Genomic Analysis of Multidrug-Resistant
Enterococcus faecium Harboring *vanA* Gene
from Wastewater Treatment Plants

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The emergence of vancomycin-resistant *Enterococcus faecium* (Efm) harboring *vanA* gene and multidrug-resistant determinants is a relevant public health concern. It is an opportunistic pathogen responsible for nosocomial infections widely distributed in the environment, including wastewater treatment plants (WWTPs). Our study addresses a genomic investigation of *vanA*-carrying Efm from WWTPs in Brazil. Samples from five WWTPs supplied with sewage from different sources were evaluated. Here we present whole-genome sequencing of eight *vanA*-Efm isolates performed on Illumina MiSeq platform. All these isolates presented multidrug-resistant profile, and five strains were from treated wastewater. Multiple antimicrobial resistance genes (ARGs) were found, such as *aph(3′)-IIIa*, *ant(6′)-Ia*, *erm(B)*, and *msrC*, some of them being allocated in plasmids. The virulence profile was predominantly constituted by *efaAfm* and *acm* genes and all isolates, except for one, were predicted as human pathogens. Multilocus sequence typing analysis revealed a new allele and five different STs, three previously described (ST32, ST168, and ST253) and two novel ones (ST1893 and ST1894). Six strains belonged to CC17, often associated with hospital outbreaks. As far as our knowledge, no genomic studies of *vanA*-Efm recovered from WWTPs revealed isolates belonging to CC17 in Brazil. Therefore, our findings point to the environmental spread of Efm carrying multiple ARGs.

Keywords: vancomycin-resistant enterococci (VRE), antibiotic resistance, whole-genome sequencing, wastewater

Introduction

ENTEROCOCCUS FAECIUM (Efm) IS AN opportunistic pathogen that has emerged as an important agent responsible for nosocomial infections.[1] It can be also found widely distributed in the environment, including in wastewater.[2] A prominent role of the genus *Enterococcus* is its propensity to acquire and disseminate antimicrobial resistance determinants.[3] Among the mechanisms of resistance to antimicrobials already elucidated in enterococci, the most relevant is related to glycopeptides, especially vancomycin, which has been widely used in the treatment of enterococcal infections.[4]

The emergence of vancomycin-resistant *Enterococcus faecium* (VREfm) that has evolved to carry multidrug-resistant determinants has led to treatment challenges in hospital settings, by hampering the therapeutic options

available for the treatment of infections caused by these microorganisms.[5,6] VREfm strains carry multiple determinants, such as antimicrobial resistance genes (ARGs) and pathogenicity islands, and the inherent antibiotic resistance and dissemination of resistance genes through conjugative transposons and plasmids.[7]

The *vanA* gene cluster is one of the most clinically relevant reported in vancomycin-resistant enterococci (VRE),[8,9] considering its phenotype of high-level resistance to vancomycin and teicoplanin.[10,11] The *vanA* operon is often located on a plasmid, including transposons or gene cassettes that facilitate mobility.[12]

Currently, a useful technique for the comprehensive understanding of antimicrobial resistance in bacterial isolates is the whole-genome sequencing (WGS), which can reveal information related to horizontal gene transfer, such as

involving plasmids, phages, genomic islands, and homologous recombination.[13] Thus, the detailed genetic study of the Efm genome becomes an important ally for knowledge about the dynamics of antimicrobial resistance.

In the present study, Efm resistant to vancomycin (*vanA* genotype) of wastewater treatment plants (WWTPs) had the genomes sequenced to better understand the determinants of antimicrobial resistance, virulence, and molecular epidemiological characteristics, considering the concern regarding the environmental resistance spreading and its relevance in the global health perspective.

Materials and Methods

Study setting and sewage sampling

Five WWTPs were selected for this study, from which samples of wastewater (500 mL) were collected at different stages of the plant treatment, including treated effluent (Table 1). The wastewater samples collected in sterile containers were transported to the laboratory under refrigeration and processed within 24 hours after collection.

The WWTP 1 and WWTP 2 samples were obtained at five points throughout the treatment. However, in the others WWTPs, it was not possible to access all points of the treatment process. Thus, in WWTP 3, WWTP 4, and WWTP 5, only raw and treated sewage samples were collected.

Identification and antimicrobial susceptibility of E. faecium

The wastewater samples were concentrated by filtration through a nitrocellulose filter (0.22 μm). To select strains resistant to vancomycin, the filters were aseptically placed on tubes containing 5 mL of brain/heart infusion (BHI) broth with 4 mg/L of vancomycin and incubated at 37°C for 24–48 hours. The antimicrobial concentration used was established according to clinical breakpoint values defined by EUCAST 2020.[14]

The gram-positive bacteria were subjected to VITEK 2 Compact (bioMérieux) for phenotypic identification using the card VITEK2 GP. The susceptibility to antimicrobials was also analyzed using the VITEK 2 Compact system with the card AST-P637. The isolates were classified as multidrug resistant (MDR), extensively drug-resistant (XDR), and non-MDR, according to Magiorakos *et al.* (2012).[15]

vanA gene detection

The extraction of genomic DNA (gDNA) was performed using the PureLink Genomic DNA Mini Kit (Invitrogen). The PCR final volume was 25 μL, including 1 μM of each primer (A1+ e A2−),[16] 1X GotTaq G2 Mastermix (Promega),

and thermal cycling profile accordingly.[16] The gDNA of reference strains *E. faecium* CBRVS 00653 (ATCC 51559) and *Enterococcus faecalis* CBRVS 00654 (ATCC 51575) was used as positive control and negative control, respectively.

The PCR fragments were sequenced with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and analyzed on the SeqStudio Genetic Analyzer (Thermo Fisher Scientific). Nucleotide similarity was carried out with BLASTn (www.ncbi.nlm.nih.gov/BLAST/) against GenBank (NCBI), and Resistance Gene Identifier (RGI) software against Comprehensive Antibiotic Resistance Database (CARD).[17]

Whole-genome sequencing and bioinformatic analysis

The *vanA*-carrying Efm was selected for whole-genome sequencing. The library was prepared using the Nextera XT DNA Library Preparation Kit (Illumina, Inc.) and the sequencing was performed on the Illumina MiSeq platform available at INCQS (Fiocruz Genomics Network). The FastQC tool[18] was used to assess the quality of generated reads. Ambiguous nucleotides based on quality score and adapter sequences were then trimmed using Prinseq,[19] and sequences with a Phred score less than 30 were removed. *De novo* assembly of trimmed reads and high-quality sequences was performed in Unicycler.[20] The quality of assembled genomes was assessed using QUAST 2.0.[21] MLST of assembled genomes was identified through PubMLST.[22]

The genomic assessment of antimicrobial resistance was performed using the RGI of the CARD[17] to investigate the presence of acquired ARGs, and ResFinder[23,24] to assess mutations that induce resistance. Plasmids were assessed using PlasmidFinder v2.1[25] and ViralVerify.[26] Mobile genetic elements were also assessed using MobileElement-Finder v1.0.3.[27] To ascertain pathogenicity and virulence factors (VFs), PathogenFinder v1.1[28] and VirulenceFinder 2.0[29] were used, respectively.

Genome sequence data availability

The draft whole-genome sequence assemblies of the Efm carrying *vanA* gene have been deposited in GenBank under BioProject PRJNA708321.

Results

Enterococci identification and antimicrobial susceptibility profile

Gram-positive isolates from 16 samples (*n*=67) were phenotypically identified by VITEK 2, resulting in 46 *Enterococcus* spp., of which 63% (29/46) were identified as *E. faecium*. A total of 44.8% (13/29) of Efm isolates were

TABLE 1. DESCRIPTION OF SAMPLING AND WASTEWATER TREATMENT PLANTS SELECTED FOR THIS STUDY

WWTP	Location	Main wastewater sources	Collection points	Collection period
WWTP 1	Rio de Janeiro, Brazil	Industrial	Five different points along the	May, 2019
WWTP 2		Industrial	wastewater treatment	February 2020
WWTP 3		Hospital	Raw sewage and final treated	October 2020
WWTP 4		Mixed	effluent	October 2020
WWTP 5	Brasília, Brazil	Domestic		October 2020

WWTP, wastewater treatment plant.

from WWTP 1, 41.4% (12/29) from WWTP 2, 3.4% (1/29) from WWTP 3, and 10.4% (3/29) from WWTP 4. No Efm isolates were recovered from WWTP 5. Among recovered isolates, 41.4% (12/29) were obtained from treated wastewater.

The antimicrobial susceptibility by VITEK 2 checked the resistance to 10 antimicrobials of different classes (Fig. 1). All isolates were susceptible to tigecycline. It was observed that 86.2% (25/29) of isolates were classified as MDR profile, two as XDR, and two as non-MDR, being resistant only to levofloxacin and nitrofurantoin.

All vancomycin-resistant isolates ($n = 18$) were considered for *vanA* gene research. Out of these isolates, 12 (66.7%) expressed *vanA*-phenotype (high-level resistance to vancomycin and teicoplanin) and were from WWTP 1 (8/12), WWTP 2 (3/12), and WWTP 4 (1/12). There were no VREfm isolates from WWTP 3.

Detection of the vanA *gene and WGS of* E. faecium

The PCR of *vanA* gene revealed eight strains (27.5%; 8/29) carrying this gene. Among them, five were from treated effluent: two from WWTP 1, two from WWTP 2, and one from WWTP 4. The other three isolates from WWTP 1 and WWTP 2 were recovered from intermediate points during the wastewater treatment. Among the isolates carrying *vanA* gene, all demonstrated MDR resistance profile and only half of them (4/8) expressed the *vanA*-phenotype. Thus, the eight MDR-Efm carrying *vanA* gene were submitted to WGS (Table 2). The sequencing statistics for these isolates are tabulated in Supplementary Table S1. Genomes in Efm isolates had a C+G low content ranging from 37.7% to 38%, and the average length of the genomes was 2.77 Mb, with 2,755 to 3,113 predicted genes.

The glycopeptide resistance was represented by the vancomycin-resistant gene cluster *vanHAX*, in addition to three isolates from WWTP 1 also presenting *vanG* (*vanRG*). Aminoglycoside resistance genes were found in all isolates. Five isolates showed genes capable of providing a high level of resistance to aminoglycosides, such as *aph(3′)-IIIa* and *ant(6′)-Ia*. The *erm(B)* macrolide resistance gene was found in five isolates, three of which were in plasmids.

Macrolide efflux pump, such as *msrC*, was found in all isolates, the same way it was found *EfrA* and *efrB*, which encode two subunits of the EfrAB efflux pump, also related to drug resistance. In addition, *lsaA* was present in all isolates. It encodes an ABC efflux pump and confers resistance to clindamycin, quinupristin/dalfopristin, and dalfopristin. The *tet(M)* gene was found in three isolates and *tet(L)* in two isolates, conferring resistance to tetracyclines. Chromosomal mutations in *gyrA* and *parC* genes were detected in one isolate from WWTP 4, attributing the resistance phenotype to nalidixic acid and ciprofloxacin. Mutations in *pbp5* were present in all isolates, being possible to confer resistance to ampicillin (Table 2).

All isolates had the gene corresponding to the adherence factor *efaAfm*. However, seven isolates presented another adherence factor (*acm*). The *hylEfm* gene was present in one strain from WWTP 4. According to the analyses on PathogenFinder, seven isolates were predicted as human pathogens with a probability ranging from 0.582 to 0.869, indicating the presence of pathogenic proteins, and one isolate from WWTP 2 was not predicted as a pathogen with a probability of 0.489 (Table 2).

The conserved areas of the rep plasmids were revealed in all isolates, with 11 different plasmids, among which rep2 [orf1 (pRE25)] was the most frequently present in six strains, followed by repUS15 [repA (pNB2354p1)] that was present in five strains. Plasmids rep1 [repE (pIP816)] and rep1 [repE (pAMbeta)] were present in four isolates. To a lesser extent, repUS43 [CDS12738 (DOp1)] was found in two isolates, as well as rep17 [CDS29 (pRUM)] and rep1 [repE (pKL0018)]. In
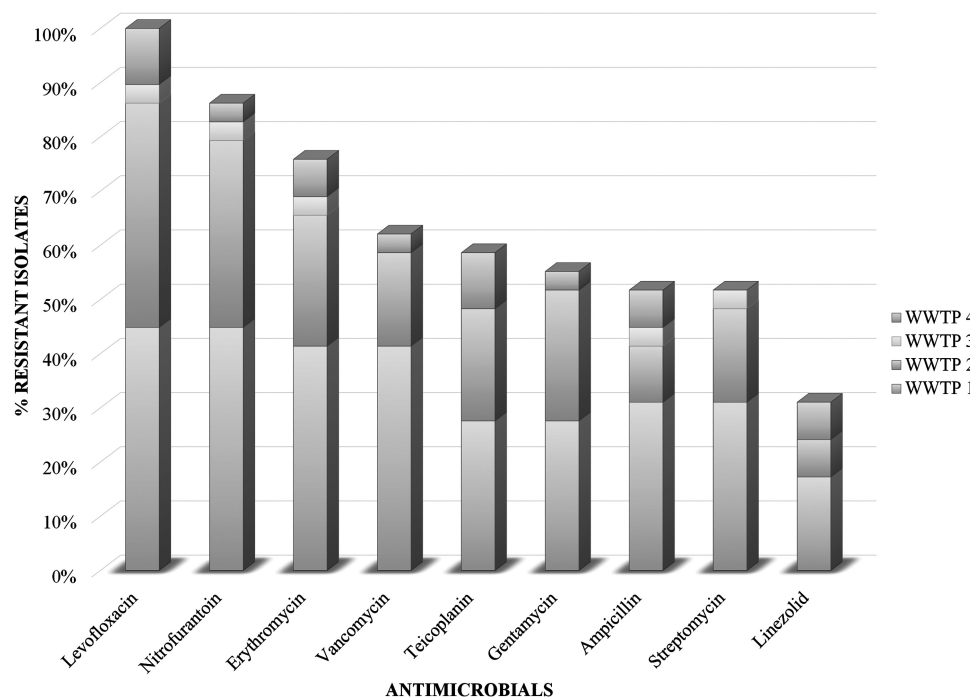

FIG. 1. Antimicrobial resistance of *Enterococcus faecium* associated with WWTP collection. WWTP, wastewater treatment plant.

TABLE 2. RESISTANCE, VIRULENCE, AND PATHOGENICITY PROFILE OF *vanA*-CARRYING *ENTEROCOCCUS FAECIUM*

Strains	WWTP	MIC		Antimicrobial resistance genes						Virulence genes			Probability of being a human pathogen
		VAN	TEI	Aminoglycoside	Macrolide	Tetracycline	β-lactam	Fluoroquinolone	Multidrug resistance	acm	efaAfm	hylEfm	
P6398	1	8	<0.5	AAC(6')-Ii, APH(3')-IIIa*, ANT(6)-Ia*	msrC, efmA, erm(B)	tet(M), tet(L)*	pbp5	—	efrA, efrB	+	+	–	0.856
P6406	1	>32	>32	AAC(6')-Ii, ANT(6)-Ia, ANT(9)-Ia*	msrC, efmA, erm(B)*	tet(M), tet(L)*	pbp5	—	efrA, efrB	–	+	–	0.582
P6407	1	>32	>32	AAC(6')-Ii, APH(3')-IIIa, ANT(6)-Ia*, ANT(9)-Ia*	msrC, efmA, erm(B)	tet(M)	pbp5	—	efrA, efrB	+	+	–	0.748
P6727	2	8	>32	AAC(6')-Ii	msrC, efmA	—	pbp5	—	lsaA, efrA, efrB	+	+	–	0.869
P6739	2	ND	<0.5	AAC(6')-Ii, ANT(6)-Ia*, ANT(9)-Ia*	msrC, efmA, erm(B)*	—	pbp5	—	lsaA, efrA, efrB	+	+	–	0.489
P6745	2	8	>32	AAC(6')-Ii	msrC, efmA	—	pbp5	—	lsaA, efrA, efrB	+	+	–	0.869
P6756	2	8	<0.5	AAC(6')-Ii	msrC, efmA	—	pbp5	—	lsaA, efrA, efrB	+	+	–	0.869
P6875	4	ND	>32	AAC(6')-Ii, APH(3')-IIIa, aad(6)	msrC, efmA, erm(B)*	—	pbp5	gyrA, parC	lsaA, efrA, efrB	+	+	+	0.799

*Genes found in plasmids.
MIC, minimum inhibitory concentration; ND, not determined; TEI, teicoplanin; VAN, vancomycin.

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TABLE 3. MLST PROFILE OF ISOLATES CARRYING *VANA* GENE

Strains	MLST allele genes							ST	CC
	atpA	*ddl*	*gdh*	*purK*	*gyd*	*pstS*	*adk*		
P6398	14	5	1	1	1	1	1	168	17
P6407	14	5	1	1	1	1	1	168	
P6727	3	3	1	2	1	1	1	32	
P6745	3	3	1	2	1	1	1	32	
P6756	3	3	1	2	1	1	1	32	
P6875	9	2	1	44	1	1	1	1,893	
P6406	3	7	3	35	1	1	1	253	ND
P6739	35	3	25	2	78	1	1	1,894	

Light gray represents the two news STs described; Dark gray represents the new allele described.
CC, clonal complex; ND, not defined; ST, sequence type.

addition, other plasmids were found in only one isolate: repUS12 [rep (pUB110)], rep11c [repA (pJS33)], rep14a [CDS2 (pEFNP1)], and rep18brepA (pEF418). Isolate P6398, from WWTP 1, showed the highest number of plasmids ($n = 8$), followed by three isolates (P6407, P6739, and P6875) that presented five plasmids.

The insertion sequences (ISs) found in the isolates were predominantly from the families IS3, IS6, IS30, IS200/IS605, IS256, IS982, and ISL3. There was one isolate from WWTP 2 that did not present any IS.

Clonal relationship

MLST analysis of eight isolates harboring the *vanA* gene revealed one new allele and five different STs, three previously described (ST32, ST168, and ST253) and two novel ones (ST1893 and ST1894). The new ''*gyd*'' allele has also been described in a new ST. It is worth highlighting that all STs belong to CC17, excepting two. Other STs were not assigned to any clonal complex according to the PubMLST database (Table 3).

Discussion

Enterococci are commensal microorganisms of the human and animal microbiota, being excreted in feces and urine. Mostly, these wastes are transported to and treated in WWTPs before being discharged into surface waters.[30] From One Health perspective, WWTPs can be considered useful surveillance sites, as they are a rich source of fecal bacteria and therefore allow the monitoring of the fecal microbiota of large human populations.[31] Gouliouris *et al.* (2019)[32] describe the association of circulating VREfm lineages in hospitals, also present in wastewater, as an example. WWTPs can also be considered ideal environments to investigate the epidemiology of antimicrobial resistance,[30] including VRE that are increasingly identified in wastewater.[2,33,34]

Our results revealed that out of 18 VRE isolates, 12 had the *vanA*-phenotype, but only 8 presented the *vanA* gene. This may be related to other vancomycin resistance genes such as *vanM*, which share the same phenotype.[8,35] In addition, it could be possible once the resistance genes may not be expressed, the antibiotic susceptibility is often related to bacterial metabolism and the metabolic regulators that modulate this phenotype.[36] It is worth noting that five of these eight isolates were recovered from treated effluents, which is worrisome, since they are discarded in aquatic environments.

Considering the importance of VREfm harboring the *vanHAX* cluster, the most prevalent glycopeptide resistance determinant in clinical settings and associated with many failures in the VRE treatment, the focus of our study was to obtain the WGS of these isolates. Expectedly, the presence of the *vanHAX* gene cluster in our eight analyzed genomes was related to Tn1546, often associated with vancomycin resistance among enterococci.[37] It is frequently carried by self-transferable plasmids, accounting for its spread.[38]

Plasmids described as possible *vanA* resistance carriers, such as repUS15 repA (pNB2354p1) and rep17 [CDS29 (pRUM)], were also observed in some of these isolates analyzed in our study, becoming important contributors to the dissemination of glycopeptide resistance.[37,39–41] In addition, seven of our isolates from industrial effluent showed the *tcrB* gene, which confers resistance to copper. The *tcrB*-carrying plasmid has also been shown to carry macrolide [*erm(B)*] and glycopeptide (*vanA*) resistance genes and it could contribute to the coselection of bacteria resistant to vancomycin and erythromycin.[42,43]

The *erm(B)* gene was found in five of our isolates, including in plasmids. Resistance to macrolides in enterococci is most often associated with a modification of the ribosomal target by 23S rRNA methylases encoded by the erythromycin-resistant methylase (*erm*) genes, providing cross-resistance to the group of macrolide antibiotics, lincosamide and streptogramin (MLS).[44,45] The *erm* gene spreading belonging to the *erm(B)* class accounts for most of the resistance caused by ribosomal methylation in enterococci.[46]

Furthermore, the presence of Tn1546 harboring *vanA* gene besides *ermB*, as occurred in some isolates of our study, has already been associated with this macrolide resistance gene in *Staphylococcus aureus*, possibly originated from Efm,[47] signaling the ARG transference and spread between bacteria. Another resistance mechanism to macrolides conferred by the *msrC* gene and expected for Efm was present in all our isolates. This gene encodes an efflux pump that is a protein of the chromosomal ABC-F subfamily that confers resistance to erythromycin and other macrolides, as well as to streptogramin B antibiotics.[2,48]

In the present study, the *aph(3′)-IIIa* gene, found in three of eight isolates, encodes the aminoglycoside

phosphotransferase enzyme APH(3′)-IIIa, conferring high-level streptomycin and kanamycin resistance in enterococci.[49,50] The gene *ant(6′)-Ia* was also found in our isolates and it is associated with high-level resistance to aminoglycoside (HLA).[50] It is already known that enterococci are intrinsically resistant to low-level aminoglycosides and the presence of the *aac(6′)-Ii* gene in all our isolates is not surprising, since *E. faecium* produces a chromosomally encoded 6′-N-aminoglycoside acetyltransferase.[51,52]

However, currently HLA mediated by the acquisition of aminoglycoside modifying enzyme-encoding genes is becoming more frequent. In addition, our results indicate that some of these aminoglycoside resistance genes were found in plasmids (Table 2). It is relevant to highlight that mobile genetic elements, such as rep2 [orf1 (pRE25)], rep18b [repA (pEF418)], and repUS15 [repA (pNB2354p1)], found in our study, are described as important determinants for horizontal transfer of antimicrobial resistance in enterococci.[2,39,40]

Three of our eight isolates presented *tet(M)* gene that acts through the binding of the gene-encoded ribosomal protection proteins to the ribosome.[53] The gene *tet(M)* is widely distributed among bacteria and this is probably due to the association of the gene with conjugative elements.[54] In addition, it has been one of the most studied tetracycline resistance genes in gram-positive bacteria and the most prevalent in enterococci.[55] Two of our isolates also showed *tet(L)* gene, which confers resistance through the effluent pump mechanisms.[56]

The mutation found in *gyrA* and *parC* present in one of our isolates has already been reported in Efm and confers resistance to ciprofloxacin.[52,57,58] However, it is important to point out that *efrA* and *efrB* were also found in this isolate and those genes encode subunits of EfrAB, which is a multiple drug efflux pump that contributes to the extrusion of fluoroquinolones in enterococci.[59] Mutations in *pbp5* have also been found in our isolates and are often related to decreased susceptibility to ampicillin and other β-lactams in Efm.[60,61] It is also worth mentioning that the *pbp5* gene has been shown to be transferable as part of large chromosomal regions and its horizontal transfer may be relevant for clinical strains to acquire β-lactam resistance.[52]

In the current study, the presence of virulence genes in multidrug-resistant enterococci has been demonstrated. The *efaAfm* gene was present in all our isolates, which is responsible for encoding cell wall adhesins. There are many reports of *efaAfm* in environmental, animal, and human clinical samples.[31,62–64] Like the *efaAfm* gene, the *acm* gene is also involved in the adhesion of Efm and it was found in all isolate genomes analyzed, excepting one. Both *acm* and *efaAfm* genes have been reported in clinical isolates in Brazil.[65,66] These genes probably play a role in the fitness of enterococci both in the human digestive tract and in WWTPs, becoming ubiquitous genes.[67]

The *hylEfm* gene, found in one of our isolates, encodes a putative glycoside hydrolase, which seems to facilitate intestinal colonization and peritoneal invasion. This virulence factor, however, presents a worrisome perspective, since an increase in the number of Efm CC17 strains carrying the *hylEfm* gene in hospitals from different countries has been documented.[68,69] Increased antimicrobial resistance is often associated with decreased virulence and fitness, although this varies according to the genera and species of bacteria.[70]

Enterococci are commonly considered to be low-virulence microorganisms and many determinants of Efm virulence are still unknown, although it could contribute to enhancing Efm capacity to cause infection.[71,72]

In our study, out of the eight Efm strains analyzed, six belong to CC17. These data are relevant because the majority of multidrug-resistant Efm isolates associated with hospital outbreaks belong to CC17.[73,74] However, in the environment, wastewater has also been frequently reported as a reservoir for CC17 Efm[1]. Accordingly, Leclercq *et al.*[75] reported in their study the association between Efm belonging to CC17 from a medical center and its respective WWTP, demonstrating that there were no significant differences in the proportions of Efm between the inflow and effluent, and therefore, the wastewater treatment does not result in the specific removal of Efm.

In addition, the study by Freitas *et al.*[76] describes the sharing between CC17 clones between animals and humans, revealing the importance of alternative routes for the spread of commensal and opportunistic bacteria.

In Brazil, data on Efm in wastewater and its potential for antimicrobial resistance spreading in aquatic environments are scarce, since studies related to this species are more directed to clinical and food samples.[9,77,78] As far as our knowledge, no genomic studies of *vanA*-Efm recovered from WWTPs revealed the presence of isolates belonging to CC17 in Brazil. Considering that this CC is associated with hospital-adapted lineages widely disseminated and responsible for human infections, our data point to the environmental spread of Efm carrying multiple resistance genes, such as resistance genes of vancomycin and HLA. In fact, some of these isolates were present in treated wastewater.

Furthermore, WGS analysis proved to be a useful tool to study antimicrobial resistance, virulence, and pathogenicity factors, as well as lineages with clinical relevance from aquatic environments.

Our results may contribute enriching data for the country's scarce scenario. It also indicates the relevance of future studies on the spread of multidrug-resistant Efm in wastewater and other aquatic environments, for the purpose of epidemiological surveillance.

Disclosure Statement

No competing financial interests exist.

Funding Information

Supplementary Material

Supplementary Table S1

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