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Low Occurrence of Virulence Determinants in Vancomycin-Resistant *Enterococcus* from Clinical Samples in Southwest Nigeria

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Abstract

Background: The virulence factors of enterococci play a major role in the pathogenicity of enterococcal strains.
Objectives: This study aimed to evaluate virulence factors and detect selected virulence and resistance genes in vancomycin-resistant *Enterococcus* (VRE) from clinical samples from southwest Nigeria.
Methods: The VRE isolates (n = 85) recovered from clinical samples were characterized using conventional microbiology techniques, and molecular identification was made with *ddlE* primers. Phenotypic screening for five virulence determinants and detection of virulence and resistance genes using a polymerase chain reaction were carried out.
Results: Phenotypic identification revealed 61 *Enterococcus faecium* and 24 *Enterococcus faecalis*. All the isolates hydrolyzed bile. Moreover, 88.2% of the isolates produced biofilm; however, 72.9% of the isolates produced gelatinase enzyme. Altogether, six isolates (7%) produced all five virulence factors. The least virulence factor expressed by the two species *E. faecium* and *E. faecalis* was DNase at 21.3% and 29.2% followed by cytolysin at 27.9% and 41.7%, respectively. Only 25 isolates (29.4%), including 23 *E. faecium* (37.7%) and only 2 (8.3%) *E. faecalis* isolates, revealed bands with molecular identification. Additionally, VRE isolates showed bands for *asa1* (16%); only one isolate (4%) each isolate had the *hyl* gene and *vanB* gene, respectively.
Conclusions: The absence of *vanA* and low detection of *vanB* resistance genes suggest the possible presence of other *van* types and emphasizes the need for further investigations on the incidence of other *van* genes using molecular screening methods in enterococci isolates in Nigeria for surveillance purposes. Moreover, the low occurrence of virulence genes implies that there might be other mediators of pathogenicity involved in *Enterococcus* virulence traits.

Keywords: Vancomycin-Resistant Enterococci, Virulence Genes, *van* Genes, *asa1*, *esp*

1. Background

Enterococci represent a substantial part of the gut flora and can exist under severe environmental conditions. They are Gram-positive cocci, facultative anaerobes, implicated in hospital-acquired infections superseded only by staphylococci as a source of Gram-positive nosocomial infections (1). Although more than 50 species have been reported (2), *Enterococcus faecium* and *Enterococcus faecalis* are the most clinically important multidrug-resistant infectious pathogens worldwide (3).

Vancomycin-resistant *Enterococcus* (VRE) particularly poses a major challenge to healthcare practitioners as its management has been trying in the hospital setting. The VRE infections have been reported to escalate cost and mortality as opposed to vancomycin-sensitive strains. Vancomycin-resistance in enterococci involves the modification of the peptidoglycan synthesis pathway.

Vancomycin attaches to the D-ala-D-ala end of the pentapeptide precursor, impeding the synthesis of the cell wall by inhibiting the cross-link of peptidoglycan chains. The VRE alters pentapeptide precursors, substituting the terminal D-ala with D-lactate or rarely D-ser (4), which now bind glycopeptides with significantly reduced affinity than typical precursors. The altered D-alanyl-D-lactate form causes loss of one hydrogen-bonding interaction and an interaction lesser than for D-alanyl-D-alanine association between vancomycin and the peptide, thereby conferring high-level resistance (1); nevertheless, D-alanyl-D-serine variation results in a six-fold affinity loss between vancomycin and the peptide, probably from steric impediment (5), conferring low-level vancomycin resistance (1).

Nine forms of vancomycin-resistance genotypes are expressed by enterococci, namely *vanA* to *vanE*, *vanG*, *vanL*,

vanM, and *vanN* (1, 4, 6), with *vanA* and *vanB* of utmost clinical importance (1). The *vanA* genotype, most common globally and linked with vancomycin-resistance in enterococci in the hospital environment (1) confers resistance to vancomycin and teicoplanin (7). Moreover, less frequently, the *vanB* gene, mostly noticed in *E. faecium*, exhibits resistance to vancomycin but susceptibility to teicoplanin (7).

Bacterial attachment to host tissues is a key phase in the instigation of any infection process. Enterococci species express diverse virulence factors, including enterococcal surface protein (*esp*), aggregation substance (*agg* and *asa1*), gelatinase (*gelE*), cytolysin (*cylA*), hyaluronidase (*hyl*), *pilA*, *pilB*, *ecbA*, *scm*, *fms8*, *efaAfs*, *efaAfm* (adhesin-like endocarditis antigen A encoded by *E. faecalis* and *E. faecium*, respectively), and *sgrA* genes, which augment colonization, subsequent binding, and invasion in the host (8-10). Therefore, enterococci have numerous possibilities as most of the virulence genes commonly harbored are associated with adherence.

2. Objectives

Within Nigeria, there are reports of VRE recovery from the clinical setting (11) and even from food samples (9). However, documentation on the virulence of these organisms remains inadequate in Nigeria. Therefore, this study was performed to evaluate virulence factors and detect the possible presence of virulence and resistance genes in VRE from clinical samples from southwest Nigeria.

3. Methods

3.1. VRE from Participants

This study assessed 85 non-duplicate VRE isolates recovered from the samples in three selected hospitals in southwest Nigeria, namely State Specialist Hospital, Osogbo (7.76958°N; 4.54999°E), State Hospital, Iwo (7.66686°N; 4.19926°E), and Oke-Baale Primary Health Centre, Osogbo (7.76516°N; 4.578°E) (12) (Figure 1). Ethical approval was obtained from the Ethical Review Board of State Specialist Hospital, Osogbo (approval no.: HREC/SSHO/11/478).

The samples were aseptically inoculated into 10 mL of sterile Tryptone Soy Broth (TSB) (Oxoid, UK) and then streaked out on Slanetz and Bartley Agar. Pale-pink/maroon colonies on Slanetz and Bartley Agar indicates the growth of enterococci species. Overnight growth from Slanetz and Bartley Agar plates were inoculated onto Bile Aesculin Agar and Mannitol Salt Agar for the speciation of enterococci into *E. faecalis* (yellow colonies on Mannitol Salt Agar [MSA]) and *E. faecium* (no growth on MSA). Screening for vancomycin resistance was through inoculation on Brain Heart Infusion Agar supplemented with 6 μg/mL of vancomycin. Recovered isolates were preserved in TSB supplemented with 15% glycerol, frozen, and stored at -20°C.

3.2. Screening for Virulence Determinants

3.2.1. Gelatinase Production

Overnight cultures of VRE isolates were stabbed 4 - 5 times 1/2 inch depth into freshly prepared nutrient-gelatin medium and incubated at 37 $\pm$ 2°C for 48 h, along with an un-inoculated tube. Afterward, 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. Control and gelatinase negative tubes remained solid.

3.2.2. Hemolysin/Cytolysin Production

The VRE isolates were streaked on freshly-prepared blood agar plates, incubated for 24 - 48 h at 37 $\pm$ 2°C, and visually observed for the patterns of hemolysis. Hemolysin production was scored as β (complete), α (partial), or γ (no clear zone) hemolysis indicated by clear/colorless zone, greenish zone, and completes absence of hemolysis, respectively.

3.2.3. Biofilm Production

The test isolate was inoculated on Congo Red Agar. The production of black colonies with a dry crystalline consistency indicated biofilm production; nonetheless, non-biofilm producers developed red colonies.

3.2.4. DNase Production

Overnight cultures of test isolates were inoculated onto DNase Agar and then incubated at 37 $\pm$ 2°C for 18 - 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.

3.2.5. Caseinase Production

Test isolates were inoculated onto Mueller-Hinton Agar supplemented with 3% skimmed milk and then incubated at 37 $\pm$ 2°C overnight. The development of clear proteolytic zones around the line of the streak was indicative of caseinase production.

3.3. Molecular Characterization of Selected Bacterial Isolates

3.3.1. Extraction of Chromosomal DNA

Deoxyribonucleic acid (DNA) was extracted from VRE isolates by thermal lysis of the cell. A 1 mL aliquot of an overnight culture of VRE was centrifuged at 5,000 rpm for 10 min, and the supernatants were discarded. The pellets were re-suspended in 500 μL of sterile DNA/DNase/RNase free water, vortexed, centrifuged at 5,000 rpm for 1 min, and then re-suspended in 200 μL of sterile water. The suspension was boiled in a water bath at 100°C for 10 min with lids closed, lysates cooled to room temperature, and

Figure 1. Map of the study areas

then centrifuged at 10,000 rpm for 10 min. The purity and concentration of the extracted DNA in the supernatant were estimated using NanoDrop One spectrophotometer (Thermo Fisher Scientific, United States) at 260 nm and stored at -20°C in sterile Eppendorf tubes to serve as DNA templates for subsequent molecular characterization.

3.3.2. Identification of Bacterial Isolates and Detection of Target Genes

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 biochemical identification of the vancomycin-resistant *E. faecium* and *E. faecalis* isolates (13). Virulence genes were detected using four multiplex PCR reactions only in isolates confirmed genotypically as VRE *faecium* and *faecalis*. Virulence genes, namely *asa1*, *gelE*, *cylA*, *esp*, and *hyl*, were amplified in one reaction (14); however, *pilA-pilB-efaAfm*, *fms8-sgrA*, and *ecbA-scm* were run in three other reactions. The resistance genes *vanA* and *vanB* were also screened for using Multiplex PCR (Table 1). Amplification was carried out using the Master Cycler Nexus Gradient 230 (Eppendorf, Germany) (Table 2) in a total volume of 25 μL solution containing 12.5 μL of 2$\times$ Master-Mix (Biolabs, England), 0.5 μL of 10 μM each of forward and reverse primers (Inqaba Biotec, South Africa), and 5 μL of each DNA template made up to 25 μL with DNAse/RNAse free sterile water (Bio-Concept, New Hampshire, United States).

Each amplicon (10 μL) was run on 1.0% agarose gel stained with SafeView Classic at 80 V for 60 min for isolate identification and resistance gene detection and at 100 V for 60 min for detection of virulence genes. The gels were visualized with the UV trans-illuminator E-BOX-CX5 TS imaging system (Vilber, France). Moreover, the 100 bp DNA Ladder (Biolabs, England) served as DNA molecular weight standard. *E. faecalis* ATCC 51299 and *E. faecalis* MMH594 were the positive control strains.

3.3.3. Plasmid Profile Analyses

Plasmid DNA from VRE isolates was extracted using Plasmid Miniprep Kit (ZymoPURE, California), and the eluted plasmid DNA was stored at $\leq$ -20°C. The *vanA* and *vanB* genes were screened with Multiplex PCR in a total volume of 25 μL reaction and observed as described above.

4. Results

4.1. Screening for Virulence Determinants

All 85 VRE isolates (100%) were observed to be catalase-negative, and all grew in the presence of 40% bile; however, 84 VRE isolates (98.8%) hydrolyzed aesculin. Only 27 VRE isolates (31.8%) could produce cytolysin on blood agar. The biofilm producers were 75 VRE isolates (88.2%). Furthermore, 72.9% of VRE isolates produced a gelatinase enzyme; nevertheless, only 20 VRE isolates (23.5%) produced DNase (Figure 2). Six isolates (7.0%) produced all five virulence factors. Only two isolates (2.4%) produced no virulence factor, as all the others produced two virulence factors or more. Biofilm was the most expressed factor at 88.5% and 87.5% for *E. faecium* and *E. faecalis*, respectively; nonetheless, the least virulence factor was DNase at 21.3% and 29.2% for *E. faecium* and *E. faecalis*, respectively (Figure 3).

4.2. Molecular Characterization

Only 25 isolates (29.4%) revealed bands for the identification with *ddlE* primers. In addition, 23 isolates (92.0%) were *E. faecium*; however, two isolates (8.0%) were *E. faecalis*. Using phenotypic identification, 23 out of 61 isolates phenotypically identified as *E. faecium* (37.7%) revealed bands

Table 1. Primer Sequences for Vancomycin-Resistant Enterococci Identification and Characterization

Target Genes Primers	Sequence (5' - 3')	Band Size (bp)	References
	Identification: *E. faecium* and *E. faecalis*		
ddlE faecium		658	(11)
F	TTGAGGCAGACCAGATTGACG		
R	TATGACAGCGACTCCGATTCC		
ddlE faecalis		941	(11)
F	ATCAAGTACAGTTAGTCTTTATTAG		
R	ACGATTCAAAGCTAACTGAATCAGT		
	Vancomycin-Resistant Gene		
van A		732	(11)
F	GGGAAAACGACAATTGCC		
R	GTACAATGCGGCCGTTA		
van B		635	(11)
F	ATGGGAAGCCGATAGTC		
R	GATTTCGTTCCTCGACC		
	Virulence Genes		
asa1		375	(12)
ASA11	GCACGCTATTACGAACTATGA		
ASA12	TAAGAAAGAACATCACCACGA		
gelE		213	(12)
GEL11	TATGACAATGCTTTTTGGGAT		
GEL12	AGATGCACCCGAAATAATATA		
cylA		688	(12)
CYT I	ACTCGGGGATTGATAGGC		
CYT IIb	GCTGCTAAAGCTGCGCTT		
esp		510	(12)
ESP14F	AGATTTCATCTTTGATTCTTGG		
ESP12R	AATTGATTCTTTAGCATCTGG		
hyl		276	(12)
HYL1	ACAGAAGAGCTGCAGGAAATG		
HYL2	GACTGACGTCCAAGTTTCCAA		
pilA		459	(6)
F	AAAACGCCACCAGAGAAGGT		
R	CATTGGCGCAATCACAACCA		
pilB		959	(6)
F	GATACCCAGCTGACGGCTTT		
R	GGTACTGCCGAAAACGAAGC		
fms8		765	(6)
F	AGACGAGCAGATGAACAGCC		
R	CCCGTCAATCGTCGTACTGT		
EfaAfm		199	(6)
F	AAAAGGCAAGCGACGCAGAT		
R	AGGTCTAGCCAAGCATGAGG		
sgrA		150	(6)
F	CTGATCGGATTGTTTATGA		
R	AATAAACTTCCCCAATAACTT		
ecbA		182	(6)
F	GGAGTGAGGCTTTTAAACCAGA		
R	GGAAACAGGGTACTTTG		
scm		1015	(6)
F	GTTTACTAGTCCTAGTTGC		
R	TCTGTACTGTCGCTTGTGTC		

Table 2. Polymerase Chain Reaction Protocols for Molecular Characterization of Vancomycin-Resistant Enterococci

Target Gene	Initial Denaturation	No. of Cycles	Temperature (°C), Time			Final Extension
			Denaturation	Annealing	Extension	
ddlE faecium; ddlE faecalis	95°C, 5 min	30	95°C, 30 s	53°C, 30 s	72°C, 60 s	72°C, 10 min
vanA-vanB	94°C, 3 min	30	94°C, 60 s	54°C, 60 s	72°C, 60 s	72°C, 7 min
asa1-gelE-cylA-esp-hyl	95°C, 15 min	30	94°C, 60 s	56°C, 60 s	72°C, 60 s	72°C, 10 min
pilA-pilB-efaAfm	95°C, 2 min	35	95°C, 20 s	58°C, 10 s	72°C, 20 s	72°C, 5 min
fms8-sgrA	95°C, 2 min	35	95°C, 20 s	58°C, 10 s	72°C, 20 s	72°C, 5 min
ecbA-scm	95°C, 2 min	35	95°C, 20 s	58°C, 10 s	72°C, 20 s	72°C, 5 min

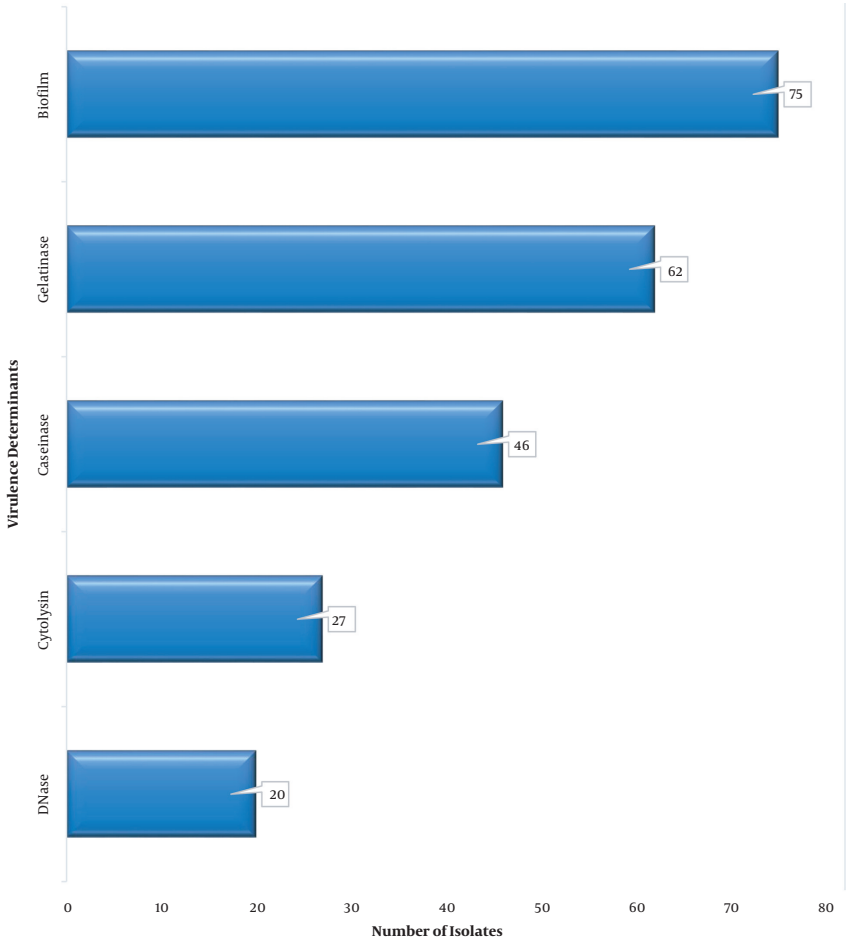

Figure 2. Frequency of occurrence of virulence factors

for *E. faecium* identification; nevertheless, only 2 out of 24 isolates phenotypically identified as *E. faecalis* (8.3%) revealed bands for *E. faecalis* identification (Figure 4). The 25 VRE isolates confirmed genotypically as *E. faecium* and *E. faecalis* were selected for screening for virulence genes. Five isolates (20%) revealed bands, including four isolates for *asa1* (16%) and only one isolate for *hyl* (4%). None of the other 10 virulence genes were detected (Figure 5). Only one isolate showed a band for the *vanB* gene at 635 bp; none of them had the *vanA* gene.

5. Discussion

The virulence factors of enterococci play a major role in the pathogenicity of enterococci and could be explained

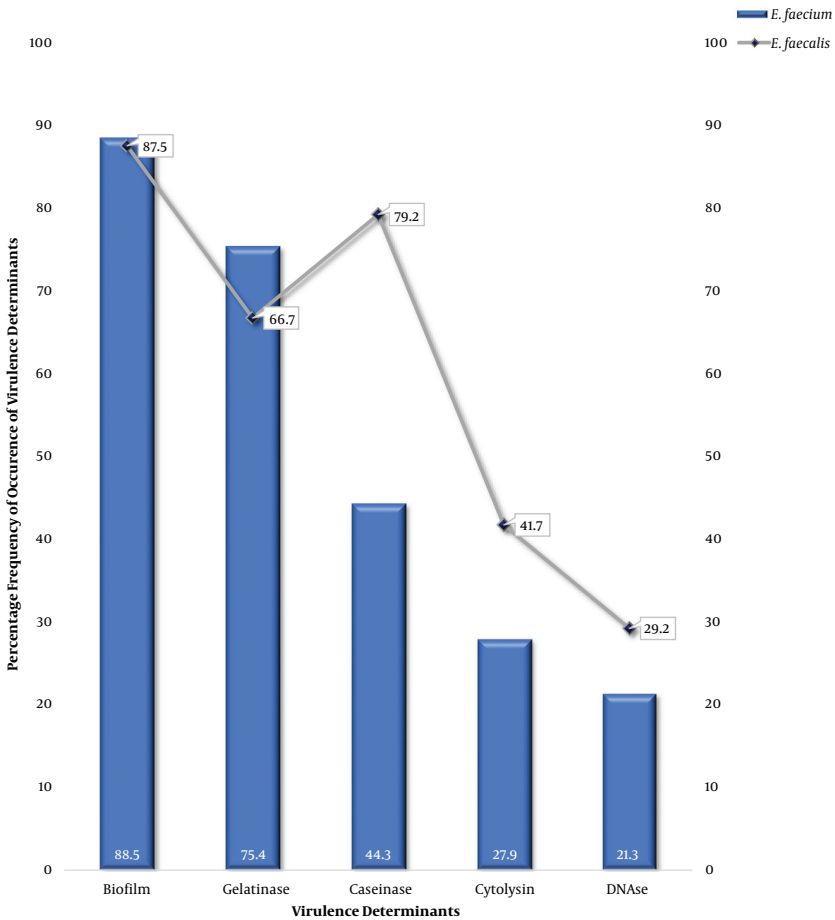

Figure 3. Percentage of occurrence of virulence factors in *E. faecium* and *E. faecalis*

not only by the presence of virulence determinants; antibiotic resistance genes play an important role in the pathogenicity of enterococcal strains (15, 16). *In-vivo* and on medical devices biofilm formation aids disease development as it boosts the persistence of infections and reduces antimicrobial activity (17). Biofilm production was 88.5% and 87.5% in *E. faecium* and *E. faecalis*, respectively, corroborating an earlier study (18) where biofilm production was 86.6% among enterococci isolates. *GelE*, a foremost virulence determinant among biofilm producers (18), facilitates signals within the quorum sensing *fsr* system resulting in biofilm production (16); however, earlier studies postulate that no correlation is observed between gelatinase and biofilm production in many *E. faecalis* isolates (19).

In this study, gelatinase production was higher in *E. faecium* than *E. faecalis*, a finding at variance with another report (20) with lower rates for both species but higher production by *E. faecalis* than *E. faecium*. Numerous *E. faecalis* isolates in the current study (64.7%; 55/85) coproduced biofilm and gelatinase. This may be adduced to environmental and genetic factors, virulence, and the existence of other mechanisms as these affect surface activity and intercellular interactions (10).

A study reported no production of gelatinase in some *Enterococcus* isolates, although *gelE* was detected (21). The activation of *gelE* expression has been reported in the late exponential growth phase at high cell concentrations, and its intracellular expression can raise the severity of infections. Biofilm formation is independent of the presence or lack of the *esp* gene (16, 18, 22); nevertheless, other authors affirmed the positive relationship between the presence of *esp* (23) and *asa1* gene with biofilm formation in enterococci as *asa1* gene promotes the adherence of microorganisms to surfaces (16, 18). However, no *gelE* or *esp* gene was detected in all the isolates of the present study, and only 4 out of 25 screened isolates had the *asa1* gene, reinforcing the complexity of the processes involved in *Enterococcus* virulence.

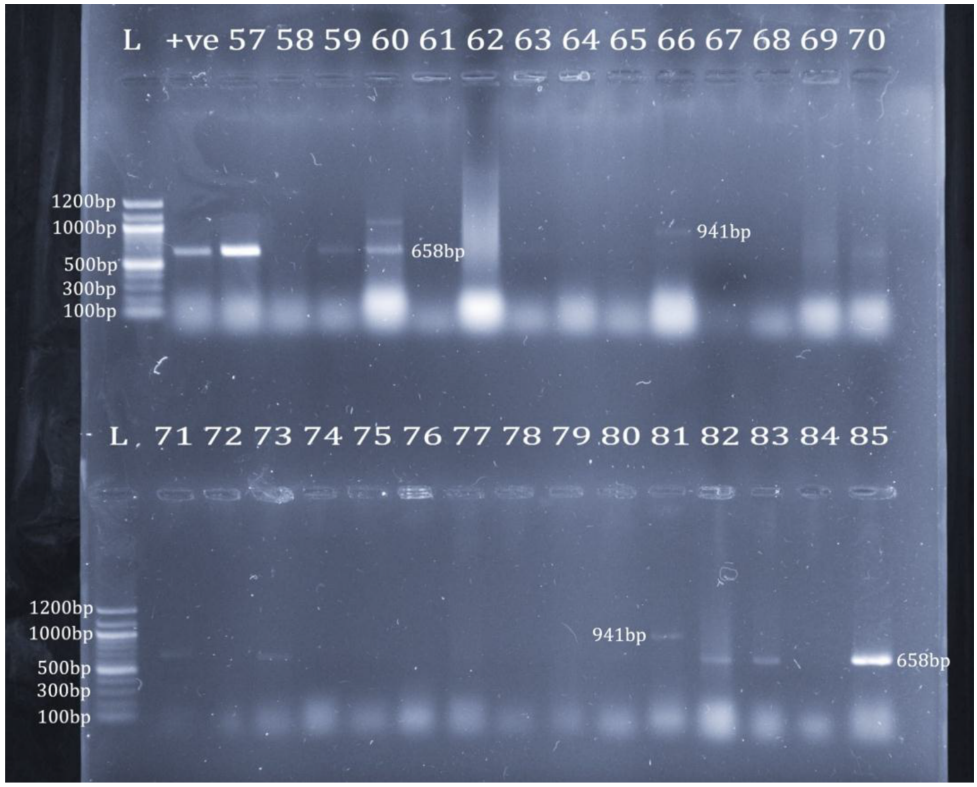

Figure 4. Identification of *E. faecium* and *E. faecalis*. Upper row: lane 1: 100 bp marker; lane 2: *ddlE. faecium* positive control; lanes 3, 5, and 6: *E. faecium* (658 bp); lane 12: *E. faecalis* (941 bp); lower row: lane 1: 100 bp marker; lanes 2, 4, 13, 14, and 16: *E. faecium* (658 bp); lane 12: *E. faecalis* (941 bp)

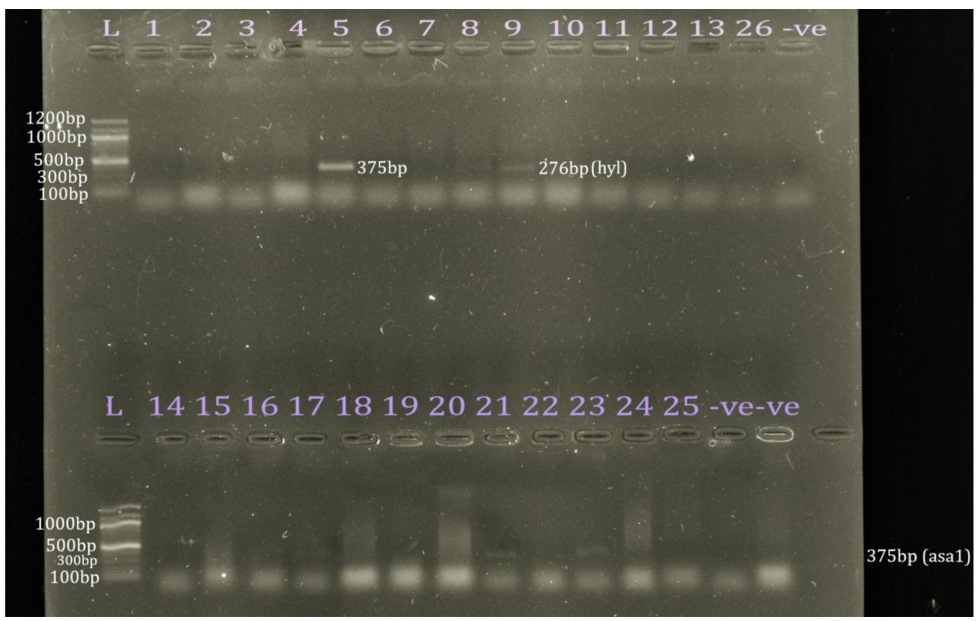

Figure 5. Detection of *asa1* and *hyl* Genes in *E. faecium* and *E. faecalis*. Upper row: lane 1: 100 bp marker; lane 6: *asa1* (375 bp); lane 10: *hyl* (276 bp); lane 16: negative control; lower row: lane 1: 100 bp marker; lanes 5, 9, and 11: *asa1* (375 bp); lanes 14-15: negative control

Cytolysin facilitates infection by damaging cell membranes (16, 20) and has been reported to enhance virulence in animal models (16). Hemolysin and/or gelatinase aids nutrient acquisition from host tissues and advances invasion, thereby increasing the severity of human infections. However, the failure to detect the *cylA* gene in the isolates of the present study, in line with other studies (18), underscores the need for phenotypic and molecular screening for virulence.

DNase production in this study was low for *E. faecium* (21.3%) and *E. faecalis* (29.2%), respectively. DNase hydrolyzes nucleic acids, contributing to bacterial virulence, although *E. faecium* is reported to be devoid of DNase activity (24). Hyaluronidase, which was detected in only 4.0% of the isolates of this study, is encoded by chromosomal *hyl* and degrades hyaluronate. Bacterial hyaluronidase behaves as endo-*N*-acetylhexosaminidase, destroys β-1-4 linkage, consequently creating unsaturated disaccharides, causing tissue damage (16).

Pathogenicity is related to the ability of virulent strains to grow profusely in the intestinal tract and invade the body. Host factors, such as underlying medical conditions, immune status, and antibiotics exposure, are thought to play a role in the pathogenicity of enterococci. Low recovery of virulence genes in this study population suggests strongly that virulence alone might not indicate infection, as other mediators of pathogenicity could be left unexplained (25).

The present study detected the *vanB* gene in only one isolate, but *vanA* in none. This result is substantiated by a study where multi-resistance *E. faecium* strains had no vancomycin-resistance genes, *E. faecalis* strains ST774 carried the *vanB* gene, and ST133 had no acquired resistance genes as confirmed by vancomycin susceptibility testing (26), a finding at variance with other reports (27). In another study, isolates screened as *vanA* and *vanB* phenotypes were negative for both genes; nonetheless, they were positive for a fragment of the *vanHM* gene (28). Therefore, the results of the current study suggest the presence of other *van* genotypes the detection of which might be missed (28). However, a major limitation of this study was the inability to confirm the identification using molecular methods and to screen all the enterococcal isolates for other virulence genes due to limited resources.

Footnotes

Authors' Contribution: FMA and NAY conceptualized and designed the study. FMA supervised the study, performed the data interpretation and analyses, and wrote the manuscript. NAY and OOO carried out the sampling and contributed to the data interpretation and the writing of the manuscript. NAY, ARR, and OOO carried out the laboratory procedures. All the authors read and approved the manuscript.

Conflict of Interests: The authors declare that there is no conflict of interest.

Ethical Approval: Ethical approval for the study was obtained from the Ethical Review Board of the State Specialist Hospital, Osogbo (approval no.: HREC/SSHO/11/478).

Funding/Support: No funding was obtained for this study.

Informed Consent: Study participants, both out-patients and in-patients, were enrolled in the study based on individual and parental consent for inclusion.

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