MAGs per sample used for resistome analysis is shown in Supplementary Table 1. Of the generated MAGs, 256 were identified to species level, 14 to genus level, 49 to family level, and 150 to order level or higher. As expected given the Kraken2 analysis of metagenomic contigs, most MAGs annotated to order level or above were members of phylum Proteobacteria.

Resistome analysis revealed a diverse reservoir of ARGs in clinical WW pipes. The highest numbers of ARGs observed were those encoding resistance to tetracyclines, fluoroquinolones, diaminopyrimidines, β-lactams and macrolides, all of which are clinically significant and commonly used antibiotic classes (Figure 3a).

PCoA comparing ARG profiles across samples showed no obvious groupings by sample type (Figure 3b,c). This is interesting given the groupings observed based on taxonomy from the same samples (Supplementary Figure 1b). This suggests that, as has been reported across many environments, ARGs are not confined to individual bacterial species but are shared across different species via divergent evolution or horizontal transfer.

Correlatin of resistome to bacterial populations

Total ARG counts by phylum are shown in Figure 4a. As expected, the highest numbers of ARGs were from phyla Proteobacteria and Actinobacteria, with proportions approximately commensurate with the relative abundance of these taxa. ARG relative abundance for each antibiotic class was compared for these two phyla (Figure 4b). As other phyla had relatively low abundance and subsequently lower numbers of ARGs, it was determined that analysing the relative abundance of ARGs by antibiotic class would not be appropriate or reliable for these phyla. As with the overall breakdown of ARGs (Figure 3a), genes encoding resistance to tetracyclines, fluoroquinolones, diaminopyrimidine, β-lactams and macrolides predominated. The proportion of ARGs for each class was similar between Proteobacteria and Actinobacteria, with counts for tetracycline- and fluoroquinolone-resistance-coding genes slightly higher in Actinobacteria (25.48 and 26.27%, respectively, vs 21.02 and 20.17%, respectively, for Proteobacteria). The next five most abundant resistance-encoding genes by antibiotic class were largely similar between Proteobacteria and Actinobacteria, constituting diaminopyrimidines (14.86 vs 15.29%), β-lactams (8.21 vs 8.12%),

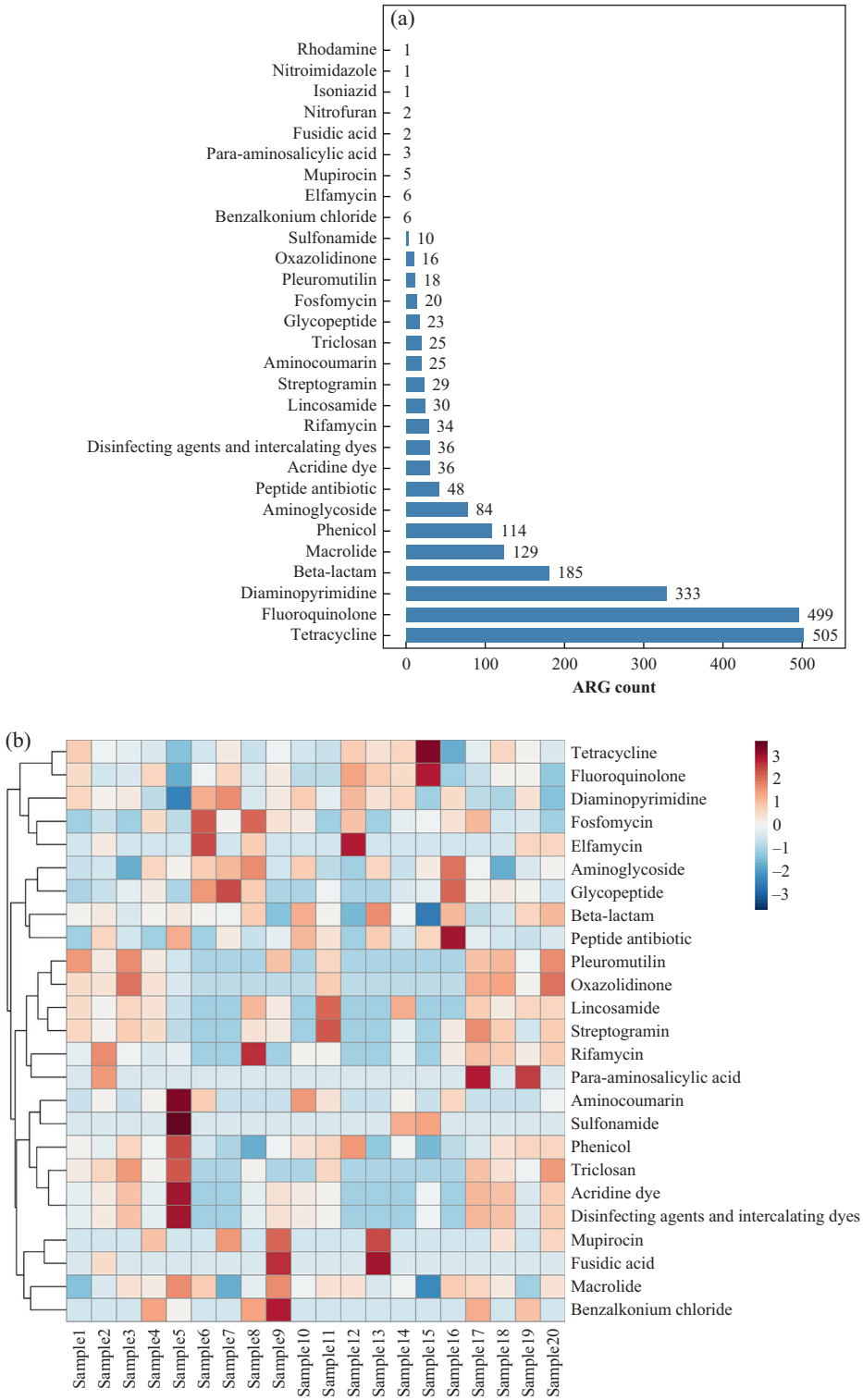

Figure 3. (a) Total combined antimicrobial resistance gene (ARG) count for each antibiotic class across all metagenomically-assembled genomes generated from wastewater pipe sections. (b) Resistome heatmap across all metagenomically-assembled genomes generated from wastewater pipe sections showing the relative density of ARG type by antibiotic class. (c) Principal co-ordinate analysis (PCoA) of resistome profiles of wastewater pipe sections generated by the Resistance Gene Identifier, grouped as showers, sinks and toilets. Heatmap and PCoA figures were generated using R v4.1.0, ggplot2 v3.3.6 and pheatmap v1.2.12.

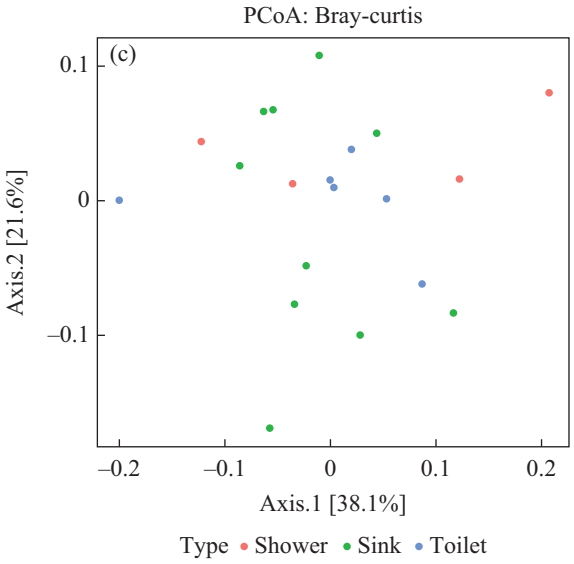

Figure 3. *(continued)*

macrolides (6.58 vs 4.94%), phenicols (5.73 vs 4.46%) and aminoglycosides (3.75 vs 3.82%).

The number of ARGs was also compared with relative abundance at family level. Figure 5 depicts the total number of ARGs identified vs mean relative abundance for each family across all metagenomes. The relationship between these variables shows a moderate positive correlation (Pearson coefficient=0.62, r^2=0.3844), suggesting that the number of ARGs carried by specific bacterial families may be determined by their relative abundance. There were several notable outliers to this finding, including Comamonadaceae, which was the fourth most abundant family (3.10%) but had only the 25th highest number of ARGs. Conversely, Erythrobacteraceae contained the third highest number of ARGs, but was only the 34th most abundant family (0.26%). This suggests that ARG carriage may be dictated by relative abundance, but this is often not the case, which may be due to associated fitness costs/burdens of carrying such genes.

Characterization of clinical isolates and location within environmental metagenomes

Initially, bacterial species were isolated from colonized patients, and their whole genomes were sequenced. These patients were being clinically managed on the ward prior to refurbishment, and were colonized with AMR bacteria. Clinical isolates (ci) 2, 3 and 5 were cultured from patients accommodated on wards with co-located ensuite shower rooms that had pipework analysed (Figure 1). All three clinical isolates, as well as isolate ci4, were from the family Enterobacteriaceae. The first clinical isolate (ci1) was identified as *E. faecium* (family Enterococcaceae). The closest match for each clinical isolate is shown in Table I. All patients from whom the clinical isolates had been sourced had recent exposure to antimicrobials: cefotaxime prior to ci1 VRE detection, ciprofloxacin prior to ci2 ESBL-producing *E. coli*, piperacillin/tazobactam prior to ci3 CPE-producing *Enterobacter kobei*, piperacillin/tazobactam prior to ci4 CPE-producing *Citrobacter youngae*, and vancomycin and piperacillin/tazobactam prior to ci5 CPE-producing *Enterobacter hormaechei*.

As with metagenomic datasets, each whole genome was subjected to ARG identification using RGI and the CARD database. A diverse range of resistance genes was identified in these isolates, including high numbers of clinically relevant ARGs (Supplementary Information SI2). ARGs present in clinical isolates were compared with those found in the environmental metagenomes from WW pipes. A local database was generated from pipe metagenome contigs, and this was interrogated using each ARG from all clinical isolates as search terms. This revealed a considerable number of identical genes, highlighting the possibility that these genes may have been passed from one source to another. These include resistance genes for many antibiotic classes commonly used in the clinical setting, including those for fluoroquinolones, aminoglycosides, β-lactams, macrolides, sulfonamides and tetracycline. Genes with ≥98% identity and ≥50% gene coverage are shown in Table II. A number of genes are present across several clinical isolates as well as several pipe metagenomes. Figure 1 shows the locations of the WW pipe sections and the patients harbouring the AMR bacteria. While these data cannot be used to determine if resistance genes were transferred from patient to WW system or vice versa, they do allow confirmation of crossover in the resistome of clinically relevant pathogens and the microbiome of the WW environment.

In order to investigate this potential crossover further, average nucleotide identity (pyani) analysis was performed to explore the similarity between clinical isolate genomes and the metagenomes of WW pipes. Pyani analysis compared the whole genome of all clinical isolates against all MAGs generated from the WW metagenomes. Assembly quality metrics for clinical isolate genomes used in pyani analysis were generated using QUAST, and these are provided in Supplementary Information SI3. This revealed exact (1) or highly similar (>0.99) matches for all clinical isolates (Figure 6). For example, ci2 returned an average nucleotide identity of 1 in samples S3, S6, S8, S9, S10, S11, S13, S15 and S16. All pyani values are shown in Supplementary Information SI4. These values suggest that hospital WW pipes

Table I
Overview of clinical isolates used in this study, showing isolate designation, closest neighbour using the National Center for Biotechnology Information database, and number of antimicrobial resistance genes (ARGs) identified in each isolate using the Resistance Gene Identifier

Isolate designation	Closest neighbour (% identity)	Ward location	No. of ARGs identified
ci1	*Enterococcus faecium* (100)	Room 3, Bed 2	24
ci2	*Escherichia coli* (100)	Main ward, Bed 5	77
ci3	*Enterobacter kobei* (99.9)	Main ward, Bed 2	26
ci4	*Citrobacter youngae* (99.8)	Room 3, Bed 2	60
ci5	*Enterobacter hormaechei* (100)	Room 6, Bed 1	54

harbour the same pathogens that have been shown to cause nosocomial infections in patients. Whilst it is difficult to determine definitively whether these patients acquired their respective infections from the WW system, or whether they contracted the infection from another source within the ward, pyani analysis suggests that these pathogens are present within the WW system, with the potential to cause infection or colonization in patients admitted to this facility. This analysis is limited somewhat by the use of MAGs for comparison, although only MAGs determined to have >50% completeness and <5% contamination by CheckM were used for comparison. With this comparison, high levels of similarity were observed in the pipe infrastructure for each clinical isolate.

Discussion

This unique cross-sectional study was made possible when a ward, endemic for CPE, underwent refurbishment, providing an opportunity to determine the microbial ecology of differing pipework elements from WW apparatus (sinks, showers and toilets). This work was instigated in the context of growing acknowledgement that the presence of AMR bacteria and ARGs in the hospital environment and its associated WW poses a potential cross-transmission threat to patients, healthcare staff and the public in the wider community setting. There are increasingly more publications defining the role of the hospital environment, particularly sanitaryware, in causing outbreaks of infection [55]. Similarly, hospital effluent and its subsequent treatment, typically in municipal WW plants, are proposed as factors contributing to the spread of ARGs in the community setting [56]. A recent meta-analysis of hospital WW as a reservoir for ARGs analysed the prevalence of ARGs in hospital WW, as well as the influencing factors in ARG distribution [57]. Resistance genes to carbapenems, macrolides, tetracyclines and quinolones were found at high abundance ($>10^{-4}$ gene copies/16S rRNA gene copies). A higher abundance of ARGs was found in hospital WW than in most other WW systems, supporting the hospital setting as an environmental hotspot for the spread of ARGs [58]. In this context, this analysis of ARGs was undertaken so it could inform infection control interventions.

Surveillance diagnostic techniques for studying the hospital environment have traditionally focused on culture methodologies, renowned for being labour intensive and time consuming. Likewise, they can be subject to isolation bias. Molecular techniques have garnered interest, initially utilizing 16S RNA sequencing [59], and the authors recently described an off-label application of using a multiplex molecular platform for the detection of CPE from environmental samples [60]. However, metagenomic analysis is playing an increasing role in elucidating hospital microbial ecology, providing insight regarding epidemiology of AMR bacteria and ARGs in environmental reservoirs, the complexity of their relationship, and transmission dynamics. In 2020, the first report was published of an extensive genomic characterization of a hospital's environmental microbiome, pathogens and antibiotic cassettes from high-touch surface sites as well as sink traps and aerators over an 18-month period [61]. The authors, from a Singaporean tertiary care hospital, described distinct ecological niches of microbes with patterns of spatiotemporal diversity. Short-read shotgun metagenomics of multiple environmental swabs of these sites over three time points were combined with enrichment culture and nanopore sequencing of antibiotic-resistant mixed cultures in this study. They found that frequently touched surfaces harboured bacteria more commonly associated with skin carriage, in comparison with sink-traps and aerators which supported the growth of biofilm-associated bacteria. Likewise, there was diversity with respect to the presence of ARGs from these respective surfaces. The findings highlight the importance of such characterization in targeting resources for prevention of nosocomial infections.

In that context, Proteobacteria was the most common phylum identified across all environmental specimens in this study, with the greatest microbial diversity present in sinks compared with toilets and showers. Moreover, 256 species were identified from the generated MAGs with differing microbial profiles present in different pipe samples, which are likely reflective of human behaviours and activities associated with each type of sample both from grey WW (e.g. wastes generated from cleaning water etc.) and black WW (i.e. wastes generated from faeculent discharge). It is well recognized that bacteria can contaminate sinks mediated by a myriad of mechanisms (i.e. mains water supply, contaminated components, poor hygienic practices during installation, maintenance, cleaning, and incorrect use including disposal of biological fluids) [62]. Anecdotally, observational environmental audits of clinical handwash basins used on the authors' wards have found both staff and patients using sinks for activities other than hand hygiene, including disposal of biological waste and pharmaceutical products.

ARG profiles across all environmental samples showed no obvious groupings by sample type or spatially throughout the different rooms of the ward. In addition, multiple ARG combinations were found, including broad-spectrum antimicrobials coupled to genes encoding resistance to disinfectants, which potentially provides favourable conditions for the evolution of microbes, even novel pathogens, with increased AMR.

In 2021, a Scottish study examined hospital WW using metagenomic technologies to determine relationships between antimicrobial usage and resistance exhibited by clinical isolates [63]. The authors concluded that resistance in hospital WW may

Table II
Comparison of antimicrobial resistance gene (ARG) identity in clinical isolates with pipe section metagenomes

Clinical isolate (query)	Environmental metagenomic sample (Hit)	Gene	Drug class resistance	% identity
1	5	tetU	Tetracycline	100
2	7	DfrA42	Diaminopyridine	100
2	2, 5, 8, 9, 11, 12, 13, 14, 15, 18, 20	APH(6)Id	Aminoglycoside	100
2	1	soxS mutation	Fluoroquinolone; monobactam; carbapenem; cephalosporin; glycylcycline; cephamycin; penam; tetracycline; rifamycin; phenicol; triclosan; penem	100
2, 4,5	1, 5, 6, 7, 9, 10, 11, 12, 13, 18, 19	qacEdelta1	Disinfecting agents: acridine and intercalating dyes	100
2	1, 2, 3, 5, 6, 7, 9, 10, 11, 12, 13, 14, 18, 20	APH(3″)	Aminoglycoside	100
2, 4,5	1, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20	sul1	Sulfonamide	100
4, 5	7	rsmA	Fluoroquinolone; diaminopyrimidine; phenicol	100
2	3	sul2	Sulfonamide	100
2	7	ANT(3″)[a]	Aminoglycoside	99.6
2	3	APH(6)Id	Aminoglycoside	99.6
2	5, 6, 7, 8, 9, 11, 12, 14, 15, 18	sul2	Sulfonamide	99.6
2	1	soxR mutation	Fluoroquinolone; cephalosporin; glycylcycline; penam; tetracycline; rifamycin; phenicol, triclosan	99.4
2	13	sul2	Sulfonamide	99.4
2	1	marR mutation	Fluoroquinolone; cephalosporin; glycylcycline; penam; tetracycline, rifamycin; phenicol, triclosan	99.3
2	1	emrR	Fluoroquinolone	99.2
2	3, 5, 8, 9, 10, 13	ANT(3″)[a]	Aminoglycoside	99.2
2	19	APH(3″)	Aminoglycoside	99.2
2	1	evgA	Macrolide; fluoroquinolone; penam; tetracycline	99
2, 4	1	cpxA	Aminoglycoside; aminocoumarin	98.7
4	13	DfrA42	Diaminopyridine	98.7
5	1	DfrA42	Diaminopyridine	98.6
2, 3, 4, 5	1	rsmA	Fluoroquinolone; diaminopyrimidine; phenicol	98.4
4, 5	1, 3, 5, 7, 9, 11, 12, 13, 14, 15, 18	AAC(6′)-Ib-cr[a]	Fluoroquinolone; aminoglycoside	98.3
4	13	mprF	Peptide	98.3
2, 4, 5	14	OXA-1	Carbapenem; cephalosporin; penam	98.2

Cut-off values for inclusion were ≥98% identity and ≥50% coverage.
Metagenome contigs were used to generate a local database, followed by gene prediction and translation to amino acid sequences. Translated ARGs identified in clinical isolate genomes were used as query terms to identify similar ARGs within the wastewater environment.
[a] Genes have been grouped as a single gene family for presentation purposes.

quantitatively reflect the resistance of clinical isolates to some bacterial species but not all. Furthermore, they stated that promotion of active antimicrobial stewardship would positively impact the burden of environmental AMR. The present findings support this view. Each ARG from the studied clinical isolates was compared with pipe ARGs using metagenome contigs to determine relatedness. A diverse range of ARGs was found in both environmental and clinical isolates, with 100% sharing of identical ARGs for disinfecting agents as well as commonly used antimicrobials such as fluoroquinolones, aminoglycosides, β-lactams, macrolides, sulphonamides and tetracyclines. There was correlation with high rates of consumption of these agents in

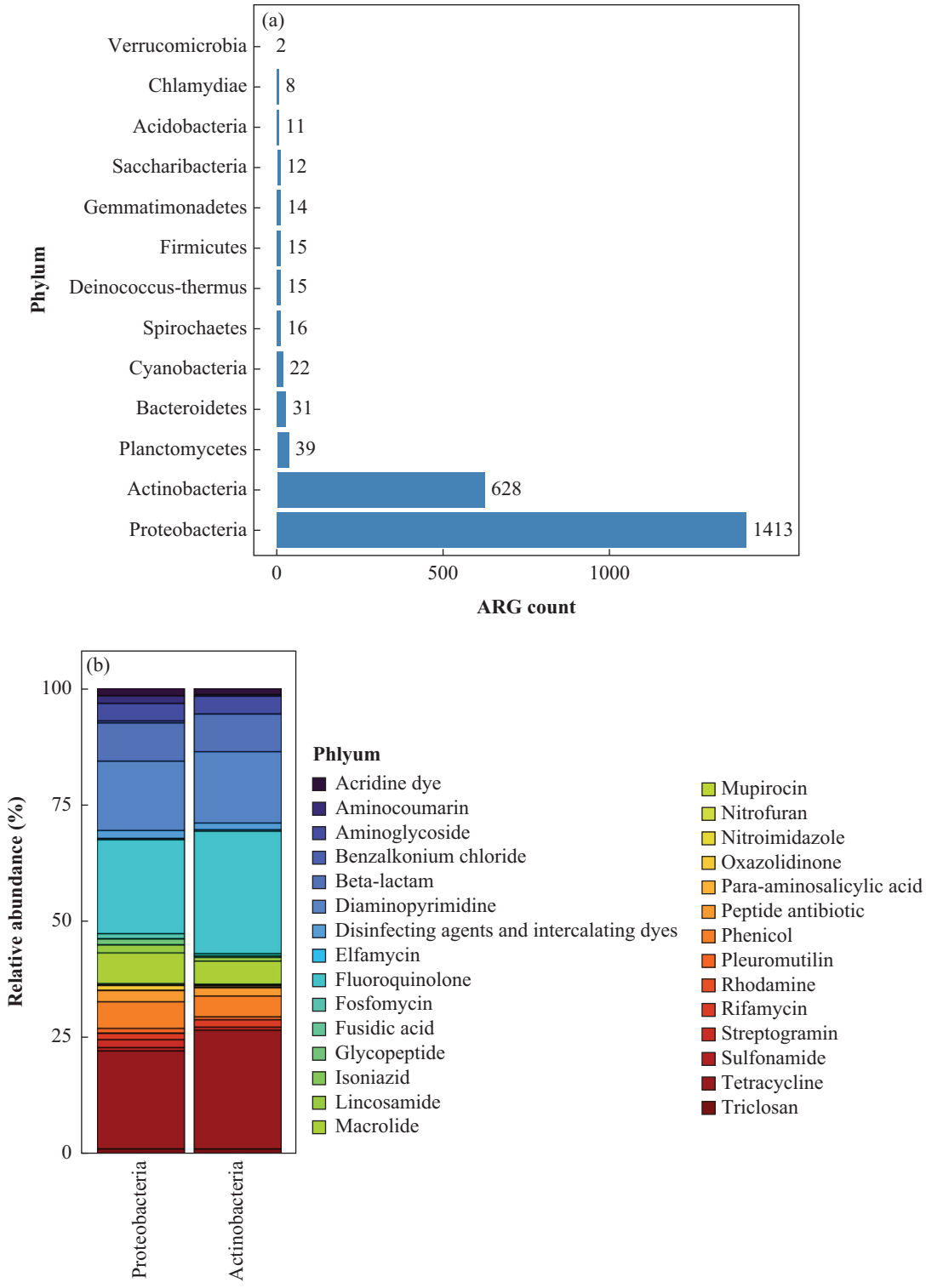

Figure 4. (a) Total combined antimicrobial resistance gene (ARG) count for each phylum across all metagenomically-assembled genomes generated from wastewater pipe sections. (b) Stacked abundance charts showing relative ARG class abundance for the two most dominant phyla, Proteobacteria and Actinobacteria.

UHL based on hospital antimicrobial consumption data and audits of antimicrobial use. Overall, the median antibiotic consumption in UHL in 2020 at the time of this study was 2.25 direct daily doses per 100 bed-days used (DDD per 100 BDU), which compared favourably with the national data of 2.25 DDD per 100 BDU [64]. Respective consumption data for fluoroquinolones, gentamicin, broad-spectrum penicillins, cephalosporins, carbapenems and oral clarithromycin showed DDD per 100 BDU of 3.87 vs 2.93, 2.88 vs 2.54, 22.32 vs 20.05, 2.95 vs 2.10, 2.88 vs 0.94, and 6.26 vs 6.31, illustrating higher overall usage of broad-spectrum antimicrobials inferring potential opportunities for improved antimicrobial stewardship.

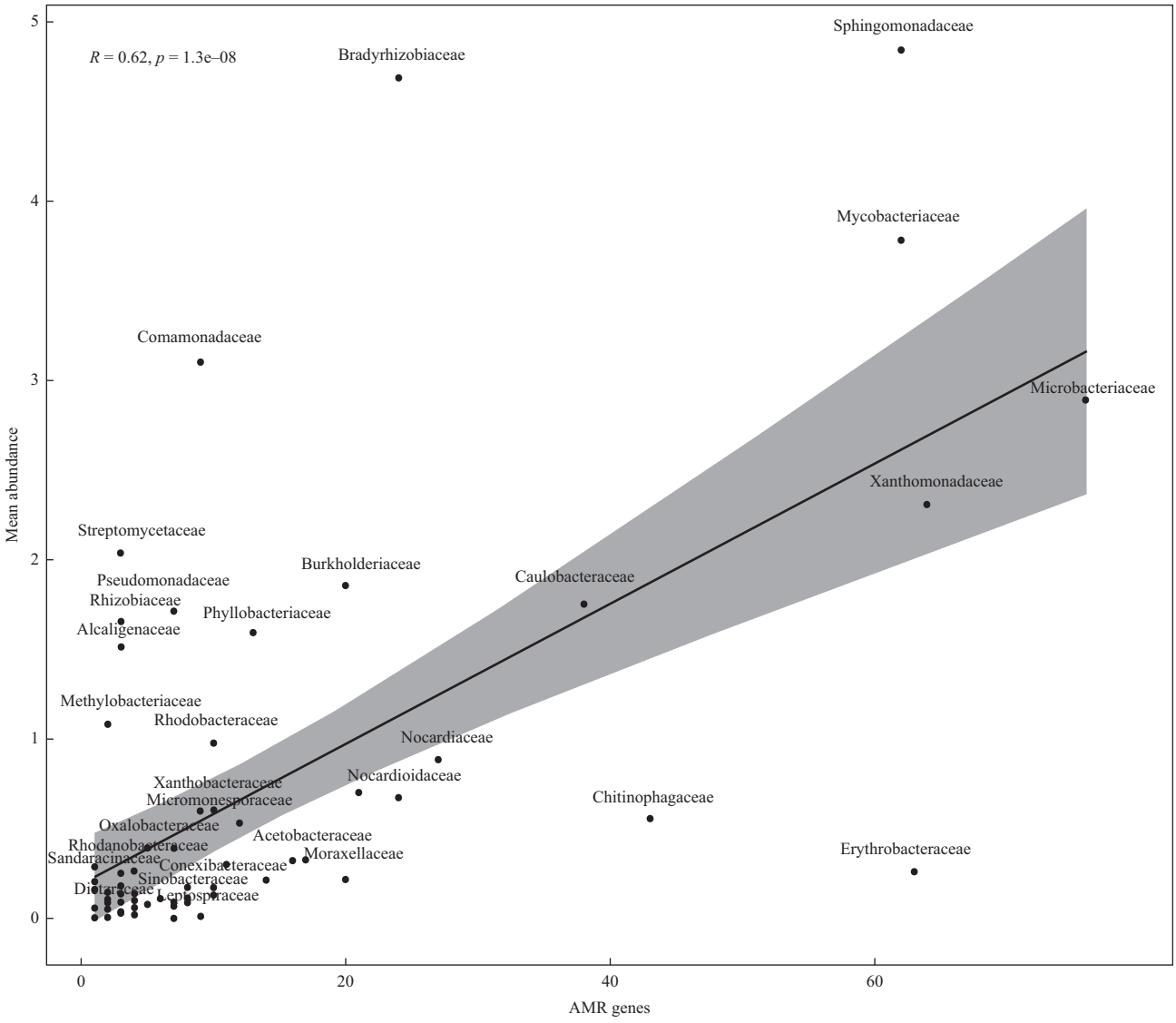

Figure 5. Correlation analysis comparing mean percentage abundance and antimicrobial resistance (AMR) gene count at family level, combined across all environmental wastewater pipe metagenomes (Pearson coefficient=0.62, r^2=0.3844). This correlation analysis figure was generated using R v4.1.0.

There are molecular data to suggest the spread of ARGs in the setting of high antimicrobial usage. Lerminiaux *et al.* [65] reviewed horizontal transfer of ARGs in clinical settings and concluded that it occurs primarily through conjugation, yet laboratory studies also implicate natural transformation and transduction as contributors to the spread of ARGs. Conditions for natural transformation and transduction are ubiquitous in clinical environments, but currently abilities to detect these gene transfer mechanisms are limited outside of controlled laboratory experiments. In the present study, three of the five patients whose clinical isolates were examined were accommodated in bays that had sections of pipework analysed in this study. These patients had been administered antimicrobials prior to the detection of their AMR clinical isolates, a well-recognized risk factor for the acquisition of AMR bacteria. These strains could have been selected for endogenously by exposure to the same strain, or could have been acquired from the WW hospital environment described herein.

As drains from all WW apparatus connect to the same sewage system, in effect forming a WW highway for the distribution of bacteria across the healthcare premise, these environmental WW sites on the ward could serve as reservoirs for AMR bacteria across the entire ward area. The study findings complement this rationale, and could influence both antimicrobial stewardship and cleaning strategies of hospitals. It is reasonable to state that the microbial communities studied were likely modified over time by repeated exposure to the microflora of multiple patients, antimicrobials and their metabolites, and cleaning agents. Furthermore, this work supports the increasing role for genomic-directed infection control strategies for tackling the

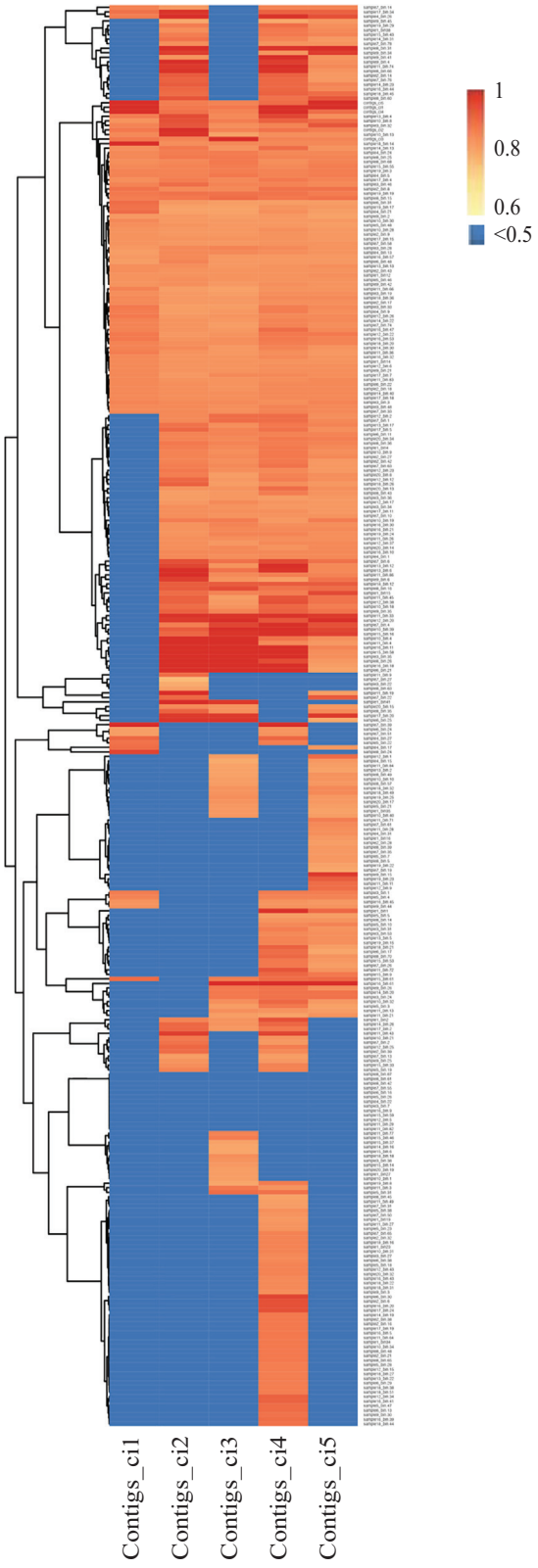

Figure 6. Average nucleotide identity analysis using pyani, showing the similarity of clinical isolate whole-genome sequences to metagenomically-assembled genomes generated from environmental DNA extracted from wastewater pipe sections. This pyani heatmap figure was generated using R v4.1.0.

spread of AMR by looking at hospital design and the location and design of WW apparatus relative to the patient zone. This study found no significant differences between the metagenomic results of patient and staff WW apparatus, which may indicate that the microbiological WW highway may be multi-directional and that there is substantial scope for contamination exposure throughout this system. A growing body of evidence suggests that WW-based epidemiology could play a role in the detection of novel antibiotic-resistant pathogens, and assist public health surveillance given the success of a Swedish study which described monitoring of severe acute respiratory syndrome coronavirus 2 and its variants over a 2-year period [66]. This work underscores the importance of characterizing antibiotic-resistant reservoirs in hospitals in this regard. As microbial diagnostics continue to advance, metagenomics may perform a pivotal routine role in surveying hospital environments to provide timely information for actionable interventions to prevent patients from nosocomial infections.

In conclusion, this study represents a unique large-scale metagenomic analysis of hospital WW pipes, at sites close to the human—WW system interface. As anticipated, analysis revealed a diverse microbiome in each of the WW pipe sections investigated, as well as a diverse resistome across all samples. Comparisons made between clinical isolates, isolates from the WW environment and associated ARGs revealed considerable degrees of similarity. When compared with the genomes of infection-causing clinical isolates, average nucleotide identity analysis suggests the presence of highly similar or identical genomes within the WW system. Whilst it is inappropriate to conclude definitively whether these isolates were transmitted from WW pipes to patients or *vice versa*, these data emphasize very clearly the potential for hospital WW systems to act as reservoirs of clinically relevant bacteria and ARGs. This evidence further supports requirements for effective infection control policies and working practices to prevent the transmission of nosocomial infections and the emergence of AMR.

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Author contributions

SAK: Writing — original draft; data curation; methodology; Writing — review and editing.
NHOC: Conceptualization; methodology; writing — original draft; writing — review and editing.
TT: Data curation; methodology; writing — review and editing.
LD: Methodology.
JW: Methodology.
CC: Methodology.
BS: Conceptualization; writing — review and editing.
PK: Writing — original draft; writing — review and editing.
JP: Methodology; writing — original draft; writing — review and editing.

BFG: Writing — original draft; data curation; methodology; writing — review and editing.
CPD: Conceptualization; writing — original draft; data curation; methodology; writing — review and editing.
All authors revised the manuscript critically and approved the final draft.

Conflict of interest statement
None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2023.09.001.

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