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Original Article

Multidrug-resistant enterococci in the hospital environment: detection of novel vancomycin-resistant E. faecium clone ST910

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Abstract

Introduction: The role of the hospital environment as a reservoir of resistant bacteria in Tunisia has been poorly investigated; however, it could be responsible for the transmission of multidrug-resistant bacteria. The objective was to study the prevalence of *Enterococcus* in the environment of a Tunisian hospital and the antibiotic resistance phenotype/genotype in recovered isolates, with special reference to vancomycin resistance.

Methodology: A total of 300 samples were taken (March–June, 2013) and inoculated in Slanetz-Bartley agar plates supplemented or not supplemented with 8 µg/mL of vancomycin. Antibiotic resistance genes were tested by polymerase chain reaction (PCR). The clonal relatedness of the *vanA* isolates was assessed using pulsed-field gel electrophoresis (PFGE) and multilocus sequence testing (MLST).

Results: Enterococci were recovered in 33.3% of tested samples inoculated in SB medium. *E faecium* was the most prevalent species, followed by *E. faecalis* and *E. casseliflavus*. Antimicrobial resistance genes detected were as follows (number of isolates): *erm*(B) (71), *tet*(M) (18), *aph*(3')-IIIa (27), *ant*(*6*)-Ia (15), *cat*(A) (4), and *van*(C2) (6). Vancomycin-resistant-enterococci (VRE) were recovered from 14 samples (4.7%), when tested in SB-VAN. The 14 VRE (one per positive sample) were identified as *E. faecium* and contained the *van*(A),*erm*(B), *tet*(M), *ant*(6)-Ia, and *aph*(3')-IIIa genes. Thirteen of the VRE strains were ascribed by PFGE and MLST to a novel clone (new ST910), and only one VRE strain was typed as ST80 included in CC17.

Conclusions: The emergence and spread of new clones of VRE, especially in the hospital environment in this country, could become particularly problematic.

Key words: *Enterococcus*; hospital environment; VRE; ST910; cross-contamination; Tunisia.

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Introduction

Enterococci are recognized now as important causes of human nosocomial infections, especially in immunocompromised patients. These microorganisms are able to cause multiple pathologies such as urinary tract infections, bacteremia, and endocarditis, among others [1]. Furthermore, the capacity of enterococci to survive outside the human body for prolonged periods of time contributes to cross-contamination through the hands of healthcare workers, leading to outbreaks [1,2]. The hospital environment can be a reservoir of resistant bacteria, and the role of a possible dissemination of these bacteria, especially vancomycin-resistant enterococci (VRE) via inanimate surfaces and equipment, has been documented by several researchers [3-6]. Moreover, the cross-transmission, between patients via contaminated hands and surfaces, of multidrug-resistant microorganisms increases length of stays in hospitals, healthcare costs, and mortality [7,8].

Over the past 20 years, epidemiological data collected have shown the emergence of enterococci, especially *E. faecium*, as pathogens responsible for nosocomial infections. The expansion of *E. faecium* isolates belonging to clonal complex (CC17) is remarkable [9]. This epidemic clonal complex is characterized by ampicillin and vancomycin resistance, as well as by the presence of virulence determinants such as hyl_{Efm} (encoding a hyaluronidase-like protein) and a putative pathogenicity island that includes esp_{Efm} (enterococcal surface protein in *E. faecium*), and is responsible for severe nosocomial infections and hospital outbreaks [10,11]. The resistance to

vancomycin in enterococci is still uncommon in Tunisia, and there are only a few previous reports of detection of *vanA*-containing enterococci in hospitals in that country [6,12].In a previous study performed by our group in the Military Hospital of Tunis in 2012 [6], the presence of *vanA*-containing *E. faecium* isolates was detected in clinical samples as well as in 5% of environmental samples of this hospital. The objective of the present work was to continue the epidemiological study on environmental samples in the hospital during 2013, to determine the prevalence of *Enterococcus* and the phenotypes and genotypes of antibiotic resistance in recovered isolates, with special reference to vancomycin resistance, as well as to analyze the evolution of environmental VRE in terms of frequency and type of detected clones.

Methodology

Samples and bacterial isolates

A total of 300 environmental samples were obtained from 17 different services of the Military Hospital of Tunis, during March–June 2013. The samples were taken with sterile cotton swabs from inanimate surfaces (beds, treatment tables, toilets, faucets, wrists, sinks) after routine cleaning (n = 250), and from the hands of patients (n = 23) and staff (n = 27). Informed consent was obtained from all individuals who participated in the study.

Samples were pre-enriched in brain-heart infusion broth for 24 hours at 37°C. Next, 0.1 mL of the suspension was seeded in plates of Slanetz-Bartley (Biolife, Milano, Italy) not supplemented (SB) and supplemented with 8 µg/mL of vancomycin (BioRad, Marnes-la-Coquette, France) (SB-VAN). The samples were then incubated for 48 hours at 37ºC for the recovery of enterococci and VRE. A colony was selected of each positive sample and was identified by species-specific polymerase chain reactions (PCRs) [13].

Antimicrobial susceptibility testing and detection of resistance genes

Antibiotic susceptibility was tested for 12 antimicrobial agents by the disk-diffusion method. The agents tested were the following (µg/disk): vancomycin (30), teicoplanin (30), tetracycline (30), chloramphenicol (30), erythromycin (15), trimethoprim-sulfamethoxazole (1.25/23.75), ciprofloxacin (5) and ampicillin (10) (BioRad, Marnes-la-Coquette, France) [14]. Detection of pristinamycin resistance (15) and high-level resistance to gentamicin (500), kanamycin (1,000), and streptomycin (500) was performed according to the Antibiogram Committee of the French Society for Microbiology [15]. Minimum inhibitory concentrations (MICs) for vancomycin and teicoplanin were determined using the agar dilution method [14].

Resistance genes were sought by PCR in enterococcal isolates that showed resistance to glycopeptides (*van*(A), *van*(B), *van*(C1), and *van*(C2)) [16], tetracycline [*tet*(M), *tet*(L)], macrolides [*erm*(A), *erm*(B), *erm*(C)], and aminoglycosides [*aph*(3')-IIIa, *aac*(6')-*aph*(2''), *ant*(6)-Ia] using primers and conditions previously reported [13].

The presence of the insertion element IS*16* (usually found in hospital-associated *E. faecium* isolates implicated in outbreaks) was performed by PCR in all the *vanA*-containing isolates [17].

Detection of virulence genes

The presence of *esp* (enterococcal surface protein) and *hyl* (encoding a hyaluronidase-like protein) virulence genes was detected by PCR in the *vanA*-containing *E. faecium* isolates, and the *esp* gene was also tested in the vancomycin-susceptible enterococci [18].

Pulsed-field gel electrophoresis (PFGE)

The clonal relatedness of the *vanA*-containing isolates was assessed using PFGE of the genomic DNA digested with *Sma*I enzyme [13]. Interpretation of banding patterns was performed according to the criteria specified by Tenover *et al.* [19]. The dendrogram of PFGE patterns of *Sma*I digested DNA of 14 VRE was prepared by FPQuest (BioRad, Hercules, USA) software using UPGMA algorithm.

Multilocus sequence typing (MLST)

Internal fragments of seven housekeeping genes (*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adk*) of our *van*A-positive *E. faecium* isolates were amplified and sequenced. The sequences obtained were analyzed and compared with those included in the website database (www.pubmlst.org) in order to assign a specific sequence type (ST). STs were grouped into clonal complexes using the program eBURST (http://eburst.mlst.net) [20]. The type of *purK* allele was analyzed in the *esp*-positive vancomycin-susceptible *E. faecium* isolates to determine if they could have the *purK*1 allele (CC17-associated).

Results

Prevalence and characterization of enterococci isolated in SB agar plates not supplemented with antibiotics

Enterococci were recovered from 100 of the 300 tested samples (33.3%) when SB agar plates were used, and one enterococci per sample was further characterized. These positive samples corresponded to 80/250 (32%) inanimate surface samples, and 20/50 (40%) of hand samples (Table 1). *E. faecium* was the most prevalent species (86%), followed by *E. faecalis* (8%) and *E. casseliflavus* (6%) (Table 1).

Antimicrobial resistance distributed by species of enterococcal strains isolated in SB medium not supplemented with vancomycin is shown inTable 2. All enterococci, including *E. faecium* isolates, were susceptible to ampicillin and to a high dose of gentamicin. The antimicrobial resistance genes detected in these isolates by PCR are shown in Table 3.

The majority of the erythromycin-resistant enterococci (71 of 73 isolates, 97.3%) harbored the *erm*(B) gene. All the 18 tetracycline-resistant enterococci (12 *E. faecium*, 4 *E. faecalis*, and 2 *E. casseliflavus*) carried the *tet*(M) gene. High-level resistance to streptomycin and kanamycin was also detected in some of the isolates; 15 of 27 streptomycin-resistant strains carried the *ant*(6)-Ia gene, and 27 of 28 kanamycin-resistant strains carried the *aph*(3')-IIIa gene. The *cat*(A) gene was found in four chloramphenicol-resistant *E. faecium* isolates. No acquired vancomycin resistance was demonstrated among the enterococci recovered in SB agar plates not supplemented with vancomycin, although 6 *E. casseliflavus* isolates were obtained (with intrinsic low-level vancomycin resistance,with the *van*C2gene).

The *esp* gene, usually associated with nosocomial isolates, was detected by PCR in only 5 strains isolated from inanimate surfaces, all of them identified as *E. faecium*. These 5 *esp*-positive *E. faecium* strains had the *purK*17 allele, indicating that they were not ascribed to clonal complex CC17.

Prevalence and characterization of VRE strains isolated in SB-VAN agar plates

To determine the potential carriage of VRE in the 300 samples taken from the environment of the hospital, the samples were inoculated in SB-VAN agar plates (supplemented with 8 µg/mL of vancomycin).

Table 1. Prevalence of *Enterococcus* strains in the hospital environment (isolated in Slanetz-Bartley medium not supplemented with antibiotics).

Origin of samples	Number of samples	Number of samples containing enterococci	Number of isolates of the species		
			E. faecium	*E. faecalis*	*E. casseliflavus*
Inanimate surfaces	250	80	73	4	3
Hands of patient	23	8	4	3	1
Hands of personnel	27	12	9	1	2
Total	**300**	**100**	**86**	**8**	**6**

Table 2. Antimicrobial resistance of enterococci strains isolated in Slanetz-Bartley medium not supplemented with antibiotics.

Antimicrobial agent	Number of enterococci resistant to antimicrobial agents of the species			
	E. faecium (n = 86)	*E. faecalis* (n = 8)	*E. casseliflavus* (n = 6)	Total (n = 100)
Vancomycin	0	0	6 *	6
Teicoplanin	0	0	0	0
Ampicillin	0	0	0	0
Streptomycin	17	7	3	27
Gentamicin	0	0	0	0
Kanamycin	20	5	3	28
Chloramphenicol	7	1	2	10
Tetracycline	12	4	2	18
Erythromycin	62	6	5	73
Trimethoprim-sulfamethoxazole	86	8	6	100
Ciprofloxacin	42	4	2	48
Pristanamycin	12	4	2	18

* 6 *E. casseliflavus* isolated in Slanetz-Bartley not supplemented with vancomycin showed a low level resistance to vancomycin (intrinsic resistance conferred by the *van*C2 gene).

VRE were recovered from 14 of these 300 samples (4.7%). The 14 VRE strains (one per positive sample) were identified as *E. faecium* and carried the *van*(A)gene, showing high-level vancomycin and teicoplanin resistance (MIC > 256 µg/mL). The characteristics of the 14 *vanA*-containing *E. faecium* isolates are shown in Table 4.

These VRE isolates were recovered from different origins (12 from inanimate surfaces, 1 from the hand of a patient, and 1 from the hand of a healthcare worker), and from 6 different services of the hospital (Table 4). All the VRE isolates showed the same multiresistance phenotype to erythromycin and tetracycline as well as high-level resistance to streptomycin and kanamycin, and harbored *erm*(B), *tet*(M), *ant*(6)-Ia, and *aph*(3')-IIIa genes. The VRE isolates showed resistance to ampicillin and ciprofloxacin, but they were susceptible to high dose
of gentamicin. All of them carried the IS*16* element.

Table 3. Resistance genes among enterococci isolated in Slanetz-Bartley medium not supplemented with antibiotics.

Antibiotic	Species	Number of resistant strains	Resistance genes detected (number of strains)
Vancomycin	*E. casseliflavus*	6	*vanC2* (6)
Erythromycin	*E. faecium*	62	*erm*(B) (60)
	E. faecalis	6	*erm*(B) (6)
	E. casseliflavus	5	*erm*(B) (5)
Tetracycline	*E. faecium*	12	*tet*(M) (12)
	E. faecalis	4	*tet*(M) (4)
	E. casseliflavus	2	*tet*(M) (2)
Chloramphenicol	*E. faecium*	7	*cat*(A) (4)
	E. faecalis	1	-
	E. casseliflavus	2	-
Kanamycin	*E. faecium*	20	*aph*(3')-IIIa (19)
	E. faecalis	5	*aph*(3')-IIIa (5)
	E. casseliflavus	3	*aph*(3')-IIIa (3)
Streptomycin	*E. faecium*	17	*ant*(6)-Ia (10)
	E. faecalis	7	*ant*(6)-Ia (3)
	E. casseliflavus	3	*ant*(6)-Ia (2)

Figure 1. Dendrogram prepared by FPQuest software using UPGMA algorithm (optimization 0.50%; tolerance 1.00%) of PFGE patterns of *Sma*I digested DNA from 14 vancomycin-resistant *Enterococcus faecium* strains isolated from the hospital environment.

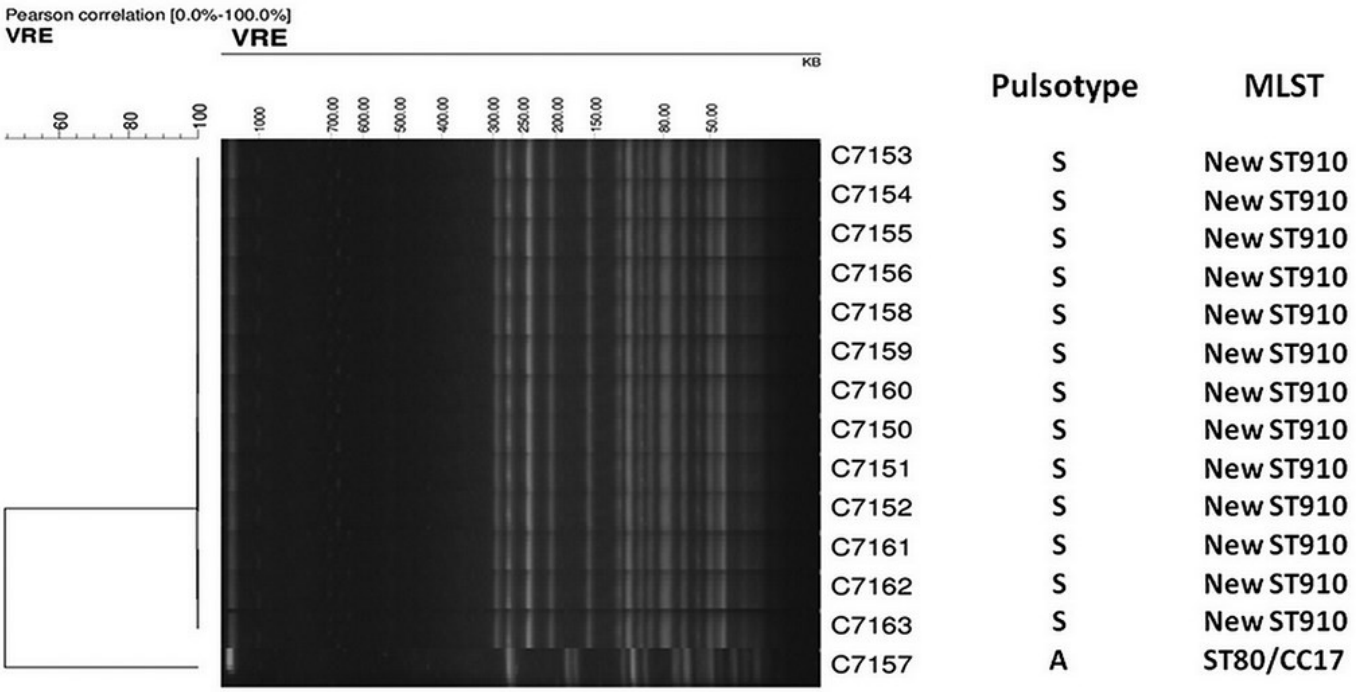

Table 4. Vancomycin-resistant *E. faecium* isolates recovered in Slanetz-Bartley medium supplemented with 8 µg/mL of vancomycin.

Strains	Origin	Service	Phenotype of resistance	Genotype of resistance	MIC (µg/mL) VA/TEC	PFGE	ST/CC
C7150	Treatment room	Cardiothoracic surgery	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7151	Treatment room	Cardiothoracic surgery	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7152	Handle door	Hemodialysis	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7153	Sink	Ophtalmology	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7154	Television	Ophtalmology	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7155	Handle door	Orthopedics and traumatology surgery	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7156	Hand of patient	Orthopedics and traumatology surgery	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7157	Bedlinen	Orthopedics and traumatology surgery	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	A	ST80/CC17
C7158	Sink	Orthopedics and traumatology surgery	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7159	Treatment table	Pneumonology	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7160	Handle door	Pneumonology	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7161	Hand of emloyee	Pneumonology	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7162	Medical apparatus	ICU	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910
C7163	Chair	ICU	VA, TEC, TE, SXT, AM, KAN, STR, E, Cp	*vanA*, *tet*(M), *aph* (3')-IIIa, *ant* (6)-Ia, *erm*(B)	> 256	S	New ST910

MIC (VAN/TEC): minimum inhibitory concentration of Vancomycin and Teicoplanin; PFGE: pulsed-field gel elecrophoresis ; ST: sequence type; CC: clonal complex VA: vancomycin; TEC: teicoplanin; TE: tetracycline; SXT: trimetoprim-sulfamethoxazole; AM: ampicillin; KAN: kanamycin; STR: streptomycin; E: erythromycin; Cp: ciprofloxacin.

The gene encoding *esp* was detected by PCR screening in all VRE isolates, but none of them contained the hyl_{Efm}virulence gene (encoding a hyaluronidase-like protein).

Analysis of clonality of vanA-containing E. faecium isolates

The clonal relatedness of isolates was assessed using PFGE and MLST. The macrorestriction analysis of genomic DNA in the 14 *vanA*-containing *E. faecium* isolates revealed two different profiles: 13 isolates showed the profile S and 1 isolate the profile A (Table 4) (Figure 1).

Two different sequences types were identified among the *vanA* isolates: the strain with PFGE-pattern A was ascribed to sequence type ST80 and clonal complex CC17, and the 13 *vanA* strains with PFGE-pattern S showed a new sequence type ST910 with the following allele combination: *atpA* (9), *ddl* (1), *gdh* (1), *purK* (1), *gyd* (16), *pstS* (1), and *adk* (18) (Table 4).

Discussion

In this study, VRE isolates were detected in 14 of the 300 (4.7%) environmental samples at the hospital setting studied. All VRE isolates obtained were *E. faecium* and contained the vancomycin resistance *vanA* gene. In Tunisia, the presence of VRE in hospitals is scarcely reported and there are only two previous reports of detection of *vanA*-containing enterococci [6,12]. VRE was first isolated in this country in 2007 [12], and it still appears to be uncommon [6]. Nevertheless, since VRE could be an emerging pathogen, further studies of VRE infection and colonization are important. Nowadays, acquired *vanA*-type vancomycin-resistant *E. faecium* are widely prevalent in hospitals around the world [21],and this situation could be similar in the future in Tunisia if appropriate control measures are not adopted.

According to previous studies, the *E. faecium* species was the most prevalent among VRE clinical isolates [22,23]. The gene *vanA* has also been detected in *E. faecalis* by other authors [18,24].Thirteen of our *vanA*-positive isolates were ascribed by PFGE and MLST to a novel clone (new ST910) and only one strain was typed as ST80 and belonged to CC17. In a previous study performed by our group, three different PFGE-types (A-C) and two sequence types (ST18 and ST80) were identified, a clone PFGE-A/ST80 being detected both among clinical and environmental *vanA E. faecium* isolates during 2012 in the same hospital [6]. It is of interest that profile A detected in one of our isolates was indistinguishable from the profile A previously detected (with the same ST80/CC17) [6], indicating that this clone is still present in the environment of the hospital. All remaining *vanA*-positive isolates belonged to a new clone, ST910/PFGE-S. These strains were vancomycin and ampicillin resistant, had *purK* (1), the *esp* gene, and all of them contained the insertion element IS*16*. All these characteristics are markers of nosocomial clones belonging to CC17. However, according to e-BURST, this ST did not belong to CC17, but was a double locus variant of ST80, from which it could be derived. The appearance of this new clone in the studied hospital could be an alert of the emergence and rapid evolution and of these clinically important resistant bacteria and the necessity for active surveillance in other hospitals and regions of the country.

In several recent studies carried out in different countries around the world (including Tunisia), VRE outbreaks seem to be related to monoclonal spread [6,25,26]. The homogeneity detected among our acquired vancomycin-resistant *E. faecium* isolates could be explained by the endemic dissemination of these VRE strains in the hospital. We found VRE in six different services of the hospital. The dissemination of *vanA*-containing *E. faecium* strains in different areas of the hospital might be due to direct contact with colonized patients or even to indirect contact via the hands of healthcare workers [4,27]. The *esp* expression on the surface of *E. faecium* seems to play a role in adherence and biofilm formation [28]. This gene could have a great role in the exchanging genetic mobile elements and could also be implicated in the adaptation of these bacteria to the hospital environment [29,30].Remarkably, our *vanA E. faecium* isolates presented a multiresistance phenotype, being resistant, in addition to glycopeptides, to erythromycin, tetracycline, ciprofloxacin, ampicillin, kanamycin, streptomycin, pristinamycin, and trimethoprim-sulfamethoxazole. In the case of resistance genes *erm*(B) and *tet*(M), it is interesting to mention that they have been previously detected in the same conjugative plasmid as the *van*(A) gene, which indicates that co-selection processes might have happened [31]. Our VRE strains were still susceptible to high dose of gentamicin, similar to what was found in a previous study reported in Tunisia showing the emergence of epidemic CC17 clones of *vanA E. faecium* that were also sensitive to high dose of gentamicin [6]. On the other hand, the detection of enterococcal species (in non-supplemented media) in one-third of samples tested (inanimate surfaces of hospital and in the intact skin hand of some patients and healthcare workers) can be explained by the fact that these microorganisms are

able to survive in the environment outside the host for a long period of time and are able to resist routine cleaning [32]. The species more frequently detected, *E. faecium* and *E. faecalis*, are implicated in most enterococcal infections [33,34]. In fact, environmental contamination may contribute to transmission of pathogens by contact of medical equipment or patients with contaminated surfaces [35].

The wide dissemination of antibiotic resistance among our enterococci isolates could be partly due to the horizontal transfer of mobile genetic elements between strains and, on the other hand, to cross-transmission of multiresistant enterococci between patients, staff, and the environment [5].

Conclusions

The dissemination of multi-resistant bacteria, including VRE of genotype *van*A, in the hospital has become particularly problematic. An active surveillance program should be carefully prepared to stop this uncontrollable evolution and emergence of these resistant bacteria. Further studies are needed and strict infection control strategies should be adopted in hospital environments to reduce potential infections by these microorganisms.

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Authors' contributions

RD and RB designed the sampling protocol and the bacteriological analyses; RD, LBS, CL, performed the molecular testing (all the PCR, MLST and PFGE); RD, NK and CL performed the analysis and the interpretation of all the data; RD, NK and CTO were involved in drafting the manuscript and revising it critically. BSK performed the analysis of the PFGE; NK, CTO and AB participated in its design and coordination and helped to draft and correct the manuscript. All authors contributed to and approved the submitted manuscript.

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