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Prevalence, Molecular Characterization and Risk Factors of Vancomycin-Resistant Enterococci: Evidence from Patients Admitted in University of Benin Teaching Hospital, Benin City Nigeria

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ABSTRACT
The aim of this study was to determine the prevalence, antimicrobial susceptibility patterns and associated risk factors of vancomycin-resistant enterococci among patients upon admission. One hundred and seventeen *samples from newly admitted patients at University of Benin Teaching Hospital were screened. Ten (8.55%) samples were positive for vancomycin resistant enterococci (VRE) isolates based on chromogenic and molecular screenings. The speciation prevalence revealed that 8/10 (80%) was E. faecalis while 2/10 (20%) was E. faecium. The prevalence based on recent histories of antibiotics usage showed that 8/74 (10.8%) of the patients with histories of antibiotics usage in the last six months appeared positive while 2/43 (4.7%) appeared positive from patients with no histories of antibiotics usage in the last six months. The prevalence based on sample types was 4/31 (12.9%) in stool samples, 2/24(8.3%) in wound swabs and 4/49 (8.2%) in urine samples. Most of the isolates 6/10 (60%) showed the presence of van A gene. The VRE isolates showed total resistance towards vancomycin and ampicillin. The MAR index of the isolates tested in this study ranged from 0.33 – 0.78. The prevalence of virulence factor formation as observed was gelatinase activity 6/10(60%), biofilm formation 8/10 (80%), β-hemolytic activity 7/10 (70%), and DNase activity 3/10 (30%). This study demonstrated the presence of VRE in healthcare settings, which is a great threat to public health. Therefore, the development of proper surveillance policies and antimicrobial sensitization programmes to monitor the use of antibiotics is advised with a view to curtailing its spread.*

Keywords: Biofilm, Virulence, *Enterococcus*, Risk factors, Public health.

INTRODUCTION

Enterococci are indigenous microflora of the gastrointestinal tracts and normal inhabitants of the oropharynx, oral cavity, vagina and human urethra (Igbinosa and Beshiru, 2019a; Igbinosa *et al*., 2020a). Common infections associated with enterococci include wound infections, endocarditis, urinary tract infections, sepsis and intra-abdominal abscesses (Igbinosa *et al*., 2020b). Currently, about 73 species of enterococci have been identified with *Enterococcus faecium* and *Enterococcus faecalis* being the prevalent in humans (Beshiru *et al*., 2017). Different risk factors have been reported to be associated with the spread of enterococci infections including concurrent infections, longer duration of hospitalization, antimicrobial resistance and underlying immunosuppressing diseases such as cancer and diabetics (European Centre for Disease Prevention and Control, 2018). The emergence and spread of antimicrobial

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resistance among *Enterococcus* species pose enormous therapeutic challenges, especially in the control of severe infections (Igbinosa and Beshiru, 2017).

The increased dissemination of multidrug resistant enterococci strains has narrowed the therapeutic options globally, as *Enterococcus* isolates significantly exhibit high resistance to aminoglycosides, penicillins, cephalosporins, sulphonamides and glycopeptides that are indeed the most historically useful anti-enterococci antibiotics (Adegoke *et al*., 2022). Vancomycin, a glycopeptide drug of choice against enterococci has become highly ineffective due to the resistance conferred on the organism by *van* genes (*A, B, C, D, E, G, L, M,* and *N*) (Yusuf-Omoloye *et al*., 2023). The *vanA, vanB,* and recently, the *vanC* phenotypes appear to be the most clinically important, capable of conferring high levels of resistance to vancomycin (Igbinosa *et al*., 2023a). Recently, *Enterococcus* species especially *E. faecium* has rapidly become a nosocomial pathogen of global importance, due to its successful adaptation to the healthcare environment and acquisition of glycopeptide resistance (Abera *et al*., 2021). A global surveillance of drug potency on Gram-positive pathogens indicated that the prevalence of vancomycin-resistant enterococci in Europe, Asia and Pacific, and Latin and North America ranges between 1% and 9.8% (European Centre for Disease Prevention and Control, 2018).

In Africa, several reports have indicated that the overall prevalence of enterococci and antibiotic resistance ranged from 2.2% to 95.5% (Abera *et al*., 2021). In southern Nigeria, the prevalence of vancomycin-resistant enterococci prevalence is alarming at estimated prevalence rates of 88.9% from environmental and 68.4% from clinical samples respectively (Igbinosa and Beshiru, 2019a). Risks factors also associated with enterococci infection other than antimicrobial resistance, is the expression of diverse virulence factors including enterococcal surface protein, aggregation substance, gelatinase, cytolysin, hyaluronidase, adhesin-like endocarditis antigen A, and colonization determinants, subsequent binding, and invasion in the host (Igbinosa and Beshiru, 2019a). Therefore, in order to curtail the spread of vancomycin-resistant related infections and related virulence factors, there is need for improved knowledge on their epidemiology especially in hospital settings that are likely to have more populations of immunocompromised individuals (Igbinosa *et al*., 2022a). Therefore, the aim of this study was to determine the prevalence, antimicrobial susceptibility patterns and associated risk factors of vancomycin-resistant enterococci among patients upon admission.

MATERIALS AND METHODS

Study Area and Samples under Study

Benin City is the capital and largest city of Edo State, Southern Nigeria. University of Benin Teaching Hospital (UBTH) is a multi-specialty provider of healthcare service in West Africa. It complement her sister institution, University of Benin, and provide secondary and tertiary care to residents in Edo and its environs. It provides facilities for the training of a high- and middle-level workforce for the health industry. One hundred and seventeen non-duplicate samples from patients newly admitted at University of Benin Teaching Hospital, Benin City were analysed in this study. The samples investigated include stool samples (n=31), blood samples (n=13), wound swabs (n=24) and urine samples (n=49).

Inclusion and Exclusion Criteria

The study included samples from adult patients newly admitted to the teaching hospital as at July 2023. Exclusion criteria were samples from patients who have been hospitalized for more than three days as previously described in previous study on healthcare admission prevalence (Bui *et al*., 2021). Duplicate samples from same patient were also excluded in the study.

Ethical Considerations

Permission to conduct this study was obtained from the authority of Medical Laboratory Unit, University of Benin Teaching Hospital, Benin City. The experimental procedure was in accordance with the ethical research method as recommended the laboratory management and the laboratory personnel obtained informed consent from the patients. Information that including gender, age, recent histories of antibiotics usage, and recent histories of hospitalization and sample types were obtained from the laboratory. The researchers had no physical contact with the patients and personal tags/confidential information was excluded.

Phenotypic Screening for Vancomycin Resistant Enterococci

One-gram (1 g) stool samples, 1 mL urine and blood samples, and the swab samples from wounds were aseptically inoculated into 10 mL of sterile tryptone soy broth (TSB) (Merck, Darmstadt, Germany) and incubated at 37 ^{o}C for 24 h. After incubation, a loopful of the bacterial growth from TSB was streaked on bile aesculin azide (BAA) agar (TM Media, Rajasthan, India) supplemented with 6 µg/mL vancomycin and the agar plates were incubated for 24 h at 37 °C. Black colonies on BAA agar were classified as presumptive vancomycin-resistant enterococci isolates (Beshiru *et al*., 2017; Adeyemi *et al*., 2021; Yusuf-Omoloye *et al*., 2023). Presumptive vancomycin-resistant enterococci were purified on nutrient agar (Lab M, Lancashire, UK). Purified isolates were characterized further via Gram staining to determine the morphology. Biochemical test such as catalase and oxidase were used to bacteriologically characterize the isolates. Presumptive vancomycin-resistant enterococci were Gram-positive, catalase negative and oxidase negative. The isolates were stored on nutrient agar slants and transported from the Teaching Hospital Laboratory to a research laboratory for further analysis.

Chromogenic Screening for Vancomycin Resistance

Chromogenic screening for vancomycin resistance was carried out on all enterococcal isolates using a chromogenic screening agar, CHROMagar™ VRE (CHROMagar Co, Paris, France). Fresh purified colonies on nutrient agar were inoculated onto CHROMagar VRE and incubated for 24 h at 37 °C. Colonies that appeared as pink were presumptively identified as vancomycin-resistant *E. faecalis* and vancomycin-resistant *E. faecium.* The blue colonies were classified as other vancomycin-resistant enterococci while agar plates showing no growth or growth with other colors were classified as negative to vancomycin resistance according to manufacturer's specifications.

DNA Extraction and Molecular Characterization of Vancomycin Resistant Enterococci

DNA extraction was carried out using the boiling method as previously described (Beshiru *et al*., 2017). The DNA templates were subjected to a polymerase chain reaction (PCR) using species-specific primers for *ddlE* genes (Inqaba Biotec, South Africa) to confirm the *E. faecium* and *E. faecalis* isolates (Nasaj *et al*., 2016). Briefly, the DNA amplification was carried out in a final volume of 20 µL containing 10 µL of Hotstart green mastermix (Thermofisher), 20 µM each of the forward and reverse primers, water and 5 µL of DNA template. The cycling condition in a thermocycler (Biorad, South Africa) consisted of an initial denaturation step of 95 °C for 5 min, 25 cycles in a denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, followed by polymerization at 72 °C for 1 min. Five microlitres of the PCR product was electrophoresed on a 2% (w/v) agarose gel using Ix TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8) stained with gel red and visualized with UV transilluminator (BIORAD, South Africa). Resistance genes *vanA* and *vanB* were screened in isolates confirmed genotypically as VRE *faecium* and *faecalis* via Multiplex PCR using specific primers *vanA*: (F: GGGAAACGACAATTGC; R: GTACAATGCGGCCGTTA) and *vanB* (F:

ATGGGAAGCCGATAGTC; R: GATTTCGTTCCTCGACC) as developed by Igbinosa and Beshiru, (2019).

Antimicrobial Susceptibility Screening

Vancomycin-resistant enterococci isolates were subjected to antimicrobial susceptibility screening using the Kirby-Bauer disc diffusion method. Suspension of the test isolates with an approximated turbidity 0.5 McFarland's standard was pipetted and aseptically spread on Mueller-Hinton agar plates (Lab M, Lancashire, United Kingdom). The antibiotics discs (Oxoid, Hampshire, United Kingdom) were aseptically placed on the Mueller-Hinton agar culture plates. The antibiotics tested include ampicillin (10 µg), rifampin (5 µg), erythromycin (15 µg), linezolid (30 µg), ciprofloxacin (5 µg), vancomycin (30 µg), chloramphenicol (30 µg), fosfomycin (200 µg) and nitrofurantoin (300 µg). The culture plates were incubated at 37 °C for 24 h. After incubation, the diameter of inhibition zones was measured and interpreted according to the Clinical and Laboratory Standards Institute (2020). Multiple antibiotic resistance index (MARI) of VRE was elucidated as follows: $\text{MAR index} = \frac{a}{b}$ where "a" represents total antibacterial agents that an isolate is resistant and "b" is represents, antibacterial agents that an isolate was exposed (Igbinosa *et al*., 2023a). Isolates with MAR index ≥0.2 was considered to be of high risk. Isolates resistant to ≥3 antibiotic class were considered multidrug-resistant (Igbinosa *et al*., 2023b).

Screening for Phenotypic Virulence Factors

Gelatinize production: Overnight cultures of vancomycin-resistant enterococci isolates were stabbed 3-4 times at about half inch depth into a freshly prepared nutrient-gelatin medium and incubated at 37 °C for 48 h with an un-inoculated tube as control. After incubation, the tubes were removed without shaking or inversion, refrigerated, then gently inverted, and visually observed for gelatinase production as indicated by partial or complete liquefaction of the test media at 4 °C (Beshiru and Igbinosa, 2023). Control and gelatinase negative tubes remained solid. Biofilm production: The vancomycin-resistant enterococci isolates were inoculated on Congo Red Agar. The production of black colonies with a dry crystalline consistency indicated biofilm production while non-biofilm producers developed red colonies (Beshiru *et al*., 2023). Hemolysin production: The vancomycin-resistant enterococci isolates were streaked on freshly prepared blood agar plates, incubated for 24 h at 37 °C, and observed for the patterns of hemolysis. The demonstration of a completely clear zone along the streaked area indicates positive β-hemolytic activity (Igbinosa *et al*., 2020b). DNase production: Overnight cultures of test isolates were inoculated onto DNase Agar and then incubated at 37 °C for 24 h. The plates were then flooded with 1N HCl for a few minutes, excess HCl tipped off, and the plates were observed within 5 min against a dark background for clear zones surrounding the line of the streak, indicative of DNase production (Igbinosa *et al*., 2022b).

Data Analysis

Data analysis was carried out on the data using the Statistical Package (SPSS) version 21.0 and Microsoft Excel 2013. Mean values were expressed using descriptive statistics.

RESULTS

Socio-demographic of the Patients under Study

One hundred and seventeen non-duplicate samples were assessed in this study. The samples investigated include stool samples, blood samples, wound swabs and urine samples. The sample sources were 38 (32.5%) males and 79 (67.5%) females as shown in Table 1. The age of the patients ranged from 18 years to 74 years. The age distribution indicated that most of the patients (33.3%) were in age group 54-65 years followed by (24.8%) belonging to age group 30-41 years. Other age groups were 18-29 [18 (15.4%)], 42-53 [23 (19.7%)] and 66-74 [8 (6.8%)]. The distribution based on recent histories of antibiotics usage showed that most of the patients

have used antibiotics in the last six months with a proportion of (63.2%) while the remaining (36.8%) reportedly recorded no histories of antibiotics usage in the last six months. Based on histories of hospitalization, 112 (95.7%) of the patients reportedly have no histories of hospitalization in the last six months while only 5 (4.3%) was reportedly hospitalized in the last six months.

Table 1: Socio-demographic and Prevalence of Vancomycin Resistant Enterococci

Variables	Groups	Frequency (%) (*n*=117)	VRE Positive (%)
Age	18-29	18 (15.4)	0/18 (0)
	30-41	29 (24.8)	2/29 (6.9)
	42-53	23 (19.7)	2/23 (8.7)
	54-65	39 (33.3)	5/39 (12.8)
	66-74	8 (6.8)	1/8 (12.5)
Sex	Male	38 (32.5)	2/38 (5.3)
	Female	79 (67.5)	8/79 (10.1)
Usage of antibiotics in the last six months	Yes	74 (63.2)	8/74 (10.8)
	No	43 (36.8)	2/43 (4.7)
Hospitalized in the last six months	Yes	5 (4.3)	4/5 (80)
	No	112 (95.7)	6/112 (5.3)
Sample Type	Stool	31	4/31 (12.9)
	Blood	13	0/13 (0)
	Wound	24	2/24 (8.3)
	Urine	49	4/49 (8.2)
	Total	**117**	10/117 (8.55)

Prevalence of Vancomycin Resistant Enterococci Isolates among Patients

A total of 10/117 (8.55%) vancomycin resistant enterococci isolates were detected based on chromogenic screening and molecular characterization from the clinical samples. The speciation prevalence revealed that 8/10 (80%) was *Enterococcus faecalis* while 2/10 (20%) was *Enterococcus faecium*. The distribution of the ten isolates identified were eight from female patients while two isolates from male patients as shown in Table 1. The level of prevalence based on age revealed a distribution of 2/29 (6.9%) in age group of 30-41 years, 2/23 (8.7%) in age group 42-53 years, 5/39 (12.8%) in age group 54-65 years and 1/8 (12.5%) in age group 66-74 years. There were no vancomycin resistant enterococci detected in age group 18-29 years (Figure 1a). The prevalence of vancomycin resistant enterococci based on sex revealed a distribution of 2 (5.3 %) in male and 8 (10.1%) in female.

The prevalence based on recent histories of antibiotics usage showed that 8/74 (10.8%) of the patients with histories of antibiotics usage in the last six months appeared positive while 2/43 (4.7%) appeared positive from patients with no histories of antibiotics usage in the last six months (Figure 1b). The prevalence based on histories of hospitalization was (5.3%) in patients with no histories of hospitalization in the last six months and (80%) in patients with hospitalization histories in the last six months (Figure 1c). The prevalence based on sample types was (12.9%) in stool samples, (0%) in blood samples, (8.3%) in wound swabs and (8.2%) in urine samples (Figure 1d).

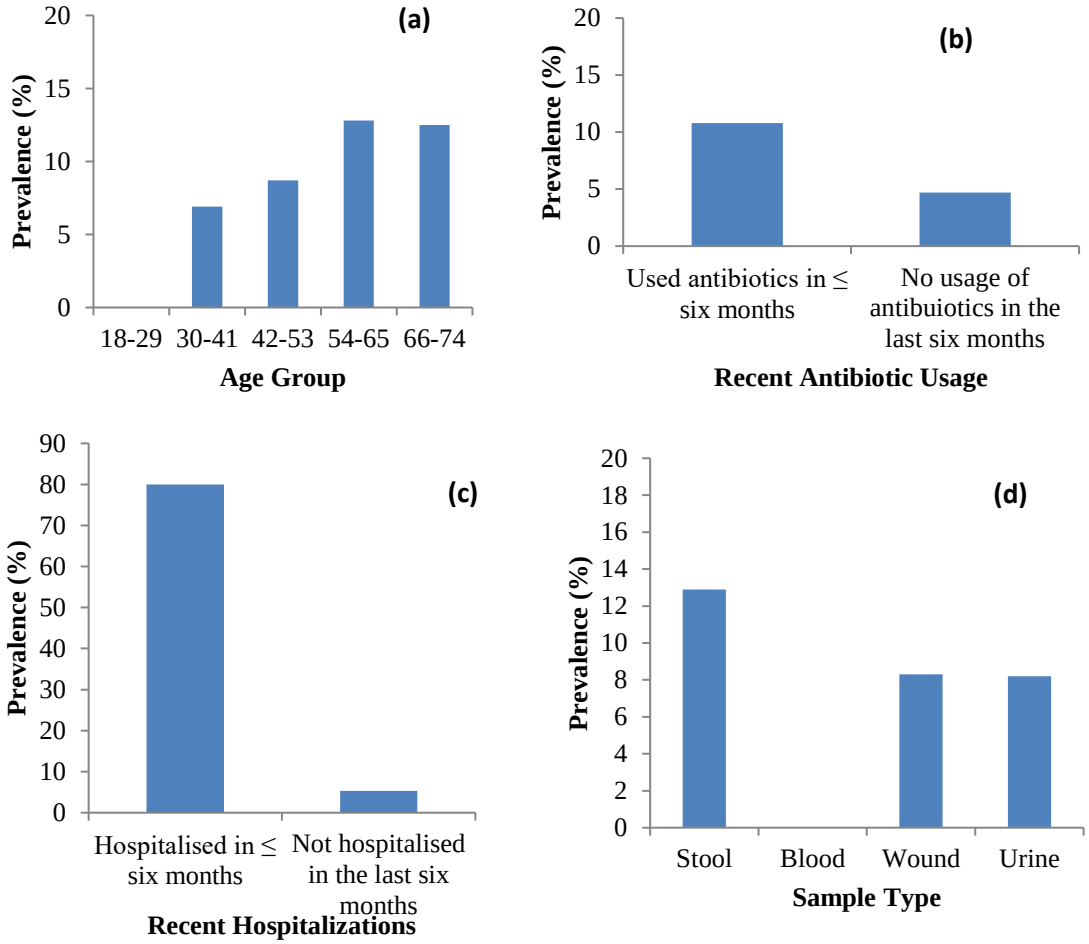

Figure 1: Prevalence of Vancomycin-Resistant Enterococci based on (a) age; (b) recent histories of antibiotics usage; (c) histories of hospitalization; (d) sample type

Prevalence of Vancomycin Genes

The ten vancomycin resistant enterococci isolates were tested for presence of *vanA* and *vanB* genes. Most of the isolates 6/10 (60%) showed the presence of *vanA* gene while *vanB* gene was detected in none of the isolates as shown in Figure 2. The distribution of van genes based on species revealed that among *E. faecalis* isolates only 6/8 (75%) showed the presence of *vanA* gene while none of the *E. faecium* isolates showed the presence of *vanA* and *vanB* gene.

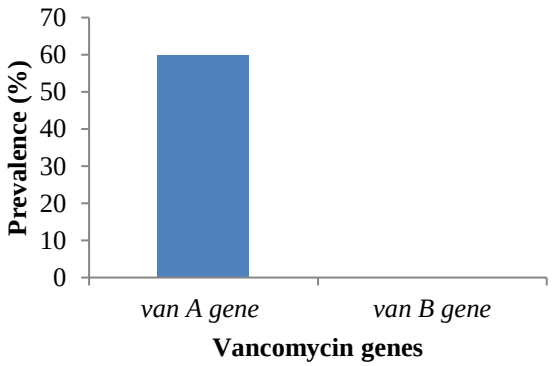

Figure 2: Prevalence of *van* genes in Vancomycin-Resistant Enterococci positive isolates

Antimicrobial Resistance Profile of Vancomycin Resistant Enterococci

The antibiogram profile of vancomycin resistant enterococci isolates as observed was ampicillin (100%), rifampin (90%), erythromycin (70%), ciprofloxacin (70%), vancomycin (100%), chloramphenicol (20%), fosfomycin (60%) and nitrofurantoin (40%) as shown in Table 2. The MAR index assessment revealed that all the detected enterococci were resistant to at least three antibiotics; four isolates were resistant to five antibiotics with an MAR index ≥ 0.56 while two isolates were resistant to seven antibiotics 0.78. The MAR index of the isolates tested in this study range from 0.33 – 0.78 as shown in Figure 3.

Table 2: Antimicrobial susceptibility profile of vancomycin resistant enterococci

Antimicrobial class	Antibiotics	Susceptibility profile of VRE (*n*=10)	
		Sensitive (%)	Resistance (%)
Penicillins	AMP	0(0)	10(100)
Ansamycins	RIF	1(10)	9(90)
Macrolides	ERY	3(30)	7(70)
Oxazolidinones	LIN	10(100)	0(0)
Fluoroquinolones	CIP	3(30)	7(70)
Glycopeptides	VAN	0(0)	10(100)
Phenicols	CHL	8(80)	2(20)
Fosfoysins	FOS	4(40)	6(60)
Nitrofurans	NIT	6(60)	4(40)

Key: AMP: ampicillin (10 µg), RIF: rifampin (5 µg), ERY: erythromycin (15 µg), LIN: linezolid (30 µg), CIP: ciprofloxacin (5 µg), VAN: vancomycin (30 µg), CHL: chloramphenicol (30 µg), FOS: fosfomycin (200 µg), NIT: nitrofurantoin (300 µg).

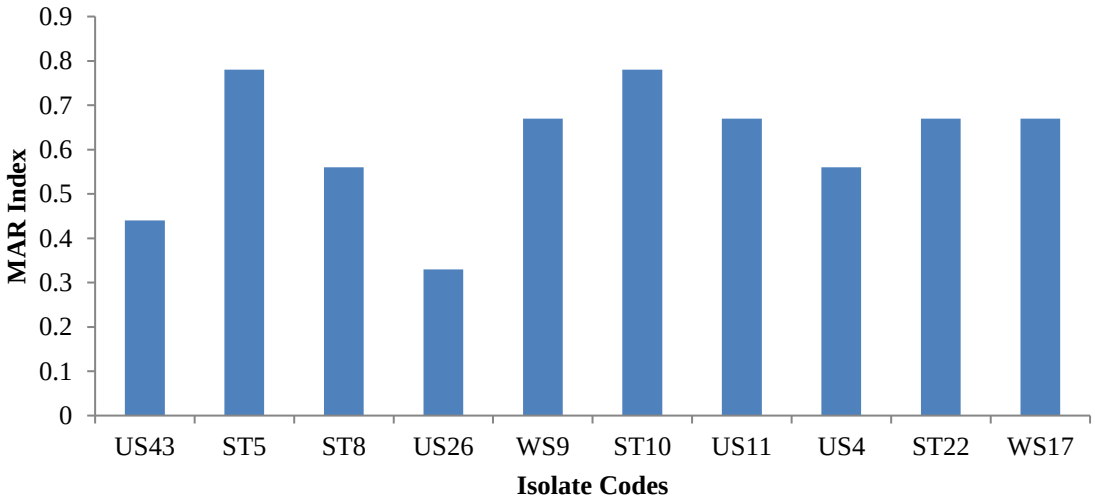

Figure 3: Multiple Antibiotics Resistance (MAR) Index of VRE Isolates

Evaluation of Virulence Factors in Enterococci Isolates

In this study, the prevalence of virulence factor formation as observed in the VRE isolates was gelatinase activity [6/10 (60%)], biofilm formation [8/10 (80%)], β-hemolytic activity [7/10 (70%)], and DNase activity [3/10 (30%)] as shown in Figure 4. Prevalence of observed in *Enterococcus faecalis* include gelatinase activity [6/8 (75%)], biofilm formation [7/8 (87.5%)], β-hemolytic activity [5/8 (62.5%)] and DNase activity [2/8 (25%)]. Prevalence observed in *Enterococcus faecium* include gelatinase activity [0/2 (0%)], biofilm formation [1/2 (50%)], β-hemolytic activity [2/2 (100%)], and DNase activity [1/2 (50%)].

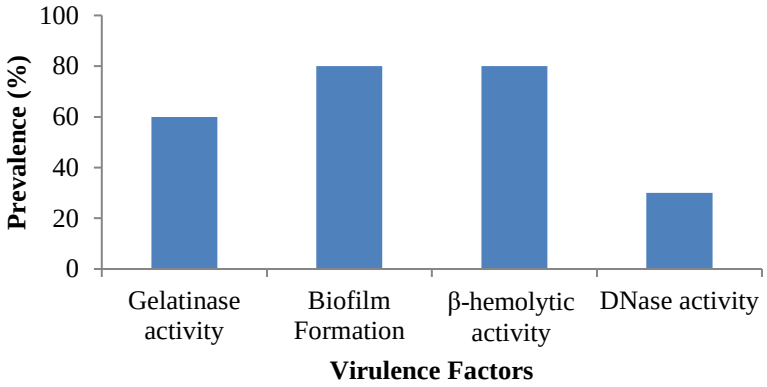

Figure 4: Prevalence of Virulence Factors in Vancomycin-Resistant Enterococci Isolates

DISCUSSION

The prevalence and persistence of enterococci related infections have been attributed globally to their ability to intrinsically resist various clinically relevant antibiotics. The prevalence observed in this study was significantly lower than 25.3% previously reported by Wada *et al*. (2020). Several authors from other countries have also reported a higher prevalence of 14.8% in Ethiopia (Melese *et al*., 2020) and 16% in South Korea (Yang *et al*., 2007). Contrarily, other investigations have reported lower VRE colonization ranging from 1.0% - 7.9% in the USA (Zacharioudakis *et al*., 2015). The variations observed among these studies can be associated with disparities in antibiotics usage study population, geographical region, sample size, sample types and screening methods.

The speciation of VRE in this study agrees with previous studies, which reported that *E. faecalis* strains are linked to most enterococcal infections (Wada *et al*., 2020). Contrarily, other study had reported higher prevalence of *E. faecium* than *E. faecalis* in healthcare settings (Davis *et al*., 2020). Differences in the geographical region can be a major factor in this variation. In this study, the highest prevalence of VRE based on age conforms with previous study which acknowledged patient age as a risk factor in enterococci infection and that younger patients are less likely get colonized by VRE (Bui *et al*., 2021). Previous studies have similarly reported higher prevalence of VRE colonization in females was higher as compared to the male patients (Yusuf-Omoloye *et al*., 2023). Nevertheless, the same study acknowledged the fact that more samples were from female, which is most likely to be urine samples from females with urinary tract infections as obtained in this study.

In this study, the prevalence based on histories of antibiotics usage showed that more patients with histories of antibiotics usage in the last six months appeared positive in comparison with patients with no histories of antibiotics usage in the last six months. Frequent use or improper use of antibiotics could be a contributing factor to VRE colonization (Igbinosa and Beshiru, 2019b). In addition, the prevalence based on histories of hospitalization revealed that prevalence of VRE colonization was significantly higher in patients with histories of hospitalization in the last six months than those that were not hospitalized. Previous study had emphasized the increased prevalence of VRE in hospitalized individuals due to their exposure to hospital environment as VRE could survive on inanimate surfaces such as beds, ventilation systems, benches, and implanted surgical devices for longer periods (Patel and Gallagher, 2015).

In this study, the *vanA* and *vanB* genes screened conforms to other studies where *vanA* genes were detected and *vanB* gene was not detected (Adegoke *et al*., 2022). This disagrees with other

studies that reported the absence of *vanA* genes (Adeyemi *et al*., 2021). Reportedly, *vanA* gene has been the most common vancomycin resistance gene (Igbinosa and Beshiru, 2019a). *VanA* resistance gene is mostly inducible and it confers higher level of resistance to vancomycin (Zacharioudakis *et al*., 2015). The *vanA* gene has been shown to be on transposons and they could be transferred easily (Adegoke *et al*., 2022). These could have contributed to the high detection rate in this study.

The high resistance to vancomycin in this study confirmed the screening procedures as the culture media were supplemented vancomycin antibiotics. Resistance to ampicillin, rifampin and other antibiotics possibly indicates multidrug resistance due to gradual spread of drug resistant strains in area under study. Moreover, of these drugs are commonly abused and self-prescription of these drugs is common, hence the emergence and spread of resistance (Beshiru *et al*., 2016; Imanah *et al*., 2017). In this study, all the VRE isolates were sensitive towards linezolid. High potency of linezolid towards VRE has also been reported previously (Igbinosa and Beshiru, 2019). In this study, the MAR index of isolates tested ranged from 0.33 to 0.78. Notably, MARI ≥ 0.2 are associated with isolates from high-risk sources, reflecting potential antibiotic abuse and high selection pressure (Adegoke *et al*., 2022).

The detection of phenotypic virulence factors have also been reported (Igbinosa and Beshiru, 2019). These virulence determinants including hemolysin and gelatinase aid nutrient acquisition from host tissues and advance invasion, thereby increasing the severity of human infections (Adeyemi *et al*., 2021). Biofilm formation aids disease development as it boosts the persistence of infections and reduces antimicrobial activity (Igbinosa *et al*., 2021). Similarly, DNase production hydrolyzes nucleic acids, contributing to bacterial virulence (Igbinosa *et al*., 2020b). Most VRE isolates in this study possessed at least one virulence factor and were resistant to various antimicrobials.

CONCLUSION

Antimicrobial resistant pathogens such as VRE are a great menace in both healthcare and community settings. This study highlighted the risk of VRE and related infections, which might increase if not curtailed. There should be nationwide VRE surveillance in healthcare facilities to determine the true risks posed by these drug-resistant pathogens and to aid the development of proper policies that will reduce their spread. Conclusively, antimicrobial sensitization programmes should be implemented in hospitals to monitor the use of antibiotics and reduce selective pressure of antibiotics on important clinical pathogens in hospital environments.

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CONFLICT OF INTEREST

The authors declare no competing interest.

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