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Antibiotic resistance and virulence potentials of *E. faecalis* and *E. faecium* in hospital wastewater: a case study in Ardabil, Iran

Elham Jannati[a,b], Farzad Khademi[c], Meysam Manouchehrifar[d], Dadras Maleki[e], Nour Amirmozaffari[f], Vajihe Sadat Nikbin[g] and Mohsen Arzanlou[c,*]

[a] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[b] Department of Microbiology, School of Sciences, Islamic Azad University, Ardabil Branch, Ardabil, Iran
[c] Department of Microbiology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran
[d] Food Control Laboratory, Ardabil University of Medical Sciences, Ardabil, Iran
[e] Microbiology Laboratory, Imam Hospital, Ardabil University of Medical Sciences, Ardabil, Iran
[f] Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[g] Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran
*Corresponding author. E-mail: m.arzanlou@arums.ac.ir; arzanlo53@gmail.com

MA, 0000-0002-4233-9497

ABSTRACT

Hospital wastewater can contaminate the environment with antibiotic-resistant and virulent bacteria. We analyzed wastewater samples from four hospitals in Ardabil province, Iran for *Enterococcus faecium* and *Enterococcus faecalis* using culture and molecular methods. We also performed antimicrobial susceptibility testing and polymerase chain reaction testing for resistance and virulence genes. Out of 141 enterococci isolates, 68.8% were *E. faecium* and 23.4% were *E. faecalis*. Ciprofloxacin and rifampicin showed the highest level of resistance against *E. faecalis* and *E. faecium* isolates at 65%. High-level gentamicin resistance (HLGR), high-level streptomycin resistance (HLSR), ampicillin, and vancomycin resistance were observed in 25, 5, 10, and 5.15% of *E. faecium*, and 15, 6, 15, and 3.03% of *E. faecalis* isolates, respectively. The *ant(6′)-Ia* and *ant(3′)-Ia* genes that were responsible for streptomycin resistance were observed in HLSR isolates and *aph(3′)-IIIa* and *aac(6′) Ie-aph(2″)-Ia* genes accounting for gentamicin resistance were detected in HLGR isolates. *vanA* was the predominant gene detected in vancomycin-resistant isolates. The majority of isolates were positive for *gelE*, *asa1*, *esp*, *cylA*, and *hyl* virulence genes. We found that drug-resistant and virulent *E. faecalis* and *E. faecium* isolates were prevalent in hospital wastewater. Proper treatment strategies are required to prevent their dissemination into the environment.

Key words: *E. faecalis*, *E. faecium*, high-level gentamicin resistance, high-level streptomycin resistance, hospital wastewater, VRE

HIGHLIGHTS

- The prevalence of *E. faecium* isolates in hospital wastewater was three times higher than that of *E. faecalis* isolates.
- Five percent of *E. faecium* and 3% of *E. faecalis* isolates were vancomycin-resistant.
- The rate of HLSR was almost four times the rate of HLGR among *Enterococcus* spp.
- Almost all of the isolates were multidrug-resistant.
- The majority of *E. faecalis* and *E. faecium* isolates contained multiple virulence genes simultaneously.

INTRODUCTION

Enterococci are known to belong to the commensal microbiota of humans and animals (Jannati *et al.* 2020). Various species of *Enterococcus* are the natural inhabitants of the oral cavity and the gastrointestinal and genitourinary tracts in both humans and animals (Ramos *et al.* 2020). *Enterococcus faecium* and *Enterococcus faecalis* are the main enterococcal species collected from clinical specimens. These species are the causative agents of approximately 80 and 20% of enterococcal infections, respectively (Wang *et al.* 2013). They are associated with several important hospital-acquired infections, such as peritoneal infections, wound infections, soft tissue and skin infections, urinary tract infections, central nervous system infections, bacteremia, and endocarditis (Hasanpour *et al.* 2021). Enterococcal infections including vancomycin-resistant

enterococci (VRE) have high mortality rates (25–50%) since they often develop in compromised hosts (Zhang *et al.* 2017). Enterococci have several virulence factors that contribute to their pathogenesis. These factors include adhesions, enterococcal surface protein (Esp), and collagen-binding protein (Ace), which allow the bacteria to attach to host cells; aggregation substances, which promote bacterial colonization; lytic enzymes, gelatinase (GelE), and hyaluronidase (Hyl), which cause the dissemination of infection; and cytolysin (CylA) which causes cell lysis (Heidari *et al.* 2017). Enterococcal infections are difficult to treat because the bacteria have the potential for resistance to virtually all clinically useful antibiotics. Enterococci express intrinsic resistance to antibiotics such as clindamycin and trimethoprim-sulfamethoxazole and show a low-level resistance to aminoglycosides and penicillins. They are capable of acquiring new resistance genes including vancomycin resistance (Sparo *et al.* 2018).

Enterococcus spp. are known for their inherent resilience to harsh physicochemical conditions in addition to their resistance to antibiotics. They can grow at pH 9.6, in 6.5% NaCl, and at 10–45 °C and withstand drying conditions for a prolonged time period (Weber & Rutala 1997; Blanch *et al.* 2003). They have also been shown to display tolerance to antimicrobial biocides (Namaki *et al.* 2022). Hence, they are found in various environments, especially the hospital environment (Wang *et al.* 2013; Strateva *et al.* 2016). Hospital settings can act as major reservoirs for the spread of highly virulent and drug-resistant *Enterococcus* spp. (Iweriebor *et al.* 2015). The dissemination of antibiotic-resistant bacteria from hospitals can occur through discharged patients, healthcare workers, and hospital wastewater effluent (Hocquet *et al.* 2016).

Hospital wastewater is one of the major sources of antibiotic-resistant bacteria and antibiotic-resistance genes (Mackuľak *et al.* 2021). Wastewater from healthcare facilities is often discharged into the sewage system without prior treatment and may act as a reservoir of antibiotic-resistant bacteria and antibiotic-resistance gene dissemination into the environment (Mackuľak *et al.* 2021). Understanding the presence of antibiotic-resistant bacteria in hospital wastewater can help mitigate the environmental and public health risks posed by these contaminants through the use of appropriate treatment strategies (Liu *et al.* 2023). In Iran, little is known about the antimicrobial characteristics of *Enterococcus* spp. in hospital wastewater.

Therefore, this study aimed to (i) investigate the prevalence of clinically significant species of enterococci *E. faecalis* and *E. faecium* in the influent of four teaching hospitals in Ardabil, Iran; (ii) assess the resistance profile of isolates against aminoglycosides, vancomycin, ampicillin, and other common antibiotics; (iii) determine the prevalence of the most common virulence genes; and (iv) evaluate the clonal relatedness of the isolates using the Enterobacterial repetitive intergenic consensus (ERIC)-polymerase chain reaction (PCR) assay.

MATERIALS AND METHODS

Sampling

Twenty-five samples of raw wastewater were collected from the wastewater influent of four teaching hospitals affiliated with the Ardabil University of Medical Science over a 10-month period between July 2017 and May 2018. The hospitals were Imam (a referral and general hospital with 500 beds and 700 m^3 daily wastewater discharge), Fatemi (a trauma hospital with 220 beds and 72 m^3 daily wastewater discharge), Alavi (a women's hospital with 220 beds and 65 m^3 daily wastewater discharge), and Bouali (a children's hospital with 150 beds and 60 m^3 daily wastewater discharge). The treatment process in these hospitals only includes the primary treatment stage. This stage involves the removal of large solids and debris, fat, and sedimentable organic matter from the wastewater through physical processes. The treated wastewater is then discharged into the municipal wastewater system.

The samples were collected in accordance with the standard operating procedures for the wastewater sampling set by the United States Environmental Protection Agency (U.S. EPA 2013). The liquid grab samples were collected from the main manhole discharging mixed wastewater from the entire hospital in sterile 250 mL bottles and immediately transferred to the microbiology laboratory in cold box containers. The samples were kept at 4 °C and microbiological analysis was performed within 3 h after sample collection.

Bacterial isolation and identification

The specimens were diluted 50 times with phosphate-buffered saline (PBS) and then filtered by 0.45 μm membranes (Millipore Corporation, USA) as described previously (Rahimi *et al.* 2007). The membranes were transferred onto Brain Heart Infusion (BHI) agar (SRL, Mumbai, India), incubated at 37 °C for 2 h, and subsequently subcultured onto *Enterococcus* agar (Becton Dickinson and Co., Sparks, MD, USA) containing 6.5% NaCl (Merck, Germany) at 37 °C for 48 h (Rahimi *et al.* 2007). Five pink/red colonies were randomly selected and transferred to bile esculin agar plates, followed by incubation

at 44 °C for 24 h. The black colonies suspected to be *Enterococcus* species were confirmed using Gram-staining, catalase, and PYR (pyrrolidonyl aminopeptidase) tests (Deasy *et al.* 2000; Martín-Platero *et al.* 2009; Jannati *et al.* 2020). *E. faecalis* and *E. faecium* species were identified by the PCR using specific primer sequences (Table 1) targeting the D-alanine: D-alanine ligase (*ddl*) gene (Dutka-Malen *et al.* 1995; Kariyama *et al.* 2000; Jannati *et al.* 2020). Briefly, genomic DNA was extracted with a commercial DNA isolation kit (DNP™, Sinaclon, Tehran, Iran) according to the manufacturer's recommendation. Amplification was conducted in a DNA thermal cycler (Bio-Rad, Hercules, CA, USA) using commercially available PCR premix (Premix Taq® mix, CinnaGen, Tehran, Iran) and temperature conditions described previously (Jannati *et al.* 2020). PCR products were analyzed via electrophoresis at 100 V for 1 h in a 1.5% agarose gel (Sinaclon, Tehran, Iran) and stained with DNA-safe stain (Sinaclon, Tehran, Iran), and DNA bands were visualized using UV illumination (Uvi Tec, Cambridge, UK). To ensure that any amplification failure was not due to poor DNA quality or to failure of the PCR itself, and the 16 s ribosomal ribonucleic acid (16s rRNA) gene amplification was used as a positive control in PCR testing (Table 1) (Kariyama

Table 1 | Oligonucleotide sequence of primers used in this study

Gene	Oligonucleotide sequence (5′ to 3′)	Product size (bp)	Annealing temperatures (°C)	Reference
16S rRNA	GGATTAGATACCCTGGTAGTCC GGATTAGATACCCTGGTAGTCC	320	56	Kariyama *et al.* (2000)
ddl E. faecalis	ATCAAGTACAGTTAGTCT′ ATCAAGTACAGTTAGTCT′	941	54	Dutka-Malen *et al.* (1995)
ddl E. faecium	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	550	54	Dutka-Malen *et al.* (1995)
vanA	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	55	Dutka-Malen *et al.* (1995)
vanB	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635	55	Dutka-Malen *et al.* (1995)
aac(6′) Ie-aph(2″)-Ia	GAGCAATAAGGGCATACCAAA GTTCCTATTTCTTCTTCACTATCTTCA	829	55	Leelaporn *et al.* (2008)
aph(2″)-Ib	TCA AAT CCC TGC GGT AGT GTA CGCCAAAATCAATAACTCCAA	428	54	Leelaporn *et al.* (2008)
aph(2″)-Ic	GAGGGCTTTAGGAATTACGC ACACAACCGACCAACAGAGG	125	54	Leelaporn *et al.* (2008)
aph(2″)Id	TAATCTGCCGAAGCAATTCA TAATCCCTCTTCATACCAATCC	550	54	Leelaporn *et al.* (2008)
ant(3″)-Ia	ACC GTA AGG CTT GAT GAA ACA GCCGACTACCTTGGTGATCTC	624	56	Leelaporn *et al.* (2008)
ant(6′)-Ia	GCC CTT GGA AGA GTT AGA TAA TT CGGCACAATCCTTTAATAACA	198	56	Leelaporn *et al.* (2008)
aph(3′)-IIIa	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	523	57	Padmasini *et al.* (2014)
asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375	56	Vankerckhoven *et al.* (2004)
gelE	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213	56	Vankerckhoven *et al.* (2004)
cylA	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	56	Vankerckhoven *et al.* (2004)
esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510	56	Vankerckhoven *et al.* (2004)
hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	276	56	Vankerckhoven *et al.* (2004)

et al. 2000). Furthermore, *E. faecium* ATCC 19434 and *E. faecalis* ATCC 29212 were used as control strains. The identified isolates were kept in BHI broth along with 15% glycerol (Merck, Germany) at −80 °C for the next analyses.

Antimicrobial susceptibility testing

BHI agar with 6 μg/mL of vancomycin antibiotic (Bio Basic, Canada) was used for the screening of vancomycin resistance. The isolates with growth on BHI agar were subjected to minimum inhibitory concentration (MIC) testing using the agar dilution technique (vancomycin concentration; 0.125–512 μg/mL). The isolates with an MIC of ≥32 μg/mL were regarded as vancomycin-resistant isolates (VRE). The MIC of ampicillin was also determined using the agar dilution method, and isolates with MIC values of ≥16 μg/mL were considered ampicillin-resistant (AR). High-level resistance to gentamicin (HLGR) and streptomycin (HLSR) were assessed by the agar dilution technique. In brief, a 0.5 McFarland bacterial suspension was spotted onto a BHI agar (SRL Diagnostics, India) with 2,000 μg/mL streptomycin and 500 μg/mL gentamicin, individually. The plates were incubated at 37 °C for 24–48 h and then assessed in terms of bacterial growth. Growth of more than one colony in a spotted region was regarded as HLGR/HLSR enterococci. Furthermore, Antimicrobial sensitivity testing of the seven other antibiotics (Padtan Teb, Tehran, Iran) was done by the disk diffusion method. The tested antibiotics were rifampicin (5 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), penicillin G (10 μg), nitrofurantoin (300 μg), tetracycline (30 μg), and teicoplanin (30 μg).

All antibiotic susceptibility testings were performed and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2023).

Determination of resistance and virulence genes

The presence of genes encoding for HLGR, i.e. *aac(6′) Ie-aph(2″)-Ia*, *aph(2″)-Ic*, *aph(2″)-Ib*, *aph(2″)-Id* and those for HLSR, i.e. *ant(3″)-Ia* and *ant(6′)-Ia*, were examined using the multiplex PCR, and the amplification of *aph(3″)-IIIa* was done using the singleplex PCR assay as described, previously (Leelaporn *et al.* 2008; Padmasini *et al.* 2014; Jannati *et al.* 2020). The vancomycin resistance encoding genes (*vanA* and *vanB*) were also identified according to the previous report (Dutka-Malen *et al.* 1995). Moreover, the presence of genes encoding for five common enterococcal virulence determinants, such as aggregation substance (*asa1*), cytolysin (*cylA*), hyaluronidase (*hyl*), *esp*, and gelatinase (*gelE*), was determined by specific primer sequences using the multiplex PCR as described previously (Vankerckhoven *et al.* 2004).

The sequence of primers used in this study along with annealing temperatures is listed in Table 1.

DNA from previously identified isolates carrying the corresponding antibiotic-resistance and -virulence genes was used as a positive control in all PCR tests (Jannati *et al.* 2020).

ERIC-PCR analysis

This method was used for the genotyping of the isolates using specific primers (Table 1) and procedures described earlier (Espigares *et al.* 2006; Jannati *et al.* 2020). ERIC patterns were analyzed by Bionumeric II 7.0 (Applied Maths, Belgium), and the resemblance between ERIC-PCR profiles was measured by the dice coefficient as well as the unweighted pair group approach with arithmetic (UPGMA). Isolates that had 80% similarity were categorized in the same clusters and regarded as clonally related.

Statistical analysis

We used descriptive statistics to summarize and present our data.

RESULTS

Distribution of *Enterococcus* species

A total of 141 *Enterococcus* isolates were obtained from hospital wastewater in Ardabil City. Through the standardized genotypic identification of the isolates, it was found that 68.8% ($n = 97/141$) of the species were *E. faecium* and 23.4% ($n = 33/141$) were *E. faecalis*.

Antibiotic-resistance pattern

Table 2 shows that the total of *E. faecium* and *E. faecalis* isolates had the highest level of resistance to ciprofloxacin and rifampicin at 65% and the lowest level of resistance to teicoplanin (1%). In the BHI–vancomycin screening agar (6 μg/mL) test, 24% ($n = 31/130$) of *E. faecium* and *E. faecalis* showed colony growth, while in MIC testing only 5% ($n = 5/97$) of *E. faecium*

Table 2 | Antibiotic susceptibility profiles of *Enterococcus* species isolated from hospital wastewater

	E. faecium ($N = 97$) *n* (%)		*E. faecalis* ($N = 33$) *n* (%)		Total ($N = 130$) *n* (%)	
Antimicrobial agent	**R + I**	**S**	**R + I**	**S**	**R + I**	**S**
Ciprofloxacin	60 (47)	35 (36)	24 (73)	9 (27)	84 (65)	44 (37)
Chloramphenicol	8 (7)	89 (92)	7 (21)	26 (79)	15 (11)	115 (88)
Nitrofurantoin	23 (15)	74 (76)	6 (18)	27 (82)	29 (22)	101 (78)
Penicillin	22 (23)	75 (77)	7 (21)	26 (79)	29 (22)	101 (78)
Ampicillin[a]	11 (11)	87 (90)	5 (15)	28 (87)	16 (12)	115 (88)
Rifampicin	54 (53)	43 (44)	30 (91)	3 (9)	84 (65)	46 (35)
Tetracycline	5 (5)	92 (95)	22 (67)	11 (33)	27 (20)	103 (79)
Vancomycin[a]	5 (5)	93 (96)	1 (3)	32 (97)	6 (5)	125 (96)
Teicoplanin	1 (1)	96 (99)	–	33 (100)	1 (1)	129 (99)

R, resistant; I, intermediate; S, susceptible.
[a]A susceptibility profile was determined using the agar dilution method.

isolates and 3% ($n = 1/33$) of *E. faecalis* isolates were confirmed as VRE strains (vancomycin MIC = 512 μg/mL). The *vanA* gene was found in 60% ($n = 3/5$) of vancomycin-resistant *E. faecium* and 100% ($n = 1/1$) of vancomycin-resistant *E. faecalis* isolates, while the *vanB* gene was not found in VRE isolates.

The resistance rate to ampicillin was 10% ($n = 10/97$) for *E. faecium* and 15% ($n = 5/33$) for *E. faecalis* isolates (MIC ≥ 16 μg/mL). In AR isolates, the MICs of ampicillin ranged from 16 to 512 μg/mL (Table 3).

In this study, 25% ($n = 24/97$) of *E. faecium* and 15% ($n = 5/33$) of *E. faecalis* isolates exhibited high-level streptomycin resistance (HLSR), while high-level gentamicin resistance (HLGR) was found in 5% ($n = 5/97$) and 6% ($n = 2/33$) of *E. faecium* and *E. faecalis* isolates, respectively. Three percent ($n = 3/97$) of *E. faecium* isolates and 6% ($n = 2/33$) of *E. faecalis* isolates showed both HLGR and HLSR phenotypes (Table 4).

Table 3 | Frequency and range of ampicillin MICs of enterococci isolates using the agar dilution method

MIC (μg/mL)	*E. faecium* ($N = 97$) *n* (%)	*E. faecalis* ($N = 33$) *n* (%)	Total ($N = 130$) *n* (%)
<0.12	5 (5)	–	5 (4)
0.25	16 (16.5)	1 (3)	17 (13)
0.5	31 (32)	9 (27)	40 (31)
1	24 (25)	16 (48.5)	40 (31)
2	3 (3)	2 (6)	5 (4)
4	6 (6)	–	6 (5)
8	1 (1)	–	1 (1)
16[a]	1 (1)	2 (6)	3 (2)
32	2 (2)	2 (6)	4 (3)
64	2 (2)	–	2 (1)
128	1 (1)	–	1 (1)
256	2 (2)	1 (3)	3 (2)
512	2 (2)	–	2 (1)
MIC_{50}	0.5	1	0.5
MIC_{90}	32	32	32

[a]MIC ≥16 μg/mL indicates resistance to ampicillin.

Table 4 | Distribution of HLSR, HLGR, AR, and VRE phenotypes among *Enterococcus* species isolated from hospital wastewater

Resistance phenotype	*E. faecium* ($N = 97$) *n* (%)	*E. faecalis* ($N = 33$) *n* (%)	Total ($N = 130$) *n* (%)
No resistant	67 (69)	25 (76)	92 (71)
AR	2 (2)	3 (9)	5 (4)
AR, VRE	1 (1)	–	1 (1)
HLGR, AR	2 (2)	–	2 (1)
HLSR	19 (17)	2 (6)	21 (16)
HLSR, AR	2 (2)	1 (3)	3 (2)
HLSR, HLGR	–	1 (3)	1 (1)
HLSR, HLGR, AR, VRE	2 (2)	1 (3)	3 (2)
HLSR, HLGR, VRE	1 (1)	–	1 (1)
AR, VRE	1 (1)	–	1 (1)
Total	97 (69)	33 (23)	130

HLSR, high-level streptomycin-resistant; HLGR, high-level gentamicin-resistant; AR, ampicillin-resistant; VRE, vancomycin-resistant enterococci.

All of the AR (100%), HLSR (96.5%), and HLGR (100%) isolates were multiple drug-resistant (MDR; resistant to ≥3 antibiotic classes) (Table 5).

Overall, the *ant(6′)-Ia* and *ant(3′)-Ia* genes encoding streptomycin resistance were detected in 96.5% ($n = 28/29$) and 24% ($n = 7/29$) of the HLSR isolates, respectively. The *aac(6′)Ie-aph(2″)-Ia* and *aph(3′)-IIIa* genes were detected in 100% ($n = 7/7$) and 57% ($n = 4/7$) of the HLGR isolates, respectively. The *aph(2″)-Id*, *aph(2″)-Ib*, and *aph(2″)-Ic* genes were not observed in HLGR isolates (Figures 1 and 2).

Virulence genes profile

Out of 97 *E. faecium* isolates, 88% ($n = 85$) were positive for *gelE*, 30% ($n = 29$) for *asa1*, 20% ($n = 19$) for *esp*, and 5% ($n = 5$) for *cylA* and negative for *hyl* genes. Similarly, out of 33 *E. faecalis* isolates, 91% ($n = 30$) were positive for *gelE*, 39% ($n = 13$) for *asa1*, 39% ($n = 13$) for *esp*, 33% ($n = 11$) for *cylA*, and 3% ($n = 1$) for *hyl* genes.

The virulence gene profile analysis showed that 87% ($n = 86/97$) of *E. faecium* isolates and 97% ($n = 32/33$) of *E. faecalis* isolates harbored at least one virulence determinant encoding gene (Table 6).

ERIC-PCR analysis

The ERIC-1R primer in *E. faecium* produced 4–15 amplicons of 100–1,600 bp. Based on the dendrogram with 80% similarity, 33 different clusters (subgroups) were found. Of the 53 evaluated isolates, 19 had unique genotypes, while the highest number of isolates was observed for genotype subgroups w, x, and d ($n = 3$) (Figure 3).

The ERIC-1R primer in *E. faecalis* produced 3–13 amplicons of 150–1,300 bp. Based on the dendrogram with 80% similarity, 11 different clusters (subgroups) were found (Figure 4). Out of the 18 isolates tested, six cases had unique genotypes (a, b, d, f, i, and k), while genotype c showed the highest number of isolates ($n = 3$). Vancomycin-resistant *E. faecalis* isolates were found in the c and f subgroups. No clonal relatedness was found between the isolates.

DISCUSSION

The dissemination of antimicrobial-resistant bacteria and antibiotic-resistance genes through untreated hospital wastewater is a growing health concern (Liu *et al.* 2023). Studies have shown that the increased use of antibiotics in hospitals admitting Covid-19 patients is associated with a higher relative abundance of antibiotic-resistant bacteria and antibiotic-resistance genes in untreated hospital wastewater (Wang *et al.* 2022). A One Health approach is needed to address this potential threat (Despotovic *et al.* 2023). In the context of the One Health approach, studying antibiotic-resistant bacteria in hospital wastewater is important because it helps to understand the growth of particular pathogenic microbes, the development and spread of antibiotic resistance in microbes, and subsequent changes in treatment efficiencies (Liu *et al.* 2023; Despotovic *et al.* 2023). In the current study, *E. faecium* and *E. faecalis* were found to compose 68.8 and 23.4% of *Enterococcus* spp. isolated

Table 5 | Antibiotic resistance (intermediate resistance + resistance) profile of HLSR-, HLGR-, VR- and AR-*Enterococcus* species

Enterococcus species	Phenotypic resistance combination pattern	Isolates *n* (%)	Antibiotic number	Antibiotic class number	Total[a] *n* (%)	MDR (%)
AR	P/CIP/V/TE/FM/C/RA/S/G/Amp	2 (13)	10	8	2 (13)	100
$N = 15$	P/CIP/TE/FM/C/RA/S/Amp	1 (7)	8	7	1 (7)	
	P/CIP/V/TE/RA/S/G/Amp	1 (7)	8	6	4 (27)	
	P/CIP/TE/FM /RA/S/Amp	1 (7)	7	6	4 (27)	
	P/CIP/TE/FM /RA/G/Amp	1 (7)	7	6	4 (27)	
	P/CIP/V/TE/FM/RA/Amp	1 (7)	7	6	4 (27)	
	P/CIP/TE/RA/G/Amp	1 (7)	6	5	5 (36)	
	P/CIP/V/TE/RA/Amp	1 (7)	6	5	5 (36)	
	P/CIP/TE/RA/S/Amp	1 (7)	6	5	5 (36)	
	P/CIP/TE/FM/RA/Amp	2 (1)	6	5	5 (36)	
	P/TE/FM/RA/Amp	1 (7)	5	4	1 (7)	
	P/CIP/RA/Amp	1 (7)	4	3	1 (7)	
	TE/RA/Amp	1 (7)	4	3	1 (7)	
HLGR	P/CIP/V/TE/TEI/C/RA/G	1 (14)	8	7	2 (29)	100
$N = 7$	P/CIP/V/TE/FM/RA/G	1 (14)	7	7	2 (29)	
	P/CIP/V/TE/TEI/RA/G	1 (1)	7	6	3 (43)	
	P/CIP/TE/FM/RA/G	2 (29)	6	6	3 (43)	
	P/CIP/TE/RA/G	2 (29)	5	5	2 (29)	
HLSR	P/CIP/V/TE/FM/C/RA/G/S	1 (3)	9	8	2 (6)	96.5
$N = 29$	P/CIP/V/TE/TEI/C/RA/G/S	1 (3)	9	7	2 (6)	
	P/CIP/TE/FM/C/RA/Amp/S	1 (3)	8	7	4 (13)	
	P/CIP/TE/FM/RA/G/S	2 (6)	7	6	4 (13)	
	P/CIP/V/TE/RA/G/S	1 (3)	7	6	4 (13)	
	P/CIP/TE/FM/RA/Amp/S	1 (3)	7	6	8 (26)	
	P/CIP/TE/FM/RA/S	7 (23)	7	6	8 (26)	
	P/CIP/TE/RA/Amp/S	1 (3)	7	5	3 (10)	
	CIP/TE/C/RA/S	2 (6)	6	5	3 (10)	
	CIP/TE/RA/S	3 (10)	6	4	5 (26)	
	CIP/TE/C/S	1 (3)	5	4	5 (26)	
	CIP/C/RA/S	1 (3)	5	4	5 (26)	
	CIP/RA/S	1 (3)	5	3	6 (19)	
	CIP/TE/S	3 (10)	4	3	6 (19)	
	TE/RA/S	2 (6)	4	3	6 (19)	
	S	1 (3)	1	1	1 (3)	
VRE	P/CIP/V/TE/FM/C/RA/S/G/Amp	2 (33)	11	8	2 (33)	100
$N = 6$	P/CIP/V/TE/RA/S/G/AMP	1 (17)	9	6	2 (33)	
	P/CIP/V/TE/FM/RA/S/G	1 (17)	8	7	2 (33)	
	P/CIP/V/TE/RA/Amp	1 (17)	7	5	1 (17)	
	P/CIP/V/RA/Amp	1 (17)	6	4	1(17)	

HLSR, high-level streptomycin-resistant; HLGR, high-Level gentamicin-resistant; AR, ampicillin-resistant; VRE, vancomycin-resistant enterococci.
P, penicillin G'; CIP, ciprofloxacin; TE, tetracycline; FM, nitrofurantoin; C, chloramphenicol; RA, rifampicin.
[a]Total number of isolates resistant to the same number of antibiotic classes.

from Ardabil hospital wastewater. Similar reports have been declared in other cities in Iran such as Tehran (*E. faecium* 80% and *E. faecalis* 17%) (Rahimi *et al.* 2019) and Sari (*E. faecium* 53.3% and *E. faecalis* 46.6%) (Asgharzadeh *et al.* 2021). This result was also in agreement with studies reported from other countries such as Poland, which showed that the occurrence of *E. faecium* isolates (42.9%) was higher than that of *E. faecalis* isolates (31%) in hospital wastewater (Gotkowska-Płachta 2021). However, the rate of *E. faecalis* isolates in clinical specimens is usually higher almost two or three times more than that of *E. faecium* isolates (Maleki *et al.* 2021; Ebrahimi *et al.* 2022). The high incidence of *E. faecium* in wastewater may be due to the higher frequency of intestinal colonization with *E. faecium* compared to *E. faecalis* in people, which their fecal material is discharged into the wastewater (Jannati *et al.* 2020).

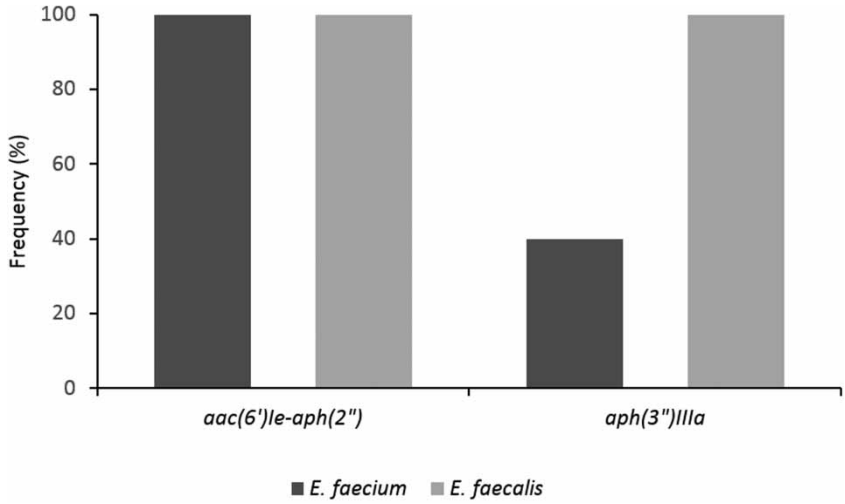

Figure 1 | Frequency of gentamicin resistance (HLGR) encoding genes in *E. faecalis and E. faecium* isolated from hospital wastewater.

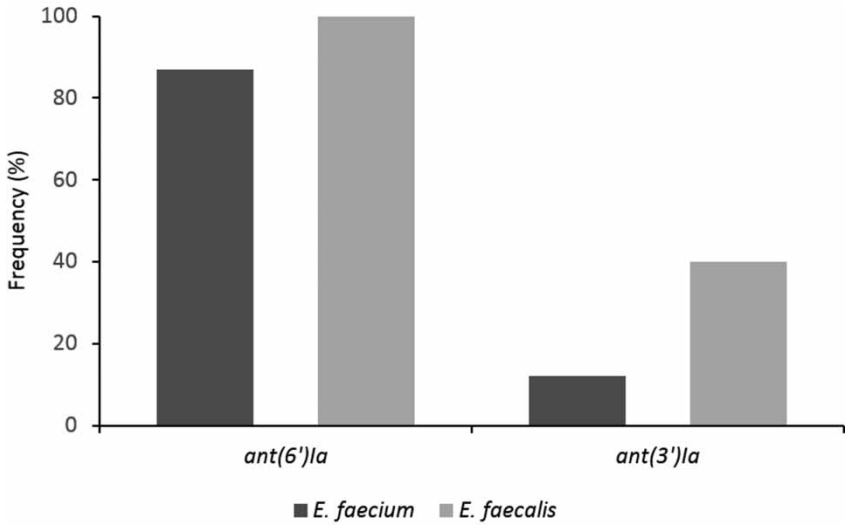

Figure 2 | Frequency of streptomycin resistance (HLSR) encoding genes in *E. faecalis and E. faecium* isolated from hospital wastewater.

Invasive enterococcal infections are typically treated with a combination of vancomycin and ampicillin along with aminoglycoside antibiotics. However, resistance to these antibiotics can make it challenging to treat such infections (Goldstein *et al.* 2003). The emergence of vancomycin resistance has been increasingly reported in clinical *Enterococcus* isolates all over the world since 1986 (Novais *et al.* 2005; Sood *et al.* 2008; Emaneini *et al.* 2016; Maleki *et al.* 2021). Nowadays, VRE isolates are reported beyond infected patients and can be found in various environments. According to a meta-analysis study conducted by Hasanpour *et al.*, the prevalence of VRE strains in non-clinical settings in Iran was found to be 33.4% in foods, 26.4% in hospital environments, and 13.1% in wastewater and surface water (Hasanpour *et al.* 2021). In comparison, in our study, the prevalence of VRE strains in hospital wastewaters in Ardabil was lower than the average reported above. However, similar results were reported from some regions in Iran (3.6%) (Talebi *et al.* 2008) and Portugal (3.4%) (Araújo *et al.* 2010). In contrast, a higher prevalence of VRE was reported in Sweden (36%) (Iversen *et al.* 2002). However, the extent of vancomycin usage and hence selective pressure imposed on bacteria and laboratory methods used to study vancomycin resistance can also affect the prevalence of VRE isolates in different geographical regions (Zakaria *et al.* 2023).

Table 6 | Virulence genes' profile in *Enterococcus* species isolated from hospital wastewater

Enterococcus spp.	Virulance genes' profile	Isolates *n* (%)	Gene number *n* (%)	Total[a] *n* (%)
E. faecium ($N = 97$)	*cylA esp asa geleE*	2 (2)	4	2 (2)
	esp asa geleE	10 (10)	3	10 (10)
	asa geleE	17 (17)	2	26 (27)
	esp geleE	6 (6)	2	
	cylA geleE	3 (3)	2	
	geleE	46 (47)	1	47 (48)
	Esp	1 (1)	1	
	–	11 (11)	0	11 (11)
E. faecalis ($N = 33$)	*cylA esp asa geleE*	4 (12)	4	4 (12)
	cylA asa geleE	5 (15)	3	7 (21)
	esp, asa geleE	1 (3)	3	
	cylA esp asa	1 (3)	3	
	esp geleE	8 (27)	2	11 (33)
	asa geleE	2 (2)	2	
	cylA hyl	1 (3)	2	
	geleE	10 (33)	1	10 (33)
	–	1 (3)	0	1 (3)

[a]Total number of isolates harboring the same number of virulence genes.

Several genes encoding D-Ala–D-Ala ligases have been identified as responsible for vancomycin resistance. Among them, *vanA* and *vanB* are the most common genes that convey high-level resistance in *Enterococcus* spp. (Eliopoulos & Gold 2001).

The overall estimate of the *vanA* and *vanB* genes in non-clinical *Enterococcus* isolates in Iran has been reported to be 35.1 and 6.6%, respectively (Hasanpour *et al.* 2021). In the current study, 66.6% of VRE strains in hospital wastewaters were positive for *vanA* and negative for *vanB* genes. Previously, we also observed that 100% of VRE clinical *Enterococcus* isolates in Ardabil were positive for the *vanA* gene (Maleki *et al.* 2021). Similar findings were reported from other countries showing the predominance of the *vanA* gene in VRE isolates (Sood *et al.* 2008; Gotkowska-Płachta 2021).

Ampicillin resistance is significantly high in clinical enterococci isolates (Hidron *et al.* 2008). However, higher rates of ampicillin resistance have also been reported in non-clinical enterococci isolates from countries such as Portugal (45%) and Iran (30.4%) (Novais *et al.* 2005; Hasanpour *et al.* 2021). In contrast, our isolates exhibited low rates of resistance to ampicillin. It is generally accepted that *E. faecium* is more resistant to penicillin and ampicillin than *E. faecalis* (Gagetti *et al.* 2019). However, in our study, ampicillin resistance was higher in *E. faecalis* (15%) than in *E. faecium* (10%) isolates. Similar results were reported by Gouliouris *et al.* on *E. faecalis* isolates collected from untreated municipal wastewater in England (Gouliouris *et al.* 2019).

In our study, we found that 15% of *E. faecalis* and 25% of *E. faecium* isolates had a HLGR phenotype and, to a lesser extent, 5% of *E. faecalis* and 6% of *E. faecium* isolates had a HLSR phenotype. Few studies have reported the occurrence of HLGR and HLSR *Enterococcus* spp. in non-clinical settings, but our frequency of HLGR isolates was lower than the rates reported for clinical isolates in Iran [67% for *E. faecalis* and 33% for *E. faecium* (Mousavi *et al.* 2020)], Italy [71% for *E. faecalis* and 29% for *E. faecium* (Zarrilli *et al.* 2005)], and South Korea [23% for *E. faecalis* and 77% for *E. faecium* (Jang *et al.* 2010)]. Similarly, higher rates of HLSR have been reported for clinical enterococci isolates worldwide (Sahm & Gilmore 1994; Jang *et al.* 2010; Dadfarma *et al.* 2013), compared to our study and a similar study from Poland, where 20.7% of *E. faecium* and 16% of *E. faecalis* isolates from marine outflow of a wastewater treatment plant (WWTP) were HLSR (Sahm & Gilmore 1994).

Resistance against aminoglycosides is commonly observed due to enzymatic changes in the drugs by aminoglycoside-modifying enzymes (Ramirez & Tolmasky 2010). However, high-level resistance to gentamicin and streptomycin is caused by different mechanisms. These antibiotics can be used interchangeably in the treatment of enterococcal infections. We found that the commonest aminoglycoside-modifying encoding genes in HLGR and HLSR isolates were *aac (6′) Ie-aph (2″) Ia* and *ant(6′)-Ia*, respectively. This is consistent with global reports, suggesting that these genes commonly encode high-level resistance to gentamicin and streptomycin (Ida *et al.* 2001).

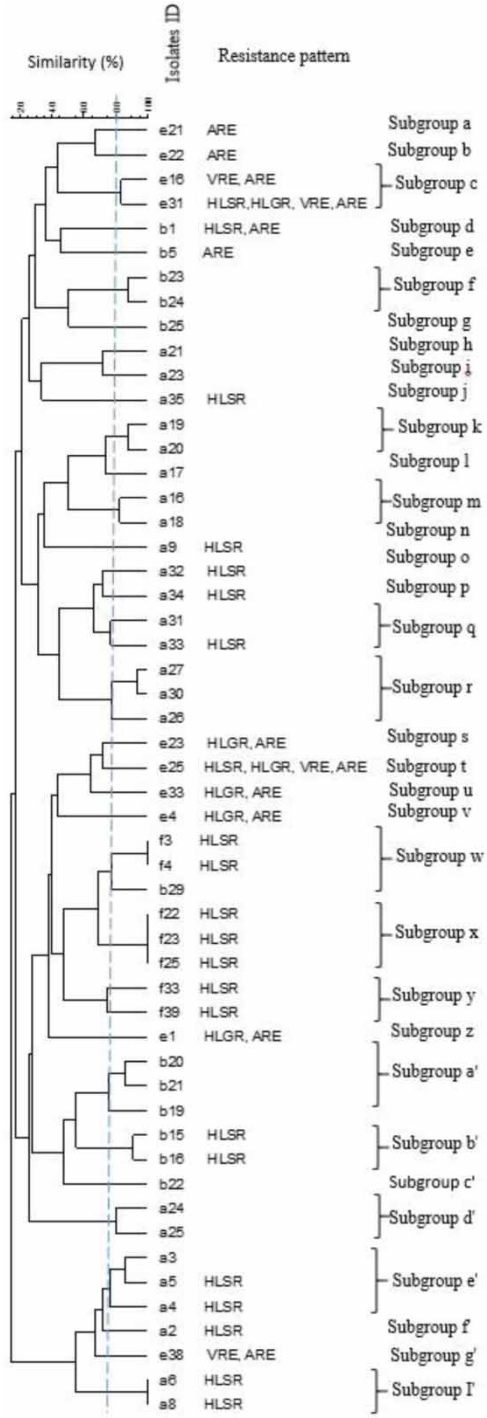

Figure 3 | Dendrogram of ERIC-PCR patterns showing the genetic relationship among the 53 *E. faecium* isolates collected from hospital wastewater. Similarities of more than 80% were considered for the clustering of the isolates.

Almost all of the HLGR and HLSR isolates were MDR. Akin to our study, high rates of MDR Enterococci in hospital sewage were reported in Poland (94.0 and 88%) and Portugal (61.5%) in the literature (Varela *et al.* 2013; Sadowy & Luczkiewicz 2014; Gotkowska-Płachta 2021). In addition to the aforementioned resistance traits, our *E. faecalis* and *E. faecium* isolates also showed significant rates of resistance to ciprofloxacin and rifampicin. Similar results have been reported in enterococci collected from hospital sewage samples in South Africa and Greece (Kotzamanidis *et al.* 2009; Iweriebor *et al.* 2015).

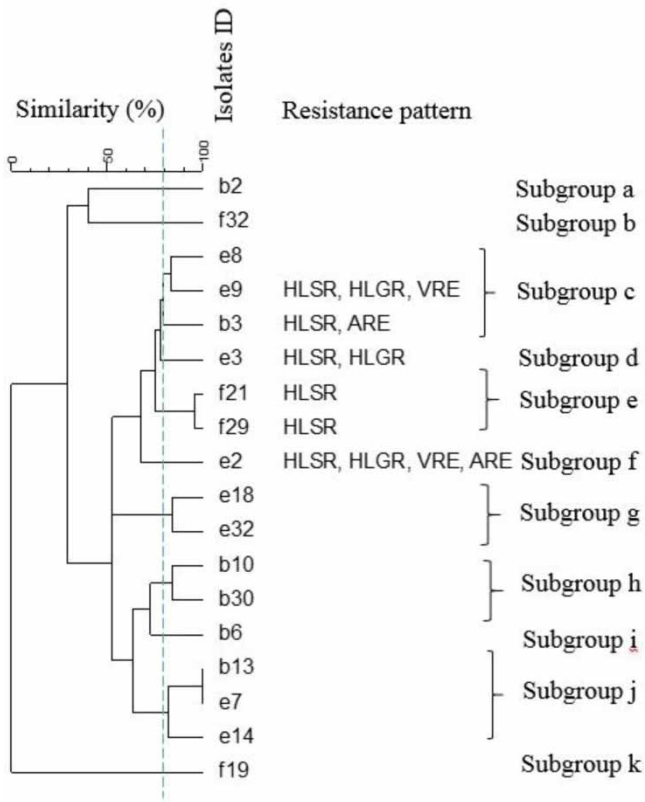

Figure 4 | Dendrogram of ERIC-PCR patterns showing the genetic relationship among the 18 *E. faecalis* isolates collected from hospital wastewater. Similarities of more than 80% were considered for the clustering of the isolates.

Enterococci were once considered microorganisms of minimal clinical impact but have now emerged as common opportunistic pathogens of humans (Ben Braiek & Smaoui 2019). The virulence factors play a significant role in the pathogenicity of enterococcal strains (Heidari *et al.* 2017). Some studies reported a lesser frequency of virulence determinants in *Enterococcus* spp. from wastewater effluent (Ferguson *et al.* 2016). However, our isolates contained multiple virulence encoding genes (*gelE* and *asa1*, *esp*, and *cyl*) simultaneously. In contrast to a general assumption that *E. faecalis* is more virulent and associated with more virulence factors than *E. faecium* isolates (Noskin *et al.* 1995), our *E. faecium* isolates harbored several virulence factors as well.

The *Enterococcus* spp. isolates in our hospital sewages showed high genotypic diversity among HLGR, HLSR, and VRE isolates as revealed by ERIC-PCR. This indicates that there is no clonal dissemination of *Enterococcus* spp. in our hospital sewages. ERIC-PCR was used to study the clonality of *Enterococcus* isolates previously (Bachtiar *et al.* 2015; Xie *et al.* 2019); however, it is generally accepted that PCR-based methods have less discriminatory power in comparison with nucleotide sequence-based methods and pulsed-field gel electrophoresis. Unfortunately, due to limited resources, we were unable to examine our isolates using these methods.

CONCLUSION

In summary, the results of this study indicate that the clinically significant *Enterococcus* spp. *E. faecalis* and *E. faecium* are prevalent in our hospital's wastewater. A significant proportion of isolates were found to be resistant to key antibiotics used for the treatment of enterococcal infections and harbor several virulence genes. Our findings show that hospital wastewater could act as a reservoir for antibiotic-resistant and virulent enterococci enabling the distribution of these organisms to the environment. Therefore, we propose that hospital effluent should be strictly examined to ensure treatment efficacy before disposal into municipal wastewater systems to manage the public health and environmental risks of antibiotic-resistant

enterococci. We also suggest conducting deep molecular studies to establish the relationship between wastewater isolates and clinical isolates and identify the circulating antibiotic-resistant clones in hospital settings.

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AUTHOR CONTRIBUTIONS

E.J. performed the experiments, analyzed the results, and prepared the initial manuscript draft. M.M. performed the experiments. F.K. contributed to writing, reviewing, and editing the manuscript. N.A. contributed to the analysis of the results. V.S.N. contributed to molecular analysis. M.A. conceived the study, led the project, and critically revised the manuscript.

CONSENT TO PUBLICATION

All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

REFERENCES

Araújo, C., Torres, C., Silva, N., Carneiro, C., Gonçalves, A., Radhouani, H., Correia, S., Da Costa, P. M., Paccheco, R., Zarazaga, M. & Ruiz-Larrea, F. 2010 Vancomycin-resistant enterococci from Portuguese wastewater treatment plants. *J. Basic Microbiol.* **50** (6), 605–609.

Asgharzadeh Marghmalek, S., Valadan, R., Gholami, M., Nasrolahei, M. & Goli, H. R. 2021 Survey on antimicrobial resistance and virulence-related genes in *Enterococcus faecium* and *Enterococcus faecalis* collected from hospital environment in the north of Iran. *Gene Rep.* **24**, 101233.

Bachtiar, E. W., Bachtiar, B. M., Dewiyani, S. & Surono Akbar, S. M. 2015 *Enterococcus faecalis* with capsule polysaccharides type 2 and biofilm-forming capacity in Indonesians requiring endodontic treatment. *J. Invest. Clin. Dent.* **6** (3), 197–205.

Ben Braiek, O. & Smaoui, S. 2019 Enterococci: between emerging pathogens and potential probiotics. *Biomed. Res. Int.* **20** (2), 296–301.

Blanch, A., Caplin, J., Iversen, A., Kühn, I., Manero, A., Taylor, H. & Vilanova, X. 2003 Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. *J. Appl. Microbiol.* **94** (6), 994–1002.

Clinical and Laboratory Standards Institute (CLSI). 2023 *Performance Standards for Antimicrobial Susceptibility Testing*, 33rd edn. CLSI; CLSI supplement M100S, Wayne, PA.

Dadfarma, N., Fooladi, A. A. I., Oskoui, M. & Hosseini, H. M. 2013 High level of gentamicin resistance (HLGR) among *enterococcus* strains isolated from clinical specimens. *J. Infect. Public Health* **6** (3), 202–208.

Deasy, B. M., Rea, M. C., Fitzgerald, G. F., Cogan, T. M. & Beresford, T. P. 2000 A rapid PCR based method to distinguish between Lactococcus and *Enterococcus*. *Syst. Appl. Microbiol.* **23** (4), 510–522.

Despotovic, M., de Nies, L., Busi, S. B. & Wilmes, P. 2023 Reservoirs of antimicrobial resistance in the context of One Health. *Curr. Opin. Microbiol.* **73**, 102291.

Dutka-Malen, S., Evers, S. & Courvalin, P. 1995 Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J. Clin. Microbiol.* **33** (1), 24–27.

Ebrahimi, T., Shahbazi, T. & Arfaatabar, M. 2022 Prevalence of vancomycin and gentamycin resistance among *Enterococci* spp. in Iran during 2007–2019: a systematic review. *Infect. Epidemiol. Microbiol.* **8** (1), 77–86.

Eliopoulos, G. M. & Gold, H. S. 2001 Vancomycin-resistant enterococci: mechanisms and clinical observations. *Clin. Infect. Dis.* **33** (2), 210–219.

Emaneini, M., Hosseinkhani, F., Jabalameli, F., Nasiri, M., Dadashi, M., Pouriran, R. & Beigverdi, R. 2016 Prevalence of vancomycin-resistant *Enterococcus* in Iran: a systematic review and meta-analysis. *Eur. J. Clin. Microbiol. Infect. Dis.* **35** (9), 1387–1392.

Espigares, E., Bueno, A., Espigares, M. & Gálvez, R. 2006 Isolation of Salmonella serotypes in wastewater and effluent: effect of treatment and potential risk. *Int. J. Hyg. Environ. Health* **209** (1), 103–107.

Ferguson, D. M., Talavera, G. N., Hernández, L. A. R., Weisberg, S. B., Ambrose, R. F. & Jay, J. A. 2016 Virulence genes among *Enterococcus faecalis* and *Enterococcus faecium* isolated from coastal beaches and human and nonhuman sources in Southern California and Puerto Rico. *J. Pathog.* **2016**, 3437214, 7.

Gagetti, P., Bonofiglio, L., García Gabarrot, G., Kaufman, S., Mollerach, M., Vigliarolo, L., von Specht, M., Toresani, I. & Lopardo, H. A. 2019 Resistance to β-lactams in enterococci. *Rev. Argent. Microbiol.* **51** (2), 179–183.

Goldstein, E. J., Le, T. & Bayer, A. S. 2003 Combination antibiotic therapy for infective endocarditis. *Clin. Infect. Dis.* **36** (5), 615–621.

Gotkowska-Płachta, A. 2021 The prevalence of virulent and multidrug-resistant enterococci in river water and in treated and untreated municipal and hospital wastewater. *Int. J. Environ. Res. Public Health* **18** (2), 563.

Gouliouris, T., Raven, K. E., Moradigaravand, D., Ludden, C., Coll, F., Blane, B., Naydenova, P., Horner, C., Brown, N. M., Corander, J. & Limmathurotsakul, D. 2019 Detection of vancomycin-resistant Enterococcus faecium hospital-adapted lineages in municipal wastewater treatment plants indicates widespread distribution and release into the environment. *Genome Res.* **29** (4), 626–634.

Hasanpour, F., Neyestani, Z., Arzanlou, M., Moradi-Asl, E., Sahebkar, A. & Khademi, F. 2021 Vancomycin-resistant enterococci in Iran: a systematic review and meta-analysis of non-clinical studies. *Gene Rep.* **24**, 101265.

Heidari, H., Hasanpour, S., Ebrahim-Saraie, H. S. & Motamedifar, M. 2017 High incidence of virulence factors among clinical *Enterococcus faecalis* isolates in Southwestern Iran. *Infect. Chemother.* **49** (1), 51–56.

Hidron, A. I., Edwards, J. R., Patel, J., Horan, T. C., Sievert, D. M., Pollock, D. A. & Fridkin, S. K. 2008 Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infect. Control Hosp. Epidemiol.* **29** (11), 996–1011.

Hocquet, D., Muller, A. & Bertrand, X. 2016 What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. *J. Hosp. Infect.* **93** (4), 395–402.

Ida, T., Okamoto, R., Shimauchi, C., Okubo, T., Kuga, A. & Inoue, M. 2001 Identification of aminoglycoside-modifying enzymes by susceptibility testing: epidemiology of methicillin-resistant Staphylococcus aureus in Japan. *J. Clin. Microbiol.* **39** (9), 3115–3121.

Iversen, A., Kühn, I., Franklin, A. & Möllby, R. 2002 High prevalence of vancomycin-resistant enterococci in Swedish sewage. *Appl. Environ. Microbiol.* **68** (6), 2838–2842.

Iweriebor, B. C., Gaqavu, S., Obi, L. C., Nwodo, U. U. & Okoh, A. I. 2015 Antibiotic susceptibilities of *Enterococcus* species isolated from hospital and domestic wastewater effluents in Alice, Eastern Cape Province of South Africa. *Int. J. Environ. Res. Public Health* **12** (4), 4231–4246.

Jang, H. C., Lee, S., Song, K. H., Jeon, J. H., Park, W. B., Park, S. W., Kim, H. B., Kim, N. J., Kim, E. C., Oh, M. D. & Choe, K. W. 2010 Clinical features, risk factors and outcomes of bacteremia due to enterococci with high-level gentamicin resistance: comparison with bacteremia due to enterococci without high-level gentamicin resistance. *J. Korean Med.* **25** (1), 3–8.

Jannati, E., Amirmozaffari, N., Saadatmand, S. & Arzanlou, M. 2020 Faecal carriage of high-level aminoglycoside-resistant and ampicillin-resistant *Enterococcus* species in healthy Iranian children. *J. Glob. Antimicrob. Resist.* **20**, 135–144.

Kariyama, R., Mitsuhata, R., Chow, J. W., Clewell, D. B. & Kumon, H. 2000 Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. *J. Clin. Microbiol.* **38** (8), 3092–3095.

Kotzamanidis, C., Zdragas, A., Kourelis, A., Moraitou, E., Papa, A., Yiantzi, V., Pantelidou, C. & Yiangou, M. 2009 Characterization of *vanA*-type *Enterococcus faecium* isolates from urban and hospital wastewater and pigs. *J. Appl. Microbiol.* **107** (3), 997–1005.

Leelaporn, A., Yodkamol, K., Waywa, D. & Pattanachaiwit, S. 2008 A novel structure of tn4001-truncated element, type V, in clinical enterococcal isolates and multiplex PCR for detecting aminoglycoside resistance genes. *Int. J. Antimicrob. Agents* **31** (3), 250–254.

Liu, A., Zhao, Y., Cai, Y., Kang, P., Huang, Y., Li, M. & Yang, A. 2023 Towards effective, sustainable solution for hospital wastewater treatment to cope with the post-pandemic era. *Int. J. Environ. Res. Public Health* **20** (4), 2854.

Mackuľak, T., Cverenkárová, K., Vojs Staňová, A., Fehér, M., Tamáš, M., Škulcová, A. B., Gál, M., Naumowicz, M., Špalková, V. & Bírošová, L. 2021 Hospital wastewater – source of specific micropollutants, antibiotic-resistant microorganisms, viruses, and their elimination. *Antibiotics* **10** (9), 1070.

Maleki, D., Manouchehrifar, M., Kheljan, M. N., Mossavi, S. H., Jannati, E., Doghaheh, H. P., Teimourpour, R., Khademi, F. & Arzanlou, M. 2021 Vancomycin-resistant *Enterococcus* species: antimicrobial resistance and virulence genes profile. *Gene Rep.* **25**, 101338.

Martín-Platero, A. M., Valdivia, E., Maqueda, M. & Martínez-Bueno, M. 2009 Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. *Int. J. Food Microbiol.* **132** (1), 24–32.

Mousavi, S. H., Peeri-Doghaheh, H., Mohammadi-Ghalehbin, B., Teimourpour, R., Maleki, D., Khademi, F. & Arzanlou, M. 2020 High-level resistance to aminoglycosides and ampicillin among clinical isolates of *Enterococcus* species in an Iranian referral hospital. *Iran. J. Microbiol.* **12** (4), 319.

Namaki Kheljan, M., Teymorpour, R., Peeri Doghaheh, H. & Arzanlou, M. 2022 Antimicrobial biocides susceptibility and tolerance-associated genes in *Enterococcus faecalis* and *Enterococcus faecium* isolates collected from human and environmental sources. *Curr. Microbiol.* **79** (6), 170.

Noskin, G. A., Peterson, L. R. & Warren, J. R. 1995 *Enterococcus faecium* and *Enterococcus faecalis* bacteremia: acquisition and outcome. *Clin. Infect. Dis.* **20** (2), 296–301.

Novais, C., Coque, T. M., Ferreira, H., Sousa, J. C. & Peixe, L. 2005 Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. *Appl. Environ. Microbiol.* **71** (6), 3364–3368.

Padmasini, E., Padmaraj, R. & Ramesh, S. S. 2014 High level aminoglycoside resistance and distribution of aminoglycoside resistant genes among clinical isolates of *Enterococcus* species in Chennai, India. *Sci. World J.* **2014**, 239157, 5.

Rahimi, F., Talebi, M., Seyfi, M. & Pourshafiei, M. 2007 Distribution of enterococcal species and detection of vancomycin resistance genes by multiplex PCR in Tehran sewage. *Iran. Biomed. J.* **11** (3), 161–167.

Rahimi, F., Torabi, M. & Barahoei, N. 2019 Isolation of vancomycin resistant enterococci from hospital sewage in Tehran 2016. *Iran. J. Infect. Dis. Trop. Med.* **23** (83), 47–53.

Ramirez, M. S. & Tolmasky, M. E. 2010 Aminoglycoside modifying enzymes. *Drug Resist. Updates* **13** (6), 151–171.

Ramos, S., Silva, V., Dapkevicius, M. L. E., Igrejas, G. & Poeta, P. 2020 Enterococci, from harmless bacteria to a pathogen. *Microorganisms* **8** (8), 1118.

Sadowy, E. & Luczkiewicz, A. 2014 Drug-resistant and hospital-associated *Enterococcus faecium* from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. *BMC Microbiol.* **14** (1), 1–15.

Sahm, D. F. & Gilmore, M. S. 1994 Transferability and genetic relatedness of high-level gentamicin resistance among enterococci. *Antimicrob. Agents Chemother.* **38** (5), 1194–1196.

Sood, S., Malhotra, M., Das, B. & Kapil, A. 2008 Enterococcal infections & antimicrobial resistance. *Indian J. Med. Res.* **128** (2), 111.

Sparo, M., Delpech, G. & García Allende, N. 2018 Impact on public health of the spread of high-level resistance to gentamicin and vancomycin in enterococci. *Front. Microbiol.* **9**, 3073.

Strateva, T., Atanasova, D., Savov, E., Petrova, G. & Mitov, I. 2016 Incidence of virulence determinants in clinical *Enterococcus faecalis* and *Enterococcus faecium* isolates collected in Bulgaria. *Braz. J. Infect. Dis.* **20** (2), 127–133.

Talebi, M., Rahimi, F., Katouli, M., Möllby, R. & Pourshafie, M. R. 2008 Epidemiological link between wastewater and human vancomycin-resistant *Enterococcus faecium* isolates. *Curr. Microbiol.* **56** (5), 468–473.

U.S. EPA Wastewater Sampling. 2013 Number: SESDPROC-306-R3 (February 28, 2013).

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D. & Goossens, H. 2004 Development of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. *J. Clin. Microbiol.* **42** (10), 4473–4479.

Varela, A. R., Ferro, G., Vredenburg, J., Yanık, M., Vieira, L., Rizzo, L., Lameiras, C. & Manaia, C. M. 2013 Vancomycin resistant enterococci: from the hospital effluent to the urban wastewater treatment plant. *Sci. Total Environ.* **450**, 155–161.

Wang, J. T., Chang, S. C., Wang, H. Y., Chen, P. C., Shiau, Y. R. & Lauderdale, T. L. 2013 High rates of multidrug resistance in *Enterococcus faecalis* and *E. faecium* isolated from inpatients and outpatients in Taiwan. *Diagn. Microbiol. Infect.* **75** (4), 406–411.

Wang, C., Mantilla-Calderon, D., Xiong, Y., Alkahtani, M., Bashawri, Y. M., Al Qarni, H. & Hong, P. Y. 2022 Investigation of antibiotic resistome in hospital wastewater during the COVID-19 pandemic: is the initial phase of the pandemic contributing to antimicrobial resistance? *Environ. Sci. Technol.* **56** (21), 15007–15018.

Weber, D. J. & Rutala, W. A. 1997 Role of environmental contamination in the transmission of vancomycin-resistant enterococci. *Infect. Control Hosp. Epidemiol.* **18** (5), 306–309.

Xie, T., Wu, G., He, X., Lai, Z., Zhang, H. & Zhao, J. 2019 Prevalence and molecular characterization of *Enterococcus faecalis* from spring water. *J. Food Saf.* **39** (6), 12694.

Zakaria, N. D., Hamzah, H. H., Salih, I. L., Balakrishnan, V. & Abdul Razak, K. 2023 A review of detection methods for vancomycin-resistant enterococci (VRE) genes: from conventional approaches to potentially electrochemical DNA biosensors. *Biosensors* **13** (2), 294.

Zarrilli, R., Tripodi, M. F., Di Popolo, A., Fortunato, R., Bagattini, M., Crispino, M., Florio, A., Triassi, M. & Utili, R. 2005 Molecular epidemiology of high-level aminoglycoside-resistant enterococci isolated from patients in a university hospital in southern Italy. *J. Antimicrob. Chemother.* **56** (5), 827–835.

Zhang, Y., Du, M., Chang, Y., Chen, L. A. & Zhang, Q. 2017 Incidence, clinical characteristics, and outcomes of nosocomial *Enterococcus* spp. bloodstream infections in a tertiary-care hospital in Beijing, China: a four-year retrospective study. *Antimicrob. Resist. Infect. Control* **6** (1), 1–11.

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