



Wastewater-based epidemiology for tracking bacterial diversity and antibiotic resistance in COVID-19 isolation hospitals in Qatar

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SUMMARY

Background: Hospitals are hotspots for antimicrobial resistance genes (ARGs), and play a significant role in their emergence and spread. Large numbers of ARGs will be ejected from hospitals via wastewater systems. Wastewater-based epidemiology has been consolidated as a tool to provide real-time information, and represents a promising approach to understanding the prevalence of bacteria and ARGs at community level.

Aims: To determine bacterial diversity and identify ARG profiles in hospital wastewater pathogens obtained from coronavirus disease 2019 (COVID-19) isolation hospitals compared with non-COVID-19 facilities during the pandemic.

Methods: Wastewater samples were obtained from four hospitals: three assigned to patients with COVID-19 patients and one assigned to non-COVID-19 patients. A microbial DNA quantitative polymerase chain reaction was used to determine bacterial diversity and ARGs.

Findings: The assay recorded 27 different bacterial species in the samples, belonging to the following phyla: Firmicutes (44.4%), Proteobacteria (33.3%), Actinobacteria (11%), Bacteroidetes (7.4%) and Verrucomicrobiota (3.7%). In addition, 61 ARGs were detected in total. The highest number of ARGs was observed for the Hazem Mebaireek General Hospital (HMGH) COVID-19 patient site (88.5%), and the lowest number of ARGs was found for the HMGH non-patient site (24.1%).

Conclusion: The emergence of contaminants in sewage water, such as ARGs and high pathogen levels, poses a potential risk to public health and the aquatic ecosystem.

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Introduction

Antimicrobial resistance (AMR) is a global public health challenge with significant clinical, social and economic consequences [1]. Among healthcare facilities, secondary care hospitals are at the forefront for the emergence and spread of

antibiotic resistance genes (ARGs), mainly due to host and pathogen factors, including immunosuppression, with the prevalence of multi-drug-resistant organisms (MDROs) accentuated by high consumption of antimicrobials [2,3]. Therefore, up-to-date monitoring and surveillance of antibiotic resistance levels are essential to guide presumptive and tailored therapy [4]. In addition, timely recorded monitoring can alert hospitals to the onset of possible outbreaks, and assist in prioritizing suitable interventions and evaluating their efficacy [5]. In modern healthcare settings, antibiotic consumption and resistance are monitored by applying the components of the antimicrobial stewardship programme (ASP), coupled with annual antibiogram monitoring and surveillance using accumulated antimicrobial consumption data and laboratory reports from clinical samples [6]. Despite enormous efforts towards the monitoring process, significant surveillance gaps exist. Foremost, surveillance concentrates on a restricted number of pathogens, which prevents the capture of complete ARG profiles often carried by commensal bacteria. Additionally, monitoring often relies on the belated reporting of infections isolated from patients, resulting in delayed outbreak detection [7]. Such significant global challenges prompted international organizations such as the World Health Organization to promote implementation of the One Health approach towards AMR, where integration of human, animal and environmental factors should be studied collectively to combat the escalating problem [8].

Nevertheless, efforts are mainly directed towards human and animal elements, compared with environmental factors. Interestingly, wastewater-based epidemiology (WBE) has been consolidated as an efficient alternative for tracking antibiotic resistance [7]. WBE is a rapid technique that provides essential qualitative or quantitative information about the inhabitants' health and behaviour within a given wastewater catchment area through the detection of urinary and faecal markers in combined municipal sewage [9]. This methodology was first proposed to evaluate the use of illicit drugs and misused therapeutic medications within a community in 2001 [10]. WBE has been applied to other indicators, including alcohol, pathogens, therapeutics and AMR markers [11–13]. Furthermore, with the emergence of the coronavirus disease 2019 (COVID-19) pandemic, many studies have shown that WBE is a practical approach for monitoring COVID-19 outbreaks [14,15]. Moreover, the COVID-19 pandemic was directly linked to the propagation of AMR in healthcare settings, but the spillover to the environment has not been widely studied [16,17]. The concept of WBE relies on the observation that wastewater is one of the primary pathways for introducing antimicrobials and resistant microbes into the environment. Testing wastewater for the presence and diversity of ARGs can reveal where, and to what degree, resistant bacteria are introduced into the environment. Wastewater from healthcare settings (e.g. hospital sewage) has been reported to be associated with higher antibiotic concentrations and a complex degree of resistance compared with other metropolitan areas [18]. Consequently, antibiotics and bacterial populations provide selective pressure for developing environmental resistance [2].

Water sampling is a non-invasive, cost-effective method for researching the concept. Furthermore, because such samples do not involve patient identification, no consent is required,

simplifying the process [2]. From a literature review, several previous studies have documented the presence of resistant bacterial species and the spread of ARGs from wastewater, including healthcare settings [19–22]. However, few publications have specifically identified these bacteria and provided in-depth analysis of their AMR profiles in hospital wastewater, despite significant concern for the One Health approach and the role of the environment as a reservoir for AMR strains. Therefore, this study focused on determining bacterial diversity and identifying embedded ARG profiles in hospital wastewater pathogens collected from COVID-19 isolation health facilities compared with non-COVID-19 facilities during the pandemic.

Methods

Settings and sampling sites

This study was conducted in Qatar, a country with a mixed, young population of approximately 3 million [23]. A universal public healthcare system is delivered through nine hospitals managed by Hamad Medical Corporation (HMC), one of the leading healthcare providers in the Middle East [24]. A schematic illustration of the sampling sites and how they are connected is shown in Figure 1. The effluent wastewater from the different hospital buildings is transported through maintenance holes of the drainage systems to the public foul sewer without prior treatment. Next, the hospital wastewater joins the community wastewater collection system, eventually leading to the wastewater treatment plant (WWTP). Upon entering the WWTP, the wastewater undergoes preliminary mechanical treatment to remove solid waste before it enters the aeration tanks for biological treatment. Next, solid waste and flocculated active sludge from sedimentation basins are digested in mesophilic digesters, and the resultant leachates are returned to the aeration tanks. Before release, the effluent undergoes a final chemical treatment to remove phosphorus using iron sulphate precipitation. Finally, the treated water is sterilized using ultraviolet radiation to reach a quality that conforms to the re-use specifications. The released treated wastewater is then re-used in different fields, including landscape irrigation, animal fodder and cooling towers.

Research approval to collect and process samples was obtained from Corporate Engineering and Health Facilities Management, HMC and Qatar University Institutional Biosafety Committee (Ref. Nos ENG/1/06/DU/JC/2 and QU-IBC-2019/066-REN1 021, respectively).

First, wastewater samples were collected from three hospitals designated solely for patients with COVID-19: Hazem Mebaireek General Hospital (HMGH), Mesaieed General Hospital (MGH) and Cuban General Hospital (CGH). In addition, samples were collected from the non-patient site of Hazem Mebaireek General Hospital and the non-COVID-19 patient site of Hamad General Hospital (HGH, a hospital assigned to non-COVID-19 patients) for comparison.

Sampling collection and quality analysis

Sampling was carried out between May 2021 and November 2021 towards the end of the second pandemic wave [25]. Three

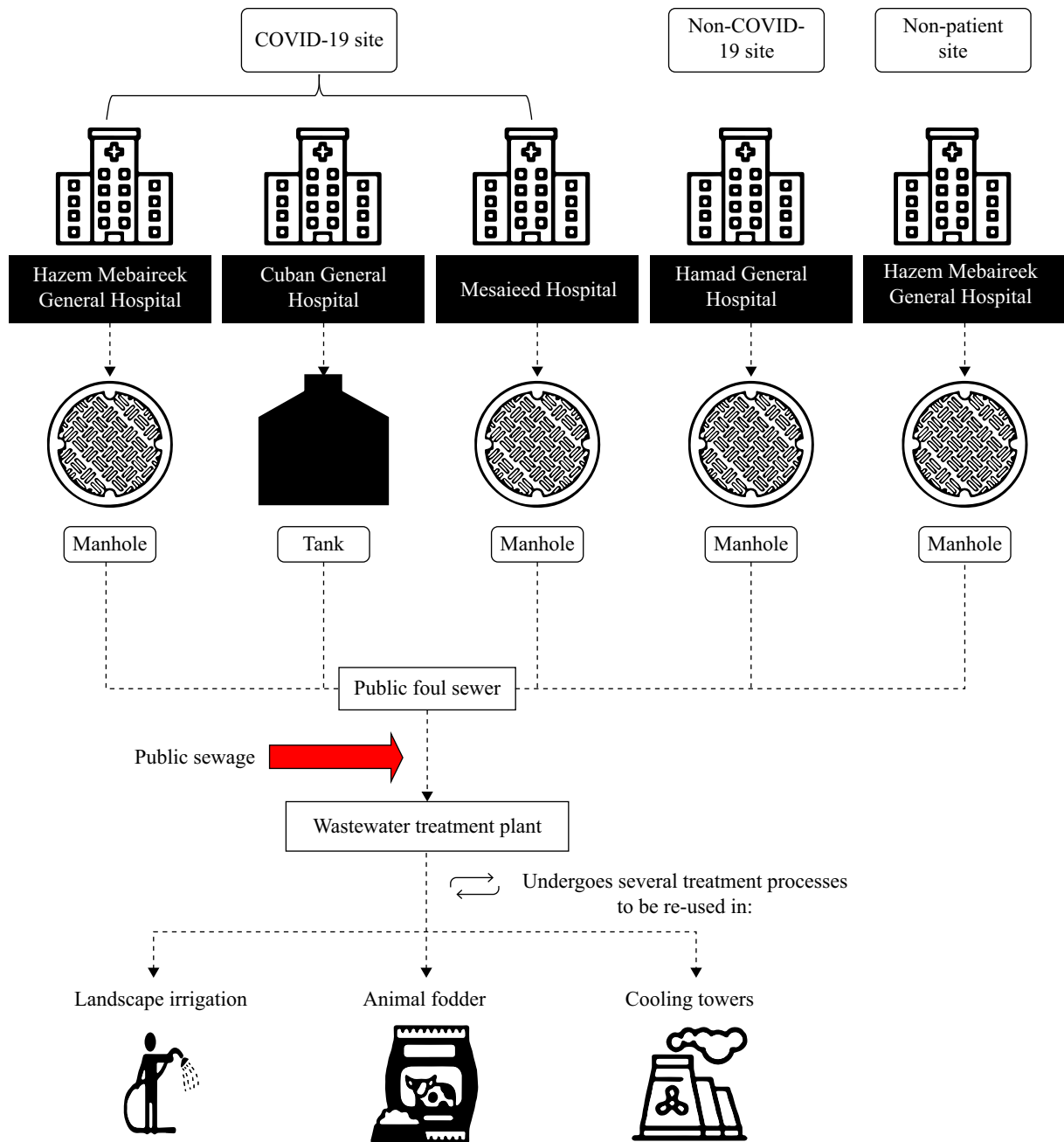


Figure 1. Illustration of the sample sites [coronavirus disease 2019 (COVID-19) and non-COVID-19] along with the wastewater system in Qatar. Wastewater from different hospital sites is transported to the public foul sewer, where it is mixed with the community's sewage until it reaches the wastewater treatment plant. Upon arrival, wastewater undergoes several treatment processes to be re-used in different fields (i.e. landscape irrigation, animal fodder and cooling towers).

independent water samples were collected from each site on different days. On the day of collection, three water samples were collected every 2 h (total of 6 h) and combined in 1-L sterile polyethylene bottles.

Physicochemical parameters were tested directly upon collection, as they are primary water quality indicators. The pH and temperature of the water were measured according to the Environmental Protection Agency (EPA) guidelines using an EcoSense pH100A meter (YSI Environmental, Yellow Springs,

OH, USA) [26]. Biochemical oxygen demand (BOD) was analysed using the standard method of the Animal Plant Health Agency for wastewater sample analysis [27]. Samples were collected in duplicate 300-mL BOD dark bottles and transported immediately to the laboratory for BOD measurement. Measurements were conducted with a YSI 5100 DO meter using a 5010 BOD probe (YSI Environmental) at day 0 and day 5 after incubation [28]. Samples collected from each location were transferred immediately to the Microbiology Laboratory at Qatar University

Biomedical Research Centre for analysis. Samples were transported in cold conditions and processed upon arrival, within 2 h of collection time.

Sample processing

In duplicate, vacuum filtration was used to filter 100 mL of water from each sample through a 0.45- μ m cellulose nitrate filter (Sartorius, Göttingen, Germany). Each filter was transferred to a 15-mL sterile Falcon tube containing 10 mL of phosphate-buffered saline and stored at -20 °C for DNA extraction. Coliform and pseudomonas counts were performed for each sample after dilution by 10^2 , 10^3 and 10^4 . Next, 10 mL of the diluted amount was filtered through a 0.45- μ m cellulose nitrate filter (Sartorius) and cultivated on chromatic coliform agar plates (Lifilchem, Roseto degli Abruzzi, Italy). After 24 h of growth at 37 °C, the distinguished colony of each species was counted, and colony-forming units (CFU) per 100 mL were calculated.

DNA extraction

The total genomic DNA of each sample was isolated using a ZymoBIOMICSTM DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) in accordance with the manufacturer's protocol. Briefly, extracted DNA samples from each site were pooled to provide a high concentration of DNA. Extracted genomic DNA samples were mixed gently with a 2.5 volume of ice-cold absolute ethanol and 10% of the total 3M sodium acetate volume, and incubated overnight at -20 °C. After incubation, centrifugation at a maximum speed of -4 °C for 10 min was carried out. The DNA pellet generated was washed twice with ice-cold 70% ethanol and left to air dry before resuspending in 50% TE buffer. DNA samples were quantified using a NanoDrop Lite spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and stored at -20 °C until further use.

Detection of bacterial species using high-throughput quantitative PCR

A water analysis microbial DNA quantitative polymerase chain reaction (qPCR) array (BAID-1405ZRA; Qiagen, Hilden, Germany) was used to investigate the microbial diversity in the wastewater, in accordance with the manufacturer's instructions. The array contains an assay for 45 bacterial species, capable of analysing two samples simultaneously. Generally, these assays target the 16S rRNA gene, and use PCR amplification primers and hydrolysis-probe detection to increase the specificity of the targeted species. For each reaction, 500 ng of template DNA was used in a 25- μ L reaction containing the Master Mix, and qPCR was performed using the 7500 Real-Time PCR System (Thermo Fisher Scientific). The PCR conditions included initial PCR activation for 10 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, and annealing/extension for 2 min at 60 °C. The cycle threshold (CT) values for all qPCR runs were calculated with a threshold value of 0.2, as recommended by the manufacturer. In addition, a no-template control was performed as a baseline for gene detection. The CT values were exported to an Excel spreadsheet (Microsoft Corp, Redmond, WA, USA) provided by the manufacturer for analysis.

Detection of ARGs by high-throughput qPCR

A high-throughput real-time PCR-based detection method was performed to detect the presence of ARGs in the wastewater, using an ARG microbial DNA qPCR array (BAID-1901ZRA; Qiagen) in accordance with the manufacturer's instructions. The array contains an assay for 87 ARGs belonging to different antibiotic classes. These qPCR assays use PCR amplification of primers and hydrolysis probes for gene-based detection. In addition, the qPCR cyclers conditions of all samples were performed as described above, in accordance with the manufacturer's recommendations.

Data analysis

Following the collection of extracted results in an Excel spreadsheet provided by the array manufacturers, species identification and ARG presence data were analysed using R Version 4.1.0. The figures were generated using ggplot2 Version 3.3.6 and ggpubr Version 0.4.0. Briefly, heatmaps were constructed for each identified species and ARGs in each location. Hierarchical clustering was used to determine the similarity between the sites in terms of species and ARGs.

Results

Preliminary assessment of water quality

The physical condition of the water is a primary requisite for water quality testing. Table I represents the characteristics of wastewater collected from various sampling sites of the four hospitals. As the sampling was conducted in summer, the water temperature at all sites was between 31 and 37 °C. The pH ranged from 6.9 to 7.9 for all hospitals, but was slightly alkaline at HGH and the HMGH COVID-19 patient site. BOD is the primary indicator of water quality, and according to the EPA recommendations, it should range between 3 and 5 ppm for a clean water supply. As all the collected samples were from wastewater, BOD had a higher range (45.7–227.2 ppm). The total coliform and pseudomonas count ranged between 9.70×10^2 CFU/100 mL and 2.96×10^4 CFU/100 mL in all locations.

Growth of diverse bacterial species in hospital wastewater

To determine the growth of essential pathogens in hospital wastewater, extracted DNA from various locations was tested for the presence of 45 bacterial species using a microbial DNA qPCR assay. The assay recorded 27 different bacterial species exhibited in the samples, belonging to five phyla: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Verrucomicrobiota. Common Gram-negative pathogens such as *Escherichia coli*, *Morganella morganii*, *Salmonella enterica* and *Citrobacter freundii*, as well as Gram-positive pathogens such as *Enterococcus faecalis*, *Enterococcus faecium* and *Clostridium perfringens* were present at all hospital locations (Figure 2).

However, *Clostridium difficile* and *Shigella dysenteriae* were only found in two hospitals (HMGH non-patient site and CGH), and *Streptococcus agalactiae* was only present in HGH wastewater (Figure 2). Interestingly, the greatest number of

Table I
Physicochemical analysis of water from different sampling sites

Hospital name	Visit	Temperature (oC)	pH	Biochemical oxygen demand (ppm)	Total coliform and pseudomonas count CFU/100 mL ^a
Hazem Mebaireek General Hospital (COVID-19 patient site)	1	33.8	8.03	223.2	9.80×10^3
	2	34.9	7.32	227.2	2.93×10^3
	3	35.6	8.11	136.1	2.46×10^4
Mesaieed General Hospital	1	34.5	7.04	87.9	2.00×10^4
	2	37.0	7.03	179.5	2.96×10^4
	3	39.0	6.99	191.2	1.26×10^4
Cuban General Hospital	1	31.7	7.79	96.1	4.80×10^3
	2	30.4	7.66	102.1	4.20×10^3
	3	30.8	7.73	66.9	2.30×10^3
Hamad General Hospital	1	34.6	8.13	119.22	2.60×10^3
	2	37.7	8.10	95.75	1.40×10^3
	3	36.9	7.90	57.1	2.30×10^3
Hazem Mebaireek General Hospital (non-patient site)	1	33.5	7.08	45.7	9.70×10^3
	2	35.8	7.83	47.85	1.00×10^3
	3	36.9	7.78	72.3	9.70×10^2
Permissible limits (EPA, 2001) [26]		25	6.5–8.5	3–5	200

COVID-19, coronavirus disease 2019; CFU, colony-forming units; EPA, Environmental Protection Agency.

^a CFU counts were conducted on isolation of *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas* spp.

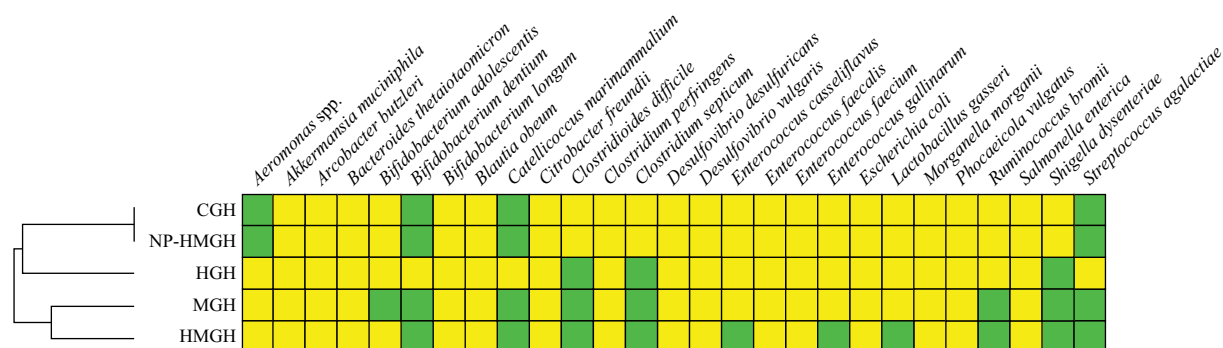


Figure 2. A heatmap that shows the bacterial species detected in the wastewater. The columns correspond to species, and the rows correspond to locations. Yellow indicates the species was detected; green indicates the species was not detected. The dendrogram shows the hierarchical clustering of the location by identified species. HMGH, Hazem Mebaireek General Hospital COVID-19 patient site; MGH, Mesaieed General Hospital; CGH; Cuban General Hospital; NP-HMGH, Hazem Mebaireek General Hospital non-patient site; HGH, Hamad General Hospital.

species were identified in HGH wastewater (24 of 27), designated for non-COVID-19 patients, but harbouring the greatest bed capacity. In comparison, the HMGH COVID-19 patient site had the fewest species (17 of 27). Based on the number of species detected, Firmicutes were prominent in all the samples, representing 44.4% of the total identified community in all the tested hospital locations. Of the detected species, the remaining community contained approximately 33.3% Proteobacteria, 11% Actinobacteria, 7.4% Bacteroidetes and 3.7% Verrucomicrobiota.

ARGs in hospital wastewater

Wastewater samples collected from different hospitals were tested for 87 ARGs conferring resistance to major classes of antibiotics. ARGs were divided into groups according to their target antibiotics (Figure 3). Specific genes detected at each

site are listed in Table II. In total, 61 of the 87 ARGs were detected. Of these 61 ARGs, 14 (22.9%) were identified at all sites and can be considered widespread in the five locations. These include genes for class A β -lactamase (*bla*_{VEB}, *bla*_{KPC}, *bla*_{GES}), class B β -lactamase (*bla*_{VIM-1}), class D β -lactamase (*bla*_{OXA-10}, *bla*_{OXA-2}, *bla*_{OXA-58}), fluoroquinolone resistance [*qnrB-1*, *qnrS*, *AAC(6)-1b-cr*], tetracycline resistance (*tetA*), macrolide-lincosamide-streptogramin B resistance (*ermB*, *mefA*) and aminoglycoside resistance (*aadA1*). The highest number of ARGs was observed at the HMGH COVID-19 patient site (88.5%, 77 of 87), while the lowest number was found at the HMGH non-patient site (24.1%, 21 of 87).

Furthermore, some genes were detected at a single site. For example, both class A β -lactamase and class B β -lactamase [*bla*_{SHV(238G240E)} and *bla*_{IMP-2} group] genes were detected at the HMGH non-patient site alone, and the class D β -lactamase (*bla*_{OXA-18}) gene was identified at HGH. Furthermore,

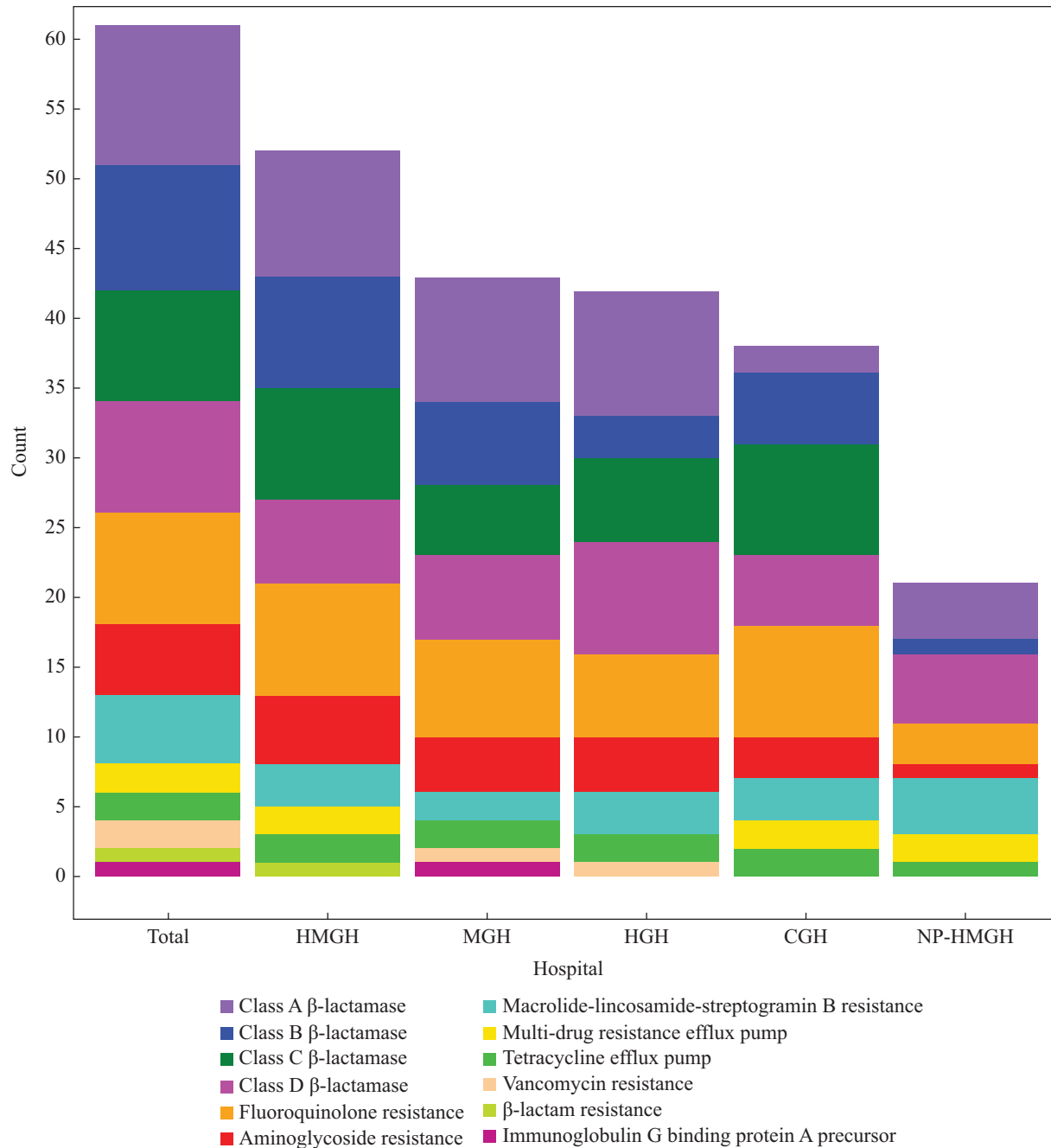


Figure 3. Counts of antibiotic resistance genes from the four hospitals. Each bar represents the number of distinct antibiotic resistance genes detected in the sites. The total bar represents the number of distinct genes detected across all sites. The colours in each bar correspond to a class of antibiotics. HMGH, Hazem Mebareek General Hospital COVID-19 patient site; MGH, Mesaieed General Hospital; CGH; Cuban General Hospital; NP-HMGH, Hazem Mebareek General Hospital non-patient site; HGH, Hamad General Hospital.

ARGs such as *aacC4* and *ermA*, as well as macrolide–lincosamide–streptogramin B resistance genes, were found at the HMGH COVID-19 patient site; at the same time, the *msrA* gene was only detected at the HMGH non-patient site (Figure 4).

Discussion

Healthcare sewage and wastewater drainage systems contain numerous harmful compounds, hazardous chemicals

and diverse micro-organisms, including MDROs harbouring ARGs [29]. As emerging contaminants in sewage water, ARGs pose a potential risk to public health and the aquatic ecosystem as they may be disseminated to the environment horizontally, and subsequently propagated vertically [30]. Hence, identifying prime ARG sources and their dissemination in highly polluted environments will aid in developing control strategies to limit their environmental spread.

Table II

Antibiotic resistance genes identified in water samples collected from five different locations in four hospitals in Qatar using a microbial DNA quantitative polymerase chain reaction assay

Gene name/ classification	Hamad General Hospital	Hazem Mebaireek General Hospital: non-patient site	Hazem Mebaireek General Hospital: COVID-19 patient site	Mesaieed General Hospital	Cuban General Hospital
Class A β-lactamase^a					
<i>bla</i> _{CTX-M-1} group	+		+	+	
<i>bla</i> _{CTX-M-9} group	+		+	+	
<i>bla</i> _{GES}	+	+	+	+	
<i>bla</i> _{KPC}	+	+	+	+	
<i>bla</i> _{Per-1} group	+		+	+	
<i>bla</i> _{SHV}	+		+	+	
<i>bla</i> _{SHV(156G)}	+		+	+	
<i>bla</i> _{SHV(238G240E)}		+			
<i>bla</i> _{TLA-1}	+		+	+	+
<i>bla</i> _{VEB}	+	+	+	+	+
Class B β-lactamase					
<i>bla</i> _{CCrA}			+		+
<i>bla</i> _{IMP-1} group			+	+	+
<i>bla</i> _{IMP-12} group			+	+	
<i>bla</i> _{IMP-2} group			+		
<i>bla</i> _{IMP-5} group			+	+	
<i>bla</i> _{NDM}	+		+	+	+
<i>bla</i> _{VIM-1} group	+	+	+	+	+
<i>bla</i> _{VIM-13}	+		+	+	+
<i>bla</i> _{VIM-7}					
Class C β-lactamase^b					
<i>bla</i> _{ACT 5/7} group	+		+		+
<i>bla</i> _{ACT-1} group	+		+		+
<i>bla</i> _{CMY-10} group			+	+	+
<i>bla</i> _{DHA}	+		+	+	+
<i>bla</i> _{FOX}			+		+
<i>bla</i> _{LAT}	+		+	+	+
<i>bla</i> _{MIR}	+		+	+	+
<i>bla</i> _{MOX}	+		+	+	+
Class D β-lactamase^c					
<i>bla</i> _{OXA-10} group	+	+	+	+	+
<i>bla</i> _{OXA-18}	+				
<i>bla</i> _{OXA-2} group	+	+	+	+	+
<i>bla</i> _{OXA-24} group	+			+	
<i>bla</i> _{OXA-48} group	+		+	+	+
<i>bla</i> _{OXA-50} group	+	+	+		+
<i>bla</i> _{OXA-51} group	+	+	+	+	
<i>bla</i> _{OXA-58} group	+	+	+	+	+
Fluoroquinolone resistance^d					
<i>AAC(6)-Ib-cr</i>	+	+	+	+	+
<i>QnrA</i>	+		+		+
<i>QnrB-1</i> group	+	+	+	+	+
<i>QnrB-4</i> group	+		+	+	+
<i>QnrB-5</i> group	+		+	+	+
<i>QnrB-8</i> group			+	+	+
<i>QnrD</i>			+	+	+
<i>QnrS</i>	+	+	+	+	+
Aminoglycoside resistance					
<i>aacC1</i>	+		+	+	
<i>aacC2</i>	+		+	+	+

(continued on next page)

Table II (continued)

Gene name/ classification	Hamad General Hospital	Hazem Mebaireek General Hospital: non-patient site	Hazem Mebaireek General Hospital: COVID-19 patient site	Mesaieed General Hospital	Cuban General Hospital
<i>aacC4</i>			+		
<i>aadA1</i>	+	+	+	+	+
<i>aphA6</i>	+		+	+	+
Macrolide–lincosamide–streptogramin B resistance^e					
<i>ermA</i>			+		
<i>ermB</i>	+	+	+	+	+
<i>ermC</i>	+	+			+
<i>mefA</i>	+	+	+	+	+
<i>msrA</i>		+			
Multi-drug resistance efflux pump					
<i>oprj</i>		+	+		+
<i>oprM</i>		+	+		+
Tetracycline efflux pump					
<i>tetA</i>	+	+	+	+	+
<i>tetB</i>	+		+	+	+
Vancomycin resistance					
<i>vanB</i>	+				
<i>vanC</i>				+	
β-lactam resistance					
<i>mecA</i>			+		
Immunoglobulin G binding protein A precursor					
<i>spa</i>				+	

β-lactamase genes that were not detected from any sample: ^a*bla*_{BES-1}, *bla*_{BIC-1}, *bla*_{CTX-M-8} group, *bla*_{IMI} & *bla*_{NMC-A}, *bla*_{KPC}, *bla*_{PER-2} group, *bla*_{SFC-1}, *bla*_{SFO-1}, *bla*_{SHV(156D)}, *bla*_{SHV(238G240K)}, *bla*_{SHV(238S240E)}, *bla*_{SHV(238S240K)}, *bla*_{SME}; ^b*bla*_{ACC-1} group, *bla*_{ACC-3}, *bla*_{CFE-1}; ^c*bla*_{OXA-23} group, *bla*_{OXA-45}, *bla*_{OXA-55}, *bla*_{OXA-60}. Antibiotic resistance genes that were not detected from any sample: ^d*qepA*, *qnrB-31* group, *qnrC*; ^e*ereB*.

To the best of the authors' knowledge, this is the first study to focus on the prevalence of bacterial diversity and the determination of ARGs in wastewater from various hospitals in Qatar, with a particular emphasis on hospitals designated for COVID-19 patients (HMGH COVID-19 patient site, CGH and MGH), compared with hospitals dedicated to other patients (HGH and HMGH non-patient site).

In this study, a baseline was established to determine the presence of bacterial species at various hospitals. The results showed no consistent trend across the tested hospital locations. Specifically, the bacterial species detected at HGH differed from those found at the HMGH non-patient site. However, hospitals designated for COVID-19 patients reported similar species in their wastewater, with some exceptions. For example, *S. dysenteriae* and *C. difficile* were only detected at CGH. At the same time, *Enterococcus gallinarum*, *Enterococcus casseliflavus* and *Lactobacillus gasseri* were absent from wastewater samples collected at the HMGH COVID-19 patient site (Figure 2). The inconsistent trend observed could be explained by differences in the number and distribution of hospitalized patients when the wastewater samples were collected. Alternatively, the capacity of the HMGH COVID-19 patient site may differ from CGH and MGH, making it challenging to compare the bacterial species detected in each hospital. The study observations highlight the need for further analysis to examine collected data against the demographics of patients admitted at each location at other times (i.e. not during the study period).

Furthermore, it should be noted that HGH is Qatar's primary and largest hospital, providing medical care for a diverse patient population, including citizens, residents and visitors. As a result, a higher bacterial load would be expected compared with other hospitals. The study findings confirmed this expectation: HGH had the greatest number of bacterial species, with *Bifidobacterium dentium*, *Catellibacoccus marimammalium* and *S. agalactiae* exclusively present in HGH wastewater samples. This disparity in bacterial species could be attributed to the widespread use of antibiotics in all other COVID-19-designated hospitals, eradicating many bacterial species and allowing only a few types of bacteria to be detected in all hospitals designated for COVID-19 patients compared with other hospitals. In addition, it is worth noting that the variation in the microbiota may be attributed to the fact that HGH hosts both male and female patients of all ages, including children. The other hospitals mainly admit male patients. For instance, the gastrointestinal and genitourinary tract pathogen *S. agalactiae* is generally harmless for human health, being a commensal common in almost one-third of healthy women [31]. As *Bifidobacterium* spp. represent one of the neonatal gut's earliest and most abundant bacterial colonizers [32], it would be expected to be identified at HGH, which houses maternity, women's health and neonatal services. Therefore, this variation in bacterial species could be due to differences in the microbial ecology of different age groups, genders and facility settings. Several studies have reported the influence of age and gender on the microbiota composition of

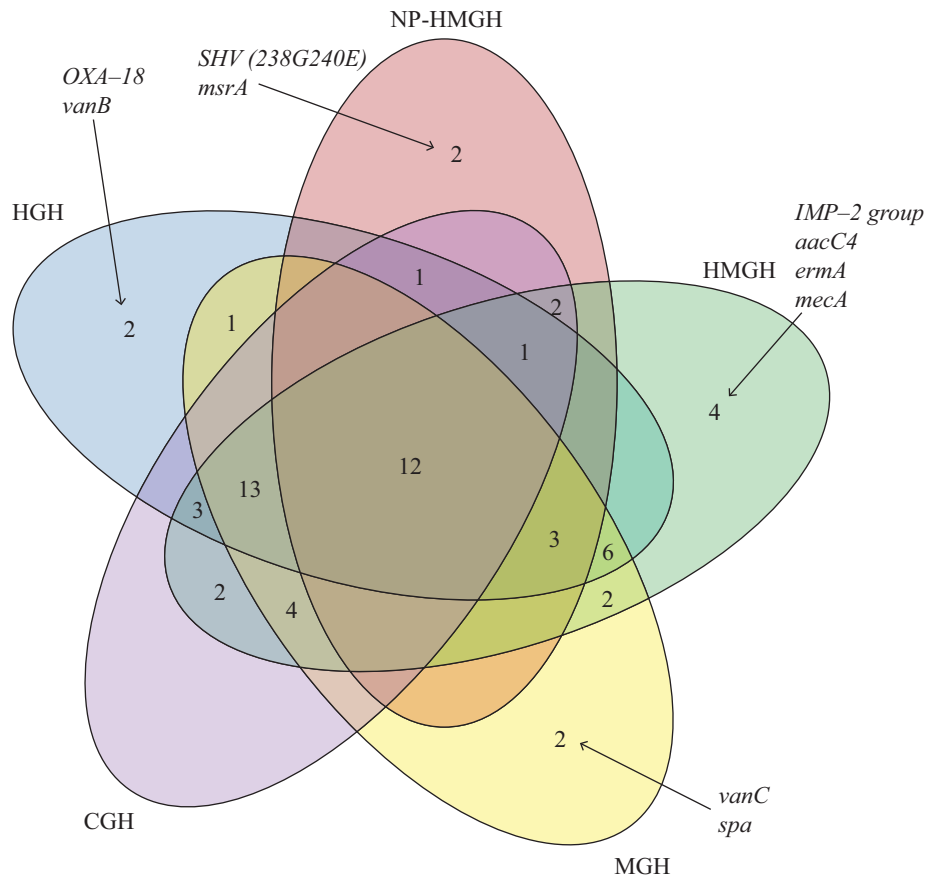


Figure 4. Venn diagram of antibiotic resistance genes detected in the hospital wastewater during the coronavirus disease 2019 (COVID-19) pandemic. This figure illustrates each hospital's unique antibiotic resistance genes, including the COVID-19 isolation hospitals, the non-COVID-19 hospital and the non-patient sewage water. HMGH, Hazem Mebaireek General Hospital COVID-19 patient site; MGH, Mesaieed General Hospital; CGH; Cuban General Hospital; NP-HMGH, Hazem Mebaireek General Hospital non-patient site; HGH, Hamad General Hospital.

various body sites (e.g. gut, skin and vaginal microbiota) [33–35]. It is plausible that the variation in microbiota observed in this study reflects the differences in the patient demographics between the sites.

Yewale *et al.* (2020) conducted a study to characterize the microbial community and ARGs isolated from diverse river water catchments in Pune City, India. Two hospitals were included among the various locations specified in the study; similar to the present findings, they found that *Escherichia* spp., *Morganella* spp., *Desulfovibrio* spp., *Salmonella* spp. and *Streptococcus* spp. were common bacteria in the two hospitals [36]. These findings were closely related to the present study which demonstrated that *E. coli*, *M. morgani*, *S. enterica*, *E. faecalis*, *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans* were dominantly present in all hospital locations. Also, the present results are comparable to those obtained by Yao *et al.* [37]. Their study analysed the bacterial community in three hospitals (primary, secondary and tertiary) located in Xinxiang in central China. Their results showed several opportunistic bacteria at high relative abundances in all hospitals, including *Klebsiella* spp. and *Pseudomonas* spp. Surprisingly, *Klebsiella* spp. and *Pseudomonas* spp. are not on the species detection panel of the microbial array used in the present study. However, chromogenic media was used to screen these species in the present study, and this showed that they had a

high colony count. However, several studies have demonstrated that the quantity of antibiotic-resistant bacteria in hospital wastewater is more significant than that in non-hospital wastewater [37,38]. Moreover, hospitals have been shown to be the primary source of antibiotic-resistant pathogens in the environment [39]. Thus, it is essential to define the risks associated with multi-drug-resistant bacteria in the wastewater outlets specific to each hospital to determine whether hospital effluents are causing significant threats to the surrounding environments.

The association between antimicrobial resistance and the COVID-19 pandemic is slowly emerging, with research studies demonstrating an imbalanced approach to antimicrobial consumption [40]. ARG evaluation in this study showed diversity of all resistance classes, ranging from β -lactamase, carbapenem, aminoglycoside, fluoroquinolone, macrolide, lincosamide and vancomycin resistance genes, as well as efflux pumps. As β -lactamases are one of the prime classes, it is essential to highlight that class A–D β -lactamase resistance genes were detected. Class A β -lactamases were dominated by *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{GES} from most hospitals, in line with regional and international epidemiology [41,42]. Regarding carbapenemases, class B *bla*_{NDM}, *bla*_{VIM} group and class D *bla*_{OXA-48} group were predominant in most facilities, in line with regional epidemiology. Still, it was intriguing to find that *bla*_{KPC} was rarely

detected in the wastewater from most hospitals, including COVID-19 and non-COVID-19 facilities, probably related to the high consumption of broad-spectrum antimicrobials witnessed during the COVID-19 pandemic [43–45]. Furthermore, vancomycin resistance genes B and C were infrequently reported, as vancomycin-resistant enterococci are rare in the region [46,47]. Regarding other detected ARGs, such as aminoglycoside, fluoroquinolone and macrolide resistance genes, it has been established that once some resistance genes are detected, the co-existence of other genes is a common observation as horizontal and vertical transmission is usually through plasmids and other mobile genetic elements that affect multiple classes, including environmental dissemination [48]. Of note, through evaluating ASPs across major HMC facilities before the pandemic, antimicrobial consumption was controlled towards a downward trend dominated by the major classes of carbapenems, glycopeptides and β -lactam- β -lactamase inhibitors, such as piperacillin tazobactam, as well as quinolones [49]. However, from the authors' unpublished records, during the course of the pandemic, there were significant upward trends for all outlined classes coinciding with observed resistance patterns.

A recent study showed a marked increase (up to 40%) in AMR patterns during COVID-19 compared with pre-COVID-19 [50]. This shift in AMR patterns, which resulted from the extensive use of antibiotics during the pandemic, allowed bacterial pathogens to gradually develop mechanisms to acquire resistance [40,50].

The present study focused on detecting ARGs from hospital wastewater collected at different locations. Overall, the high prevalence of ARGs observed at the HMGH COVID-19 site and other hospitals designated for COVID-19 patients (CGH and MGH) suggests the overuse and excessive consumption of antibiotics and antimicrobial agents by COVID-19 patients. In addition, these patients may receive antibiotics as part of their treatment, even if not needed, which can contribute to developing antibiotic-resistant bacteria. A systematic review highlighted that almost 70% of COVID-19 patients received broad-spectrum antimicrobials with little supporting microbiological identification, which may facilitate AMR [45]. Secondly, the absence of some ARGs from the wastewater from the HMGH non-patient site could be because the sample was collected from a location restricted to healthy individuals, such as the security and maintenance team, who are less likely to be exposed to antibiotics and antimicrobial agents. This suggests that the source of ARGs in hospital wastewater is expected to be from patients and their activities within the hospital environment, such as using antibiotics and personal hygiene products that contain antimicrobial agents.

Khan *et al.* [51] studied the prevalence and diversity of ARGs in Swedish aquatic environments. They showed that the aquatic environment of Sweden was mainly impacted by hospital wastewater, as specific class B β -lactamase genes such as *bla*_{IMP-1}, *bla*_{IMP-2} and *bla*_{OXA-23} were highly detected. Similarly, the present findings indicated that class B β -lactamase genes were dominant in all hospital locations. This may indicate high use of β -lactamase antibiotics for COVID-19 patients. The number of ARGs identified in wastewater samples from COVID-19-designated hospitals was higher than that of HGH and the HMGH non-patient site, reflecting the overuse of antibiotics, mainly during the pandemic.

Similarly, a study was conducted in Saudi Arabia (mainly Jeddah) to investigate the AMR patterns in hospital wastewater during the COVID-19 pandemic [52]. The results showed a positive selection of specific ARGs (mainly carbapenem) within hospital wastewater due to the prevailing treatment given to COVID-19 patients [52]. This expedites the potential dissemination of ARG resistance to last-resort antibiotics through horizontal gene transfer, causing a significant risk to ecological sustainability and public health [53].

In the context of the One Health initiative, it is critical to monitor and manage the release of treated wastewater from hospitals to the community environment, including surface waters, soils and agricultural systems, and to consider applying further assessment of the absence of any ARGs before re-use in the community. Paulus *et al.* illustrated the presence of ARGs in treated hospital wastewater, demonstrating the advantage of insight hospital wastewater treatment in reducing the spread of these genes to the community [54].

In conclusion, WBE is promising for disease monitoring and surveillance of ARGs within sewage. It can aid in detecting outbreaks early, inform public health interventions, and track the effectiveness of these interventions over time. Also, the qPCR array procedure is effective for detecting bacterial species in hospital wastewater samples, is simple, can be performed in any laboratory with a real-time PCR, and does not require bioinformatics to analyse the results. In addition, it can be used to recognize diverse ARGs belonging to class A, B, C and D β -lactamases, tetracycline, fluoroquinolones and aminoglycosides. Successful screening was achieved among hospitals designated for COVID-19 patients and other hospitals for different patients. Thus, further WBE investigations should be conducted to eliminate the factors that drive the dissemination and spread of AMR bacteria from hospital wastewater, and maintain low resistance to the surrounding community via the environmental waters.

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Conflict of interest statement

None declared.

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