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Original article

Molecular determination of *van* genes among clinical isolates of enterococci at a hospital setting

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ABSTRACT

Vancomycin-resistant enterococci (VRE) poses a formidable challenge to public health due to its inherent resistance to multiple antibiotics coupled with the ability to transfer genetic determinants to dangerous pathogens like Methicillin-resistant *Staphylococcus aureus* (MRSA). The purpose of this study was to investigate the incidence of vancomycin resistance in enterococci among clinical isolates at a tertiary care military hospital in the eastern region of Saudi Arabia and to detect *van* genes using multiplex-PCR. Overall, 246 isolates of enterococci were collected from various clinical specimens. The isolates were identified, and antimicrobial susceptibility testing was done using the Vitek 2 system. Multiplex PCR was performed on the VRE isolates, thus identified to determine the *van* genes harbored. A total of 15 VRE were identified, of which 14 (93.3%) were *Enterococcus faecium*, and 1(6.7%) was *Enterococcus casseliflavus* with intrinsic *vanC* resistance. Of the 14 vancomycin-resistant *Enterococcus faecium*, 8 (57.1%) harbored *vanB* genes, while 6 (42.8%) harbored *vanA* genes. All the VRE were susceptible to linezolid and tigecycline. Our study detected a low prevalence (6.1%) of VRE among clinical isolates of enterococci and that the *vanB* gene predominates in such strains. Susceptibility profiles indicated that linezolid and tigecycline are still effective against these multidrug-resistant pathogens. Pus specimens yielded the highest percentage (53.3%) of isolates from which VRE was obtained, and this finding is novel among studies done in Saudi Arabia.

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1. Introduction

The genus *Enterococcus* consists of gram-positive cocci that top the list of ubiquitous, profoundly adaptable normal microbiota harbored by humans, animals, and even insect populations (Murray, 1990). Worldwide, they have emerged as a multidrug-resistant pathogen plaguing the community and hospital population alike. This genus' evolution and endurance contemporaneously as a commensal and a formidably untreatable pathogen have bewildered the scientific community. According to the List of Prokaryotic names with Standing in Nomenclature, as of 2020, the genus *Enterococcus* consists of 59 validly published species with the correct name (Parte, 2018). However, *Enterococcus faecalis* and *Enterococcus faecium* remain the most prevalent species cultured from humans, accounting for >90% of clinical isolates (Cetinkaya et al., 2000).

After initial identification in 1986 in Europe, Vancomycin-resistant enterococci (VRE) have been recognized over the years to be a significant threat to public health (Leclercq et al., 1988). This problem has been further compounded by the transmission of *vanA* resistance genes to methicillin-resistant *Staphylococcus aureus* (MRSA) to form vancomycin-resistant *Staphylococcus aureus* (VRSA) (Chang et al., 2003). As part of the global efforts to address growing resistance to antimicrobial drugs, the World Health Organization has listed VRE in its priority pathogens list as HIGH (Tacconelli et al., 2017).

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Several authors have reviewed enterococcal resistance to glycopeptides thoroughly and established that modification of antimicrobial target forms the basis of resistance to glycopeptides like vancomycin (Arthur and Courvalin, 1993; Arthur and Quintiliani, 2001; Courvalin, 2006; Kristich et al., 2014). Glycopeptide-resistant enterococci synthesize altered peptidoglycan precursors in which the D-Ala-D-Ala termini have been altered such that they terminate in either D-Ala-D-Lac or D-Ala-D-Ser. Specific operons mediate this modification, and vancomycin resistance operons are named based on the genes with ligase activity in these operons. These operons usually are encoded on mobile genetic elements and thus donated to otherwise susceptible bacteria. Specific types of ligases are also encoded in the chromosome as part of certain enterococcal species' core genome.

Currently, nine operons that confer resistance to glycopeptides have been identified among enterococci (Lebreton and Cattoir, 2019). They are classified into two categories based on the ligases they encode. The first category comprises of the operons *vanA, vanB, vanD*, and *vanM* that encode for D-Ala-D-Lac ligase. The second category encodes for D-Ala-D-Ser ligase and consists of the operons *vanC, vanE, vanG, vanL*, and *vanN*. These operons act by decreasing the antibiotics' binding affinity for the peptidoglycan precursors (~1000 fold reduction for D-Ala-D-Lac; ~7 fold for D-Ala-D-Ser). The altered precursors can still serve as substrates for cell wall biosynthetic enzymes that enable functional peptidoglycan construction, but the reduced affinity of glycopeptides renders the drugs unable to inhibit peptidoglycan biosynthesis (Cetinkaya et al., 2000).

Of the nine *van* gene clusters, eight have been identified to be of the acquired glycopeptide resistance type (*vanA, vanB, vanD, vanE, vanG, vanL, vanM,* and *vanN*), while one is of the intrinsic resistance type (*vanC*), which is unique to *E. gallinarum* and *E. casseliflavus* (Boyd et al., 2008; Courvalin, 2006; Lebreton et al., 2011; McKessar et al., 2000; Xu et al., 2010). Another *van* gene cluster (*vanF*) has been described in a biopesticide, *Paenibacillus popilliae*, but has not yet been found in enterococci (Lebreton and Cattoir, 2019; Patel et al., 2000).

Among the vancomycin resistance gene clusters, two are more prevalent globally and are predominantly carried by *E. faecium*. They are the *vanA* operon, and the *vanB* operon typically carried on the transposable elements Tn1546 and Tn1549 (Arthur et al., 1993; Rangberg et al., 2019; Wardal et al., 2014). These resistance genes can be transferred between enterococci and other bacterial strains by plasmids and through conjugative transposons. This ability has resulted in the emergence of VRSA. As of 2019, 52 VRSA strains carrying van genes have been reported from countries like the US, India, Iran, Brazil, and Portugal. Investigations have detected the inducible expression of the *vanA* gene cluster in all these VRSA strains (Cong et al., 2020).

The first report of VRE from Saudi Arabia was in 1993 in Riyadh (Qadri et al., 1993). However, there are only a few studies of VRE from Saudi Arabia enumerating the specific genes harbored by this pathogen in different parts of the country, especially in the eastern region. Hence, this study aims to evaluate the prevalence of VRE among the clinical isolates at the King Fahd Military Medical Complex (KFMMC), a tertiary care military hospital in the eastern region of Saudi Arabia, and to elucidate the *van* genes harbored by them.

2. Materials and methods

2.1. Study design and collection of enterococcal isolates

This cross-sectional and analytical study was done on 246 *Enterococcus* species isolated from various clinical specimens received in the microbiology laboratory of the KFMMC from June 2018 to December 2019. Patient information was anonymized. Repeated positive cultures of the same patient were eliminated. The isolates were transported to the microbiology laboratory of PSMCHS, located in the same complex for further study.

2.2. Bacterial identification and antimicrobial susceptibility testing

Here, following subculture and preliminary identification, the isolates were subjected to species identification and antimicrobial susceptibility testing using the automated Vitek®2 Compact system (bioMérieux, Marcy L'etoile, France) as per CLSI guidelines. Gram-positive, GP cards containing different substrates were utilized for species identification, and AST-GP67 cards were used for susceptibility. The VRE isolates obtained were also subjected to the E-test (bioMérieux, USA) for reconfirmation. *E. faecalis* ATCC 29212, *E. faecium* ATCC 700221, and *E. faecalis* ATCC 51299 were used as controls.

2.3. Detection of van genes by Multiplex PCR

According to the manufacturer's instructions, the VRE isolates obtained were then subjected to DNA extraction using the Bacterial DNA kit (MOLEQULE-ON, New Zealand). The extracted DNA was analyzed for purity using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific) and stored at −70 °C before testing.

Using specific primers (MOLEQULE-ON, New Zealand), the extracted DNA was subjected to Multiplex PCR on T100™ Thermal Cycler (Bio-Rad Laboratories, USA) (Table 1). The final concentrations were as follows: 1X PCR buffer, 1.5 mM MgCl2, 0.12 pmol of each primer, 1.25U Taq Polymerase, variable volumes of DNA templates (≤250 ng/reaction) all adjusted to 25 µl with sterile distilled water.

The PCR conditions consisted of an initial denaturation step at 95 °C for 15 min, followed by 35 cycles of 1 min at 94 °C, 30 sec at 54 °C, and 1 min at 72 °C. A final extension step was performed at 72 °C for 10 min. Amplified products were analyzed by electrophoresis on 1% agarose. DNA bands were visualized by staining with VisualaNA (A) DNA Stain (MOLEQULE-ON, New Zealand) and photographed using gel documentation system (U: Genius, Syngene U.K).

2.4. Statistical analysis

The study's result was analyzed using IBM SPSS Statistics for Windows, version 21(IBM Corp., Armonk, NY, USA). The Pearson Chi-Square test of independence was performed to assess any significant relationship between the various parameters. The statistical significance was set at $p > 0.05$.

3. Results

Of the 246 *Enterococcus* species isolated, 220 were *E. faecalis* and 25 *E. faecium*, and 1 *E. casseliflavus*. As there was only one isolate of *E. casseliflavus* obtained from a pus specimen from the male med-

Table 1
PCR primer sequence.

Primer name	Sequence (5′ − 3′)	PCR product size (bp)
vanAF	CATGAATAGAATAAAAGTTGCAATA	1030
vanAR	CCCCTTTAACGCTAATACGATCAA	
vanBF	ATGGGAAGCCGATAGTC	635
vanBR	GATTTCGTTCTTCGACC	

ical ward, it was excluded from analysis, and a description of its findings is added separately. So the analysis is based on 245 enterococcal isolates.

Of the 245 enterococcal species isolated from various hospital wards, most isolates were recovered from urine and pus specimens (Table 2).

Following antimicrobial susceptibility testing by the Vitek system and keeping in view the various inherent resistant characteristics of enterococci, the susceptibility patterns of a set of 10 antibiotics were taken into consideration (Table 3). Susceptibility results of vancomycin revealed that 14 enterococci were resistant with a high MIC (>256 mg). Linezolid and tigecycline were the only antibiotics that showed susceptibility to all *Enterococcus* species isolated, including VRE. VRE isolates were mostly (53.3%) recovered from pus samples, and this percentage also includes the *E. casseliflavus* (Fig. 1).

Among the 245 Enterococci, only *E. faecium* strains exhibited vancomycin resistance. A chi-square test of independence was calculated comparing the frequency of antimicrobial Susceptibility testing results with vancomycin for *E. faecalis* and *E. faecium*. A significant interaction was observed (Pearson Chi-Square: 130.34, df:1, $p < 0.05$) (Table 4). Odds for developing resistance to vancomycin for *E. faecium* species are 278 times more than those for *E. faecalis.*

On Multiplex PCR analysis of 14 *E. faecium* isolates, it was noted that eight strains harbored *vanB* genes, while six isolates harbored *vanA* genes (Figs. 2 and 3). The lone *E. casseliflavus* with the intrinsic *vanC* gene did not harbor either *vanA* or *vanB* genes. The prevalence percentage of VRE among the total 246 enterococcal isolates was 6.1%.

4. Discussion

Glycopeptide resistance among enterococci exhibits a marked adverse impact not only in treatment failure of enterococcal infections but also in their resistance transmissibility to other multidrug resistant pathogens like MRSA, which significantly hampers therapeutic options to an array of ailments, especially in the immunocompromised. Humans and animals excrete both antibiotics and antibiotic-resistant bacteria into the environment. This dissemination's far-reaching effect is even more pronounced in enterococci, which are resident intestinal flora that exhibits antibiotic resistance by intrinsic and acquired mechanisms.

In the current study, 246 clinical isolates of enterococci were studied for *van* genes in a modern military hospital in the eastern region of Saudi Arabia. This sample size is comparatively more substantial than other studies done on enterococci in Dammam (N = 138), which is also in the eastern region of Saudi Arabia, and two other studies in Riyadh (N = 206 and N = 231 respectively) (Alotaibi and Bukhari, 2017; Salem-Bekhit et al., 2012; Sirkhazi et al., 2014). However, it has to be also noted that a study on the molecular epidemiology of vancomycin-resistant enterococci done at Riyadh in 2013 investigated 378 clinical enterococcal isolates (Somily et al., 2016).

Table 2
Distribution of frequency and percentage of specific clinical specimens used for Enterococcal isolation.

Specimen	Frequency	Percent
Urine	137	55.9
Pus	102	41.6
Rectal Swab	5	2
Blood	1	0.4
Total	**245**	**100**

Table 3
Distribution frequency and susceptibility pattern percentage of different antimicrobials for the 245 enterococcal isolates tested.

		Count	Column N %
Benzylpenicillin	Susceptible	209	85.30%
	Resistant	36	14.70%
Ampicillin	Susceptible	209	85.30%
	Resistant	36	14.70%
Gentamicin HL	Susceptible	164	66.90%
	Resistant	81	33.10%
Streptomycin HL	Susceptible	179	73.10%
	Resistant	66	26.90%
Quinupristin/ dalfopristin	Susceptible	20	8.20%
	Resistant	225	91.80%
Linezolid	Susceptible	245	100.00%
	Resistant	0	0.00%
Vancomycin	Susceptible	231	94.3%
	Resistant	14	5.7%
Tetracycline	Susceptible	62	25.30%
	Resistant	183	74.70%
Tigecycline	Susceptible	245	100.00%
	Resistant	0	0.00%
Nitrofurantoin	Susceptible	126	92.60%
	Resistant	10	7.40%

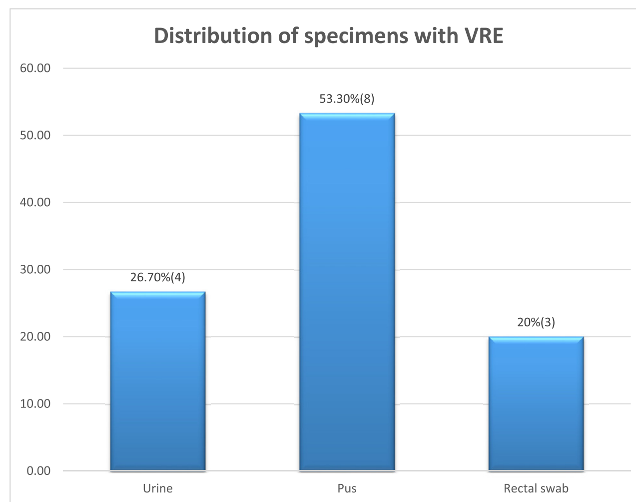

Fig. 1. Bar graph depicting the isolation of VRE from various clinical specimens.

This study found a prevalence rate of 6.1% of VRE among the 246 enterococcal isolates, which is relatively similar to the mean national rate of 7.8%, according to a review report that comprehensively evaluated the available studies on VRE in Saudi Arabia from February 1992 to November 2015 (Abdallah and Al-Saafin, 2019). Our finding is higher than two other studies done in Riyadh, 3.9% and 4.5% but is lower than the most recent phenotypic study on VRE that revealed a rate of 17.3% (Alotaibi and Bukhari, 2017; Salem-Bekhit et al., 2012; Somily et al., 2016).

The prevalence rate of vancomycin resistance among enterococci remains low in Saudi Arabia, and resistance is represented by *E. faecium* rather than other enterococcal species as identified elsewhere. Nevertheless, the situation is alarming in many countries as the prevalence rate from data sourced from the Center for Disease, Dynamics Economics & Policy (CDDEP) shows, especially among *E. faecium*, Argentina topped the list with 69% followed closely by the US with 68%. In Europe, Ireland, with 38%, has the highest prevalence rate, followed by Romania with 34%. Among the Asian countries, Vietnam and India have the highest

Table 4
Frequency distribution of isolates by species type and susceptibility testing status with vancomycin.

Susceptibility testing for vancomycin				Chi-square tests		
Species type	Resistant	Susceptible	Total	Pearson chi-square	df	P-value
Enterococcus faecium	14(56%)	11(44%)	25(100%)	130.34	1.00	**0.000**
Enterococcus faecalis	0(0%)	220(100%)	220(100%)			
Total	14 (5.7%)	231(94.30%)	245(100%)			

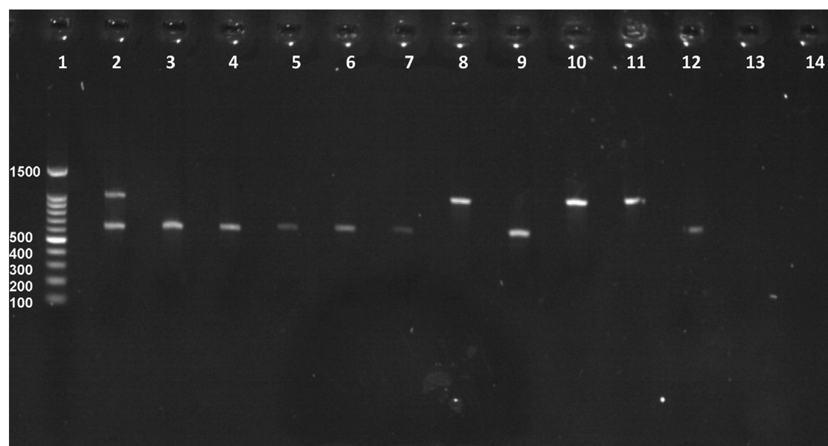

Fig. 2. Lane 1, ladder (100 base pairs); Lane 2, positive control *vanA*(1030 bp) and *vanB*(635 bp); lane 3,4,5,6,7,9,12, positive samples of *vanB* (635 bp); lane 8,10,11, positive samples of *vanA* (1030 bp); lane 13, negative control; lane 14 negative sample for both *vanA* and *vanB*.

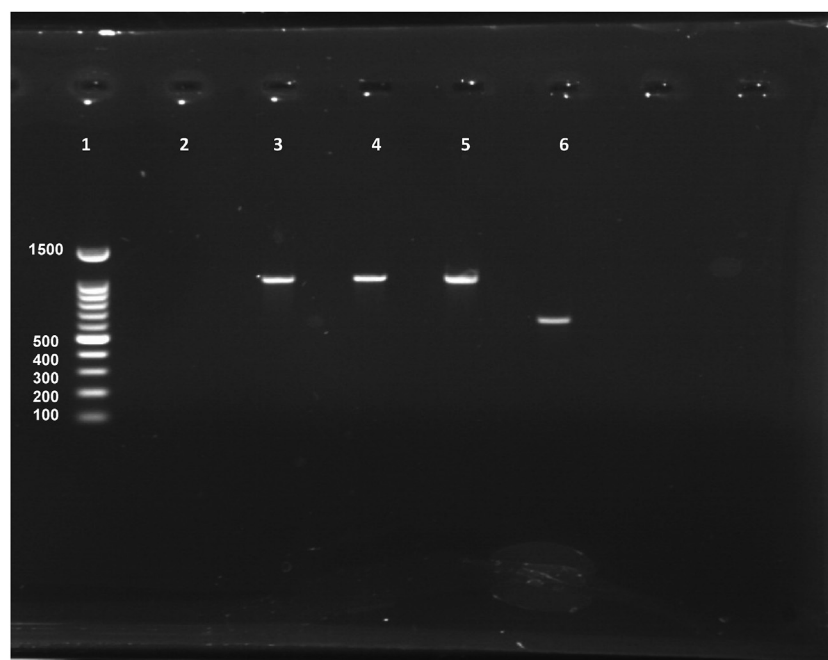

Fig. 3. Lane 1, ladder (100 base pairs); Lane 2, negative control; lane 3, 4, 5, positive samples of *vanA* (1030 bp); lane 6, positive samples of *vanB* (635 bp).

rates, with 27% each, whereas Australia has 50% (ResistanceMap, n.d.).

This study also isolated more *E. faecalis* than *E. faecium* from clinical samples documented globally in other studies. Furthermore, vancomycin resistance due to *van* genes was demonstrated mainly in *E. faecium*. Our study underlines that this trend remains unchallenged, and *E. faecium* harbors resistant determinants much more than any other enterococci species (Lebreton and Cattoir, 2019; Miller et al., 2014). On molecular characterization, our study found that the majority of the resistant isolates harbored *vanB* genes, and this in agreement with a previous study in Saudi Arabia (Somily et al., 2016).

We found out that most of the enterococcal isolates were recovered from urine, comparable with other studies in Saudi Arabia

(Salem-Bekhit et al., 2012). However, we found that pus specimens yielded the highest percentage (53.3%) of isolates from which VRE was obtained. This finding is considered a new observation as all previously reviewed studies over 23 years reported before have shown blood samples as a significant source of VRE in Saudi Arabia (Abdallah and Al-Saafin, 2019).

The vancomycin sensitive enterococci and VRE isolated in our study were uniformly susceptible to tigecycline and linezolid but demonstrated varied susceptibility patterns to other anti-enterococcal antibiotics like quinupristin/ dalfopristin and high-level aminoglycosides. This observation is a relief as reports of linezolid and tigecycline resistance has been documented lately from different parts of the world (Bender et al., 2018; Bi et al., 2018). Furthermore, it also highlights that these antibiotics' rational use is critical to prevent an upsurge of resistance.

A feasible paradigm for VRE treatment can be ascertained by involving more hospitals around the eastern region. The resulting increase in sample size would provide a better understanding of the precise nature of VRE and their susceptibility patterns. Furthermore, this will offer much needed scientific clarity to patient caretakers and policy implementers, and epidemiologists. Such a structured model will partly alleviate the difficulties faced in this study to ascertain whether the isolates obtained were from infections or resident colonization.

5. Conclusion

Our study found a low prevalence of VRE, and all of them were *Enterococcus faecium* with a predominance of *vanB* genes among them. However, this ostensibly low prevalence is a serious challenge to the treatment of enterococcal infections. This finding is of particular significance in hospitalized patients, given these bacteria's ability to transmit *van* genes to other Gram-positive organisms such as *Staphylococcus aureus*. We advocate further studies in the eastern province that involves multiple centers. The data gathered from the resulting increase in sample size will augment control measures to prevent the spread of VRE among the community and hospital settings.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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