Antibiotic Resistance in Municipal Wastewater: A Special Focus on Hospital Effluents

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Contents

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Abstract Hospital effluents contain a hazardous amalgam of drug residues and infectious agents. Qualitative and quantitative evidence shows that hospital effluents are enriched in antibiotics, multidrug-resistant bacteria, antibiotic resistance genes and genetic vectors which could facilitate the horizontal transfer of these genes. This chapter provides an overview of the current status of antimicrobial resistance (AMR) surveillance in hospital effluents and draws comparisons to other AMR monitoring studies in domestic wastewaters and natural aquatic environments. We discuss approaches and standard tools that have been used to measure levels of AMR contamination and provide insights to the latest developments in the detection and profiling of AMR which have yet to gain traction in present surveillance programs.

Keywords Antibiotic residues, Antibiotic resistance genes, Antibiotic resistant bacteria, Hospital effluent, Resistome

Abbreviations

AMR	Antimicrobial resistance
ARB	Antibiotic-resistant bacteria
ARGs	Antibiotic resistance genes
CLSI	Clinical and Laboratory Standards Institute
CRB	Carbapenem
ESBL	Extended spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GC	Gene copy
HPLC-MS/MS	High-performance liquid chromatography coupled with tandem mass spectrometry
HT-qPCR	High-throughput quantitative polymerase chain reaction
MGE	Mobile gene element
MGEs	Mobile genetic elements
MLST	Multilocus sequence typing
qPCR	Quantitative polymerase chain reaction
SPE	Solid phase extraction
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization

1 Introduction

Antimicrobial resistance (AMR) is a menace in both community and healthcare facility settings. Hospital hygiene limitation and the overuse and misuse of antibiotics are factors contributing to the spread of antimicrobial drug resistance in hospitals [1]. The mode of transmission is complex and can occur between patients through the healthcare environment (surface, air, clothes), contaminated healthcare workers or others [2, 3]. Other sources of transmission are mediated through the use of invasive medical devices during surgical procedures which may result in hospital-acquired infections [4]. The selection pressure is imposed by the constant presence of antibiotics, which in turn accelerates the transfer of antibiotic resistance genes (ARGs) between bacteria by mobile genetic elements (MGEs) [1, 5, 6]. A transmission model of antibiotic-resistant bacteria (ARB) developed by Almagor et al. [6] showed that frequent antibiotic usage heightens the risk of transmission by increasing the vulnerability of susceptible patients and the contagiousness of colonized patients who are treated with antibiotics. The highest likelihood of AMR emergence and dissemination is through human transmission; however, hospital effluents that are loaded with microbes, infectious agents and pharmaceutical waste, originating from human sources, pose a significant public health risk if not sufficiently treated and discharged into receiving environments [7, 8].

On-site hospital wastewater treatment using advanced technologies (membrane bioreactor treatment, ozonation, granulated activated carbon, UV treatment) is capable of reducing ARGs and eliminating antibiotics in hospital effluents [9]; however, in most countries, there are no specific recommended or standardized treatments of hospital wastewaters. Hospital effluents are often routed for release into community wastewater treatment plants and co-treated with domestic wastewaters [10]. In rural areas of India, Nepal and Bangladesh, where wastewater management is inadequate, domestic effluent is directly discharged into receiving water bodies that are used as drinking water sources [11]. Despite the recognized risk of antibiotic resistance emergence and transmission, there is currently a lack of AMR surveillance in hospital wastewater. There is a clear need to survey current methods and practices, which can be applied to assess the spread of AMR from hospital effluents to the environment. This chapter reviews analytical chemistry methods, microbiological techniques and next-generation sequencing platforms which can be used to measure AMR loads in hospital effluents.

2 Antibiotic Residues

2.1 Antibiotic Residues in Hospital Effluents

Hospital effluents are important sources of antibiotics entering into the aquatic environment [12–19]. To date, analytical methods for determination and

quantification of target antibiotics in hospital effluents have been well developed and validated, in which high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) is widely used to identify and quantify antibiotic residues in hospital effluents as well as environmental water samples [20, 21].

2.2 Challenges in Quantification of Antibiotic Residues in Hospital Effluents

To have better understanding of the occurrence, fate and environmental risk of antibiotics in hospital effluents to public health and aquatic ecosystems, the development of robust sensitive analytical methods for simultaneous extraction and analysis of the target antibiotics is critically needed [21]. One of the challenges in the determination of antibiotics in hospital effluents is related to simultaneous extraction of antibiotics from hospital effluent samples, since antibiotics are often present at very low concentrations (ng/L–μg/L) under complex matrices [21]. To date, solid phase extraction (SPE) is widely employed to enrich and purify a wide variety of target antibiotics from environmental samples [21–23]. However, apart from enrichment of target antibiotics, solid phase extraction may enrich some interferences that affect HPLC separation and MS/MS detection. In addition, it is challenging to extract simultaneously multiple classes of antibiotics using a single SPE cartridge as the antibiotics belonging to different classes tend to have different physicochemical properties (i.e. log K_{ow} and pK_a) and molecular structures. For these reasons, it is difficult to select a suitable single cartridge to extract simultaneously all target antibiotics in environmental samples [21].

The selection of a suitable SPE cartridge plays an important role in enhancing recovery of target antibiotics in environmental samples. Normally, the selection is often based on the physicochemical properties of target antibiotics and SPE adsorbent characteristics [21, 24, 25]. For example, Kasprzyk-Hordern et al. [25] chose a strong cation-exchange mixed-mode polymeric sorbent (Oasis MCX) for the simultaneous extraction of selected antibiotics (including ciprofloxacin, doxycycline, sulfamethoxazole, trimethoprim and erythromycin) and other pharmaceuticals and found that recovery for these analytes ranged from 61.6 to 82.5%. In another study, Babić et al. [24] used the Oasis HLB cartridge to extract seven antibiotics (sulfamethazine, sulfadiazine and sulfaguanidine, trimethoprim, oxytetracycline, enrofloxacin and penicillin G). Theoretically, the use of a specific SPE cartridge for each class of antibiotics may provide a good extraction recovery. However, this approach is time-consuming when analysing a large number of antibiotics with different physicochemical properties, and this approach is quite expensive due to SPE cartridge consumption. In a recent effort, Tran et al. [21] optimized the simultaneous extraction of 20 antibiotics and 2 antimicrobial agents belonging to 10 different classes in environmental water samples via using dual cartridges, Chromabonds HR-X (500 mg, 6 mL) [HR-X] coupled with Chromabonds SB (500 mg, 6 mL) [SB].

In addition to extraction, the detection and quantification of antibiotics in hospital effluents are challenging. To date, the use of HPLC-MS/MS is considered to be the best analytical instrument for the detection and quantification of antibiotics in wastewater matrices as it has high sensitivity and selectivity for target analytes compared to other instruments (i.e. HPLC-UV, HPLC-FID, etc.). However, the matrix effect in wastewater samples may lead to reduced detection sensitivity [13]. For example, in a previous study, Gómez et al. [13] found that significant signal suppression (ca. 85%) was observed for erythromycin when using LC-MS/MS for quantification. Hitherto, matrix effects are often corrected using a matrix-matched standard calibration method [13, 26–29], but this approach is challenging to apply for routine monitoring of environmental samples because matrices of environmental samples vary from place to place and from time to time. In such circumstances, the selection of a representative blank with a matrix composition similar to the samples is impossible. Therefore, the accuracy of the analytical methods based on matrix-matched standards calibration approach is limited. To tackle the issues regarding the losses of antibiotics during sample preparation (i.e. storage and extraction) and matrix effects during HPLC-MS/MS, the use of isotopically labelled surrogate/internal standards is deemed to be more accurate for quantification of antibiotics in environmental samples in general and hospital effluents in particular [21].

In short, the use of HPLC-MS/MS coupled with isotope dilution is a recommended option to detect and quantify antibiotics in hospital effluents as well as other environmental water samples (i.e. municipal sewage and surface water), because it allows correcting the losses, matrix effects and instrumental fluctuations during analytical processes.

2.3 *Occurrence of Antibiotics in Hospital Effluents*

The occurrence of multiple classes of antibiotics in hospital effluents has been well documented [13, 14, 17, 30–32]. For example, Gómez et al. [13] reported that the concentrations of trimethoprim and erythromycin in hospital effluents in Spain varied from 10 to 30 ng/L. In another study, Duong et al. [14] found that the concentrations of ciprofloxacin and norfloxacin in hospital wastewater in Vietnam ranged from 1.1 to 44 μg/L and from 0.9 to 17 μg/L, respectively. In a recent study, Thai et al. [17] measured the occurrence of beta-lactams, sulfonamides, macrolides, trimethoprim and fluoroquinolone in hospital effluents in Vietnam and found that the concentrations of detected antibiotics ranged substantially from below detection limit (beta-lactams) to over 40 μg/L (fluoroquinolone antibiotics). Similarly, in an earlier study in Singapore, Le et al. [32] found the presence of macrolides, fluoroquinolones, sulfonamides, beta-lactams, lincosamides, tetracycline and trimethoprim in hospital effluents, in which the maximum concentration of macrolide (clarithromycin) and fluoroquinolone (ciprofloxacin) was greater than >70 μg/L while other antibiotic classes such as lincosamides, tetracyclines and beta-lactams were rarely detected, even though

beta-lactams are known to be one of the most consumed antibiotic classes. The presence and concentrations of antibiotics in hospital effluents tend to depend on the compound and type and size of hospitals.

3 Antibiotic-Resistant Bacteria (ARB)

3.1 ARB in Hospital Effluents

Hospital wastewater contains a mixture of antibiotic residues, disinfectants, metabolized and non-metabolized drugs and bacterial shedding from patients' excreta [33–35]. As a result, hospital wastewater discharged to receiving waters could contribute to AMR dissemination in the natural environment if insufficiently treated [36].

Gram-negative bacteria are of particular concern in hospital settings, with the ability to cause pneumonia, bloodstream, wound, or surgical site infections [4]. In 2017, the World Health Organization (WHO) responded to the burgeoning antimicrobial resistance threat by publishing a priority list of antimicrobial-resistant pathogens which pose problems in human infections, failure to respond to current antibiotic treatment and transmissibility between humans and animals. Within the list, of highest priority are carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* and carbapenem-resistant, extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* [37]. These guidelines provide a context to bacterial targets and patterns of resistance which should be incorporated into surveillance strategies.

3.2 Methods to Detect ARB and Commonly Used Susceptibility Testing Method

To isolate ARB in hospital effluents, wastewater samples are serially diluted and filtered through nitrocellulose membranes to trap biomass or spread-plate on media at dilutions required to capture viable bacterial populations within a countable range. Typically, Luria-Bertani medium [32] or selective media such as MacConkey agar [38] or CHROMagar [39] are used to support growth of viable bacteria. Colonies can then be sub-cultured and taxonomically characterized using Sanger sequencing targeting the 16S rRNA gene, multilocus sequence typing (MLST), MALDI-TOF bacterial identification or whole genome sequencing. Isolates identified, subjected to antibiotic susceptibility testing to determine resistance patterns, permit the determination of the ratio between ARB and total number of viable bacteria, which can be designated as prevalence. Alternatively, antibiotics are supplemented into media to directly select for the ARB growth and expressed as total concentrations of viable ARB.

To determine antibiotic minimal inhibitory concentrations (MIC) of bacterial isolates, manual procedures include the broth dilution tests, the antimicrobial gradient method and the disk diffusion test. Amongst the automated instrument systems, the BD Phoenix Automated Microbiology System (BD Diagnostics), the VITEK 2 System (bioMerieux) and the Sensititre ARIS 2X (Trek Diagnostic Systems) are the most commonly used [40]. Manual procedures such as the disk and gradient diffusion methods allow customization and cost savings. All these techniques provide qualitative assessments using the categories: susceptible (S), intermediate (I) or resistant (R). However, reliable interpretation of MIC values requires constant updating of current clinical breakpoints using either the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines of specific bacterial pathogens [40].

3.3 Occurrence of ARB in Hospital Effluents

The AMR selective pressure is particularly high in hospitals. For example, 20–30% of European inpatients receive an antibiotic treatment during their hospitalization [41], and antibiotics as well as antibiotic-resistant bacteria (ARB) excreted from inpatients receiving treatment contribute to the composition of hospital effluent [42, 43].

A study by Le et al. [32] described various taxa of viable ARB cultured from the effluents of two hospitals in Singapore, which showed resistance to different classes of clinically relevant antibiotics. Concentrations of ARB resistant to amikacin (1.06×10^6 CFU/mL), clindamycin (1.37×10^6 CFU/mL), erythromycin (1.24×10^6 CFU/ml), ciprofloxacin (1.14×10^6 CFU/mL) and tetracycline (1.30×10^6 CFU/mL) were at least one order of magnitude higher than those of meropenem (4.79×10^5 CFU/mL), ceftazidime (8.22×10^5 CFU/mL), vancomycin (9.19×10^5 CFU/mL), chloramphenicol (6.08×10^5 CFU/mL) and co-trimoxazole (2.54×10^5 CFU/mL). Using the same hospital effluent samples, Haller et al. [39] used a selective culture-based screening approach on chromogenic agar to specifically target Gram-negative extended-spectrum beta-lactamase (ESBL)-producing bacteria and bacteria with a decreased susceptibility to carbapenems (carbapenem-resistant bacteria, CRB). Concentrations of ESBL producers ranging from 10^3 CFU/mL to 10^6 CFU/mL and mean concentrations of CRB ranging between 10^3 CFU/mL and 10^5 CFU/mL were detected in the hospital wastewaters. Amongst the isolated bacterial strains, 35% were resistant to ceftazidime, and 39% were resistant to ceftriaxone (third-generation cephalosporins), while resistance to ertapenem and meropenem were 19 and 26%, respectively [39]. Another study by Korzeniewska and Harnisz [44] described a ceftazidime resistance rate of 81.6% in *Enterobacteriaceae* strains isolated from hospital wastewater in Poland. Picão et al. [45] measured resistance levels to third-generation cephalosporins ranging between 35 and 79% in hospital sewage in Brazil and levels of meropenem resistance of about 22%, on average.

A wide range of environmental bacteria as well as opportunistic pathogens from the gut microbiota of humans and other animals has been described in hospital wastewater discharge, but this chapter will specifically focus on those published on the WHO priority list.

3.3.1 Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli*

The concentration of *E. coli* in hospital and community wastewaters typically lies within the same range (10^7–10^8 CFU/100 mL), but concentrations of ESBL-producing *E. coli* are generally higher in hospital effluents with percentages ranging between 3.8 and 39% [33, 35, 44, 46, 47]. This is attributed to higher incidence and density of carriage amongst inpatients compared to community carriers [48]. In addition, hospital effluents contain large quantities of antibiotics and antiseptic residues that might favour as well as further the development of ESBL-producing *E. coli.*

3.3.2 Multidrug-Resistant *Pseudomonas aeruginosa*

P. aeruginosa is increasingly recognized as an emerging opportunistic pathogen of clinical importance. It is a widespread hospital-acquired pathogen responsible for respiratory and urinary tract infections especially in intensive care units, where 15% of healthcare-associated infections are attributed to this pathogen [49]. One of its most worrying characteristics is its low antibiotic susceptibility due to its concerted action of multidrug efflux pumps and low permeability of bacterial cellular envelope, as well as the liability to acquire and express antibiotic resistance genes through plasmids, integrons or other mobile genetic elements. *P. aeruginosa* is noted for its intrinsic resistance to certain antibiotics and for its ability to acquire genes encoding resistance determinants [50]. Multidrug resistance in *P. aeruginosa* occurs mainly in clinical settings, which is a result of chromosomal mutations or horizontal gene transfer. As opposed to *E. coli*, *P. aeruginosa* is not a commensal human bacterium, and the frequency of carriage amongst inpatients is low [51]. *P. aeruginosa* is ubiquitous in wastewater; however, the proportion of antibiotic-resistant *P. aeruginosa* is much higher in hospital than in urban wastewater [49, 52–55]. Recent studies reported the presence of multidrug-resistant ESBL-producing *P. aeruginosa* in hospital effluents [39].

3.3.3 Antibiotic Resistant *Acinetobacter baumannii*

A. baumannii can cause various infections like nosocomial pneumonia, bacteraemia, meningitis, and skin and soft tissue and urinary tract infections. The incidence of serious infections (blood stream infections and ventilator-associated pneumonia)

caused by multidrug-resistant *A. baumannii* ranges between 47 and 93%, with mortality rates between 30 and 70% [56]. The overuse of carbapenems has rapidly resulted in the worldwide dissemination of carbapenem-resistant *A. baumannii* strains, as reported in studies from Croatia and China [57]. The observation of multidrug-resistant *A. baumannii* in hospital wastewater has also been previously reported in Brazil, China and Zagreb in Croatia [58–60].

3.3.4 Vancomycin-Resistant Enterococci (VRE)

Enterococci are Gram-positive bacteria, which are part of the natural intestinal microbiota of animals and humans, and are released to the environment through sewage or wastewater [61]. Some members of the genus, such as *Enterococcus faecalis* and *Enterococcus faecium*, are amongst the major causes of nosocomial infections worldwide [62]. One factor contributing to the pathogenesis of enterococci is their resistance to a broad range of antibiotics. This resistance trend has increased in recent years [63]. Vancomycin is a glycopeptide antibiotic used for serious infections by Gram-positive bacteria when treatment with other antibiotics has failed. The excessive use of this antimicrobial agent has led to the appearance of vancomycin-resistant enterococci (VRE). Concentrations of enterococci in urban and hospital wastewater have been found to be similar, although the proportion of VRE was detected in higher concentrations in hospital than in urban effluents [63–66]. Varela et al. [66] reported concentrations ranging between 2.50×10^1 and 2.30×10^3 CFU/mL and between 1.60×10^1 and 2.20×10^3 CFU/mL of enterococci resistant to ciprofloxacin and vancomycin, respectively, in hospital effluents. Hospital effluent constitutes a source of enterococci having multiple resistances to antibiotics, presumably from the faeces of patients, because the rules of biosecurity in medical centres would impede other sources of contamination [67].

4 Antibiotic Resistance Genes (ARGs)

4.1 ARGs in Hospital Effluents

One of the major aspects of understanding antibiotic resistance in hospital effluents is to detect and quantify ARGs. Molecular techniques such as real-time quantitative PCR (qPCR), including singleplex, multiplex and high throughput, and metagenomics have been employed to identify and quantify ARGs in hospital effluent samples.

4.2 Application of High-Throughput qPCR (HT-qPCR) to Measure ARGs and MGE

Characterizing and quantifying resistomes are a rapid method of assessing AMR pollution. Probes and primers designed to target ARGs that confer resistance to different classes of antibiotics provide quantitative information on evaluating the abundance of genes. Unlike the traditional qPCR approach which is limited to a few targets in one assay, high-throughput qPCR (HT-qPCR) arrays are able to simultaneously quantify hundreds of ARGs and other related MGEs in one run [68]. There are a few commercially available platforms, which includes the Fluidigm Array, the Qiagen Antibiotic Resistance Genes Microbial DNA qPCR Array, OpenArray by Applied Biosystems and the WaferGen Biosystems SmartChip Real-Time PCR. Each system allows customization of primers depending on the ARGs or MGE of interest. The utility of HT-qPCR arrays has been demonstrated in environmental surveys aimed at comparing relative concentrations of ARG contamination across different aquatic sources such as lakes and estuaries [69–71], sediments of fish farms [72], drinking water [73] and wastewater treatment plants [74, 75]. Monitoring efforts of ARGs and MGE in hospital wastewaters are predominantly based on data generated from traditional qPCRs targeting the few clinically relevant beta-lactamase genes (e.g. bla_{KPC}, bla_{NDM}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{TEM}, bla_{SHV}) [32, 76–79] with a shifting trend towards using upscaled customized HT-qPCR with increased capacity to detect more ARG targets and markers of MGE [80].

4.3 Prevalent ARGs and MGEs in Hospital Wastewaters Globally and Comparisons with Other Water Sources

High prevalence of sulfonamide (*sul*) and tetracycline (*tet*) genes has been detected in various environments and deeply studied by many groups [32, 81, 82]. In addition, investigations on MGEs such as *intl*1 (class 1 integron-integrase) were included in many studies as integrases have been statistically correlated with anthropogenic sources of ARGs and are potentially involved in ARGs integration in chromosomes or plasmids [32, 81]. However, owing to the rise in importance of beta-lactam resistance and WHO's recent announcement of the global priority list that consists of beta-lactam-resistant pathogens, there is a shift in trend towards studies focused on the detection and quantification of beta-lactamase genes (e.g. bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{OXA}) [9, 82, 83]. Emerging genes such as bla_{KPC}, $bla_{NDM\text{-}1}$ and *mcr-1* are of concern in recent years due to their possible origin from hospitals, their occurrences in plasmids residing in multidrug resistance "superbugs" and the potential for these genes to spread amongst the bacteria community via horizontal gene transfer [84].

A comparison of resistomes in the final effluents of seven European countries (Portugal, Spain, Ireland, Cyprus, Germany, Finland, Norway) using HT-qPCR showed that AMR profiles mirrored patterns of clinical antibiotic resistance prevalence, providing insightful information on country- or region-specific trends of AMR distribution [85]. In a study conducted in China by Li et al. [81], ten *tet* genes (A, B, C, G, L, M, O, Q, W, X), *sul1*, *sul2* and *intl*1 were detected in hospital effluents using singleplex qPCR, with *intl*1 concentrations as high as 10^{11} gene copies (GC) per mL. Compared to residential area effluents studied in parallel with hospital effluents, the total gene abundances from hospital effluents (1.81×10^{11} GC/mL) were slightly lower as compared to residential effluents (2.79×10^{11} GC/mL). In contrast, Lamba et al. [83] compared 12 hospitals and residential effluents in New Delhi, India, where the gene targets were bla_{TEM}, bla_{OXA}, $bla_{\text{CTX-M}}$ and $bla_{\text{NDM-1}}$, and found that all the ARG concentrations had higher relative abundance (normalized to 16S rRNA genes) in hospital effluents than in residential effluents. The differences in ARG abundance could be attributed to antibiotic usage and human demographics where healthy asymptomatic individuals within the community could serve as carriers of ARGs [86].

The effects of discharging untreated hospital effluent into other environments have been evaluated in a few studies. In Tamil Nadu, India, samples were taken from five hospital effluents and five points upstream and downstream of the Cauvery River Basin where genes encoding for beta-lactamase (bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV}, $bla_{\text{NDM-1}}$) and aminoglycosides (*aadA*) were quantified [79]. Results showed that bla_{SHV} and $bla_{\text{NDM-1}}$ were not present upstream but were detected downstream of the river, indicating that these genes were likely introduced by wastewater. However, it was inconclusive if the genes were derived directly from hospital effluents as the source of wastewater discharge was from a combination of effluents originating from residential areas, industries and hospitals. Rodriguez-Mozaz et al. [82] quantified five ARGs (bla_{TEM}, *ermB*, *qnrS*, *sul1*, *tetW*) from hospital effluent; influent and effluent of a nearby wastewater treatment facility located in Girona, Spain, that receives the hospital effluent; and water upstream and downstream of the river that receives treated wastewater effluent. All the ARG targets in the hospital effluents were found to be of similar concentrations as compared to wastewater influents, ranging from 3 to 7 log GC/mL, but were significantly higher as compared to the other locations (wastewater effluent, river upstream and downstream) sampled. This implies that the ARG concentrations in domestic wastewater were of similar concentrations as compared to the hospital effluents. On the contrary, a massive study done in the Netherlands by Pallares-Vega et al. [87] concluded that healthcare facilities such as hospitals had minimal impact on the concentrations of ARGs entering wastewater treatment facilities. This could be an effect of dilution by domestic wastewater that have lower ARG concentrations resulting in an overall reduction in the abundance of ARGs. It is however worthwhile to note that within the same study, there was an increase in the relative abundance of broad-host-range *IncP-1* type plasmids which are known to carry broad-spectrum ARGs and are

transmissible between Gram-negative bacteria. The demographics and antibiotic usage patterns in humans and animal and differences in regulations governing the sale and use of antibiotics differ from one country to the next which could explain varying global trends in AMR.

To understand AMR trends and occurrence patterns, research groups have designed studies to compare ARG concentrations of effluents derived from different ward types across different hospitals locally to facilitate stewardship efforts. For example, a study by Le et al. [32] concluded that effluents from clinical isolation wards had higher concentrations of ARGs compared to general wards. Another study by Li et al. [81] noted significant differences in total resistance gene abundance across seven hospitals, with concentrations ranging from 10^7 to 10^{11} GC/mL. A detailed study by Lamba et al. [83] correlated the concentration of $bla_{\text{NDM-1}}$ across 12 different hospitals of different capacities and found that larger hospitals were discharging higher concentrations of $bla_{\text{NDM-1}}$. The authors concluded larger hospitals that receive higher volumes of inpatients likely result in higher AMR output in wastewaters.

In contrast to singleplex qPCR, HT-qPCR is able to profile a wider number of ARG and MGE targets in one run which provides higher throughput to comprehensively assess vectors of AMR in hospital effluents. In contrast to metagenomic profiling, HT-qPCR is more sensitive and requires less starting DNA material (PCR reactions at the nanolitre scale) with the ability to detect concentrations of 10^{-4} ARGs/16S rRNA gene [72, 88]. There are four main HT-qPCR platforms currently available in the market, with Biomark Dynamic Array (Fluidigm) requiring the lowest reaction volumes (~10 nL) followed by OpenArray (Biosystems ~35 nL reactions), WaferGen SmartChip (WaferGen ~100 nL reactions) and Bio-Rad CFX384 (Biorad ~3,000 nL reactions) [88].

In a study done in Xinxiang City, Central China, Wang et al. [80] fabricated 258 qPCR primers and utilized a HT-qPCR platform to detect 178 unique ARG targets that confer resistance to seven classes of antibiotics and two MGE targets (*intl1* and *Tn916/Tn1545*) to compare concentrations of wastewater from three tertiary public hospitals in the city. A core of 126 ARGs were detected in all three hospital effluents. Concentrations of 12 frequently detected ARGs (*tetM*, *tetO*, *tetX*, *ereA*, *ermA*, *ermB*, *sul1*, *sul2*, *sul3*, *qnrA*, *qnrB*, *oqxB*) were validated by qPCR yielding results of highest concentrations of *tetO* detected in effluents of two hospitals, with *sul1* detected at high abundance in the effluents of the third sampled hospital. Amongst the five MGEs (*intI1*, *intI2*, *intI3*, *Tn916/Tn1545*, *ISCR1*), *ISCR1* had the highest abundance ranging from 10^7 to 10^8 GC/mL. It would be advantageous to use HT-qPCR for routine monitoring of hospital effluents as it is time-efficient, with a low sample volume requirement per reaction, without the reliance on complicated downstream bioinformatics analyses when compared to using metagenomics.

5 Resistomes and Mobile Genetic Elements (MGEs)

5.1 Uncovering Resistomes by Metagenomics

In the field of water research, integrated multi-omics approaches have been used as bio-monitoring tools for water quality assessment to investigate microbial composition, their functional roles and involvement in water contamination [89]. One of the advantages of metagenomics in AMR surveillance is the collective recovery of genomes from microbes in environmental samples which provides genetic insights to microbial composition (bacterial and viral), ARGs and other MGEs (e.g. plasmids, integrons, transposons). The ability to capture entire genomic profiles to track the distribution of ARGs and MGEs in a variety of environments has made it possible to assess and identify AMR hotspots in different aquatic compartments [96], sources and sinks of ARGs in environmental waters [90], ARG removal in the wastewater treatment process [91, 92] and fate and transport of ARGs in environments receiving treated wastewater effluents [93–95]. There are a handful of studies which have applied metagenomics as an opportunity to create ARG and microbiome catalogues of hospital wastewaters to identify novel carbapenemases [96] and to classify and resolve differences between municipal wastewaters [97, 98] and waters receiving treated hospital wastewaters [99].

Environmental resistomes are profiled by interrogating metagenomic reads or assembled contigs against one of the publically available ARG databases such as the Comprehensive Antibiotic Resistance Database [100], the Antibiotic Resistance Database [101], Resfams [102], ARG-ANNOT [103], ResFinder [104], MEGARes [105] and ARGs-OAP [106]. The relative abundance of ARGs identified from metagenomic datasets is then calculated by normalizing to the ARG reference sequence length (nucleotide) and to the number of 16S rRNA genes [107] or by coverage normalized to the ARG reference gene and size of metagenomic dataset [108].

5.2 Identifying MGEs

Intercellular mechanism of exchange mediated by MGEs such as plasmids, transposons and integrons play a major role in AMR dissemination as they facilitate the capture, transfer and expression of exogenous ARGs [109, 110]. The mobility of plasmid-borne ARGs and the rates of inter- and intraspecies transfer in hospital effluents are largely unknown. In a laboratory-scale experiment, Chen et al. [111] demonstrated plasmid transferability through mating a ceftazidime-resistant strain of *A. baumannii* isolated from hospital wastewaters with a ceftazidime susceptible *E. coli* as a recipient. Whole genome sequencing of plasmids in donor and transconjugants showed highly similar sequences, concluding that plasmid-mediated intraspecies transfer of ARGs had occurred. Interspecies transfer of ESBL-encoding

plasmids between microbial community within a hospital sink environments has been inferred [112, 113]. However, there is limited data available on the frequency and environmental cues that trigger ARG transfer in clinical wastewaters.

Class 1 integron gene cassettes, which are frequently carried by human pathogens, often include ARGs acquired by genetic recombination. Hence, insight to their presence in hospital wastewater may contribute to infer about the risks associated with ARG dissemination [96]. There are a range of bioinformatics tools designed for in silico detection of integrons (I-VIP [114], MARA [115], INTEGRALL [116]) and plasmids which are carried by *Enterobacteriaceae* and Gram-positive bacteria (PlasmidFinder [117]). Inspecting assembled metagenomic datasets (contigs) for co-localization of ARGs and MGE features provides a means of exploring specific mechanisms that mediate the spread of certain ARG types, with this analysis approach proposed as a method to predict ARG mobility incidence in environmental resistomes [118].

5.3 *Examples of the Application of Metagenomics to AMR Monitoring in Hospital Wastewaters*

There is currently more literature on resistome profiles in hospital wastewaters using either qPCR or HT-qPCR, rather than metagenomics. This could be attributed to better sensitivity (detection limits) offered by qPCR platforms that are target specific and easily interpreted [88]. A metagenomics approach in contrast seizes information of known and unknown DNA sequences in a single run, thus providing a greater depth of sequence information without the restraint of a specific targeted sequence [119]. For example, within the context of AMR monitoring in wastewaters, Le at al. [32] used qPCR to detect the relative abundance of four beta-lactamase ARG targets (bla_{NDM}, bla_{KPC}, $bla_{\text{CTX-M}}$, bla_{SHV}). However, a more in-depth metagenomics analysis of the same samples gave a snapshot of the entire ARG diversity within the hospital wastewater samples and allowed the assembly of entire scaffolds providing information on ARG arrangements and co-occurring MGEs within the same gene neighbourhood [97]. Leveraging on the latest DNA sequencing technologies by combining long-read data from third-generation sequencing platforms (Oxford Nanopore) with Illumina short-read data has yielded better sequencing coverage and assembly of ARG bearing plasmids as described in a study of wastewater treatment plants [120].

AMR metagenomics studies conducted in Singapore [97], the Netherlands [99] and France [98] appear to have a consistent pattern of a core microbiome specific to hospital wastewaters. All three studies reported the predominance of anaerobic human gut bacteria belonging to the order *Clostridiales*, *Bifidobacteriales* and *Bacteroidales*. There were however other dominant bacterial taxa (e.g. *Acinetobacter baumannii*, *Enterobacteriaceae*) that contributed to differences in hospital wastewater microbiomes originating from different countries [96–99], which could explain AMR variations globally [119].

As an extension of the utility of rapid AMR and microbiome profiling, Li et al. [121] demonstrated that by integrating metagenomics datasets from different sources with complementary metadata into machine learning classification models, AMR source contribution could be identified to predict putative sources of ARG contamination. This would be particularly useful in tracking the dissemination of AMR originating from hospital effluents.

5.4 *Targeted Metagenomics for Qualitative and Quantitative Resistome Analysis*

One of the challenges with the application of metagenomics within complex microbial communities is the detection sensitivity of low abundant bacterial populations that harbour ARG [122]. To overcome the limitation of heterogeneity, Lanza et al. [123] adopted an in-solution targeted capture platform (TCP), a technique used for diagnosis of human-inherited diseases [124] to develop a targeted metagenomic resistome analysis method coined "ResCap", a TCP based on SeqCapEZ (NimbleGen) technology. The TCP is designed to target ~78,000 nonredundant genes, comprising of ARGs, genes conferring resistance to metals/biocides and relaxase genes as plasmid markers [123]. Briefly, whole-metagenome shotgun libraries are constructed, and DNA captured by the probes are sequenced using Illumina platform and analysed using the ResCap bioinformatics workflow. Comparison of resistomes identified by metagenomic shotgun sequencing versus the ResCap platform showed improved gene abundance detection of 2–83% and increase of gene diversity detection by 300-fold [123]. This underscores the large proportion of ARGs that go undetected by relying on just metagenomics alone. The sensitivity and specificity of the ResCap technology provide qualitative and quantitative means of measuring the levels of ARG contamination which could potentially meet the needs of AMR monitoring and tracking from source to sink.

5.5 *Other OMIC Strategies to Study ARG Expression Levels in Hospital Wastewaters*

To understand the activity and contribution of ARB to AMR dissemination in linked aquatic environmental sources, a combined OMIC approach of metagenomics and metatranscriptomics was used to detect ARG transcripts in wastewaters from hospital and farm effluents into a receiving river in Cambridge, United Kingdom [125]. The authors reported a significant overexpression of bla_{GES} and bla_{OXA} in hospital effluents over a consistent period of 5 months relative to the two other sampled waters which was considered to be due to the levels of antibiotic usage in hospitals [125].

6 Curbing the Spread of AMR

Antimicrobial stewardship in hospitals is a prescribed intervention strategy by the WHO Global Action Plan to contain the spread of AMR beyond the clinical setting [126]. National or regional surveillance networks that monitor antibiotic usage and resistance using standardized methods will enhance knowledge on the extent of AMR severity, region-specific prevalence trends and associated health outcomes [127]. Antimicrobial peptides, probiotics, phage therapy and phage endolysins have been proposed as alternative replacements of antibiotics. However, safety and efficacy in vivo in humans have yet to be determined [128, 129].

7 Possible Treatment Technologies of Hospital Wastewaters

As hospital effluents are recognized as sources of AMR, there is increased awareness in possibly pretreating effluents before discharging them into municipal sewage. Two published studies have attempted to evaluate the removal efficiency of ARB and/or ARGs and MGE.

In Riyadh, Saudi Arabia, Timraz et al. [38] investigated the removal efficiency of wastewater treatment systems placed on site at two different hospitals. Although both treatment plants utilized conventional activated sludge process followed by chlorination, one plant outperformed in terms of log removal values of total viable bacteria and ARGs. The ARGs *sul1* and *intl*1 remained detectable at concentrations of up to 10^5 GC/mL in hospital effluent from the plant with a more interior removal performance. This observation suggests that operational parameters of wastewater treatment plants play a vital role in removal efficiencies of vectors of AMR.

Paulus et al. [9] compared the removal efficiency between an advanced on-site treatment facility (membrane bioreactor/ozonation/activated carbon/UV treatment), in two Dutch cities which received hospital effluent directly, and a municipal wastewater treatment facility, which received both hospital and residential effluents. Data showed significantly higher removal efficiency for the 13 ARG targets by the advanced treatment as compared to the municipal treatment facility. The study recovered the ARGs bla_{KPC} and *vanA* only in hospital effluents, which suggests that healthcare facilities are potential sources of these clinically important ARGs.

Other known methods, such as coagulation [130] and the use of biochar [131], have been demonstrated to effectively remove ARB, ARGs and antibiotic residues, although these methods have only been used to treat other types of waste other than hospital effluents. Nevertheless, these methods have the potential to pretreat hospital effluents before discharge into the main sewers.

8 Conclusion

This review covers the detection and quantification of the three main aspects of AMR (antimicrobial residue, antibiotic resistance bacteria and genes) and their occurrences in multiple hospital discharges. There is a need to step up surveillance systems of wastewater discharged from hospitals which are potential drivers for the spread of AMR. Factors which influence differences in AMR occurrence are dependent on the age and size/capacity of the hospital and the severity and types of infections amongst inpatients. The implementation of the latest molecular and OMIC techniques reviewed in this chapter could provide new standardized methods of qualitatively and quantitatively assessing the dissemination of a wider array of ARGs in different aquatic sources. Physical and chemical treatment processes can be put in place to pretreat hospital discharges in order to reduce the spread of AMR into receiving domestic wastewater treatment facilities or natural water bodies.

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