Original Article

Antimicrobial Resistance Pattern of Enterococci Isolated from Stool Samples in a Tertiary Hospital in Nigeria

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Abstract

Background: Enterococci cause infections both in and out of the hospital setting and have demonstrated resistance to almost all classes of drugs. A combination of cell wall acting agents and high-level aminoglycosides is a commonly used regimen for serious infections, but resistance to either renders the synergism ineffective. Vancomycin is the drug of choice for life-threatening infections, but there have been increasing reports of resistance to the drug. Vancomycin-resistant enterococci (VRE) infection is usually preceded by gastrointestinal colonization. **Aim:** This study was carried out to determine the antimicrobial resistance profile of *Enterococcus* species isolated from stool and the prevalence of VRE. **Materials and Methods:** Enterococci were identified from stool samples based on characteristic growth patterns on Bile Esculin Agar and MacConkey agar and growth in 6.5% sodium chloride broth. Speciation was by conventional biochemical identification. Antibiotic susceptibility testing and screening for high-level aminoglycoside resistance (HLAR) were done by modified Kirby–Bauer disk diffusion technique. Susceptibility of isolates to linezolid, penicillin, nitrofurantoin, high-level gentamicin and streptomycin, tetracycline, ciprofloxacin, vancomycin, and teicoplanin was tested. VRE screening was done using a chromogenic agar. The polymerase chain reaction was used for confirmation. **Results:** Nine species of *Enterococcus* were identified from 561 isolates. The most common species were *Enterococcus faecium* (46.0%), *Enterococcus faecalis* (21.6%), *Enterococcus gallinarum* (18.5%), and *Enterococcus casseliflavus* (5.2%). Resistance was highest to ciprofloxacin, tetracycline, and nitrofurantoin. Lowest resistance was to vancomycin, teicoplanin, gentamicin, and linezolid. VRE prevalence rate was 1.1% and that of HLAR was 20.7%. All VRE had *vanA* gene. **Conclusion:** Overall, *E. faecium* was the predominant species. Highest resistance was to ciprofloxacin and tetracycline.

Keywords: *Enterococcus,* high-level aminoglycoside resistance, vancomycin-resistant enterococci

Received on: 03-01-19 **Review completed on:** 24-03-19 **Accepted on:** 02-04-19 **Published on:** 06-12-19

Introduction

Enterococci are multidrug-resistant opportunistic pathogens that have been implicated in serious and life-threatening healthcare-associated infections such as catheter-related urinary tract infection (UTI), intra-abdominal and pelvic infections, surgical site infections, and bacteremia.[1-4] Due to their remarkable ability to adapt to the environment, they acquire antibiotic resistance determinants either by a mutation in deoxyribonucleic acid (DNA) or by acquisition of new DNA through plasmids or transposons.[5-7]

Treatment is often challenging[8] and depends on the species, resistance patterns, location, and severity of infection. Gastrointestinal colonization often precedes infection; hence,[9] species identification and knowledge of antibiotic resistance profile of gastrointestinal commensals are essential for formulation of guidelines for empiric and targeted therapy.[10,11] In addition, targeted surveillance for identification of vancomycin-resistant enterococci (VRE) is necessary for institution of proper infection control measures to avoid dissemination of resistant strains.[12] Early detection of VRE through highly sensitive screening methods is also important for preventing the emergence of vancomycin-resistant *Staphylococcus aureus*.[13]

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Quick Response Code:

Website: www.atpjournal.org

DOI: 10.4103/atp.atp_1_19

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How to cite this article: Shettima SA, Iregbu KC. Antimicrobial resistance pattern of enterococci isolated from stool samples in a tertiary hospital in Nigeria. Ann Trop Pathol 2019;10:126-31.

Since colonized patients are the primary reservoir of VRE,[14] the use of stool sample for screening will give a better reflection of the prevalence, because rectal swab samples have been associated with a high false-negative rate.[15]

Materials and Methods

A cross-sectional study conducted over 2 years (August 2013–August 2015). Enterococci were isolated from stool samples of adult patients (18 years and above) after written informed consent was obtained. Stool samples were self-collected in clean, dry, and leak-proof plastic containers. They were transported to the laboratory immediately, kept at room temperature, and processed as soon as possible. They were identified based on the observation of tiny brown/black colonies on Bile Esculin Agar and tiny magenta-colored colonies on MacConkey agar. Further identification was done by Gram staining and growth in 6.5% sodium chloride broth (Oxoid, Basingstoke, UK).

Isolates were inoculated onto nutrient agar slants and stored as working cultures at 4°C–8°C for subsequent antibiotic susceptibility testing and chromogenic VRE screening.

Speciation was conducted using conventional biochemical methods based mainly on carbohydrate fermentation reactions using 1% solution of (sucrose, sorbose, mannitol, sorbitol, raffinose, and arabinose) as described by Facklam and Collins.[16]

Antibiotic susceptibility testing of all confirmed *Enterococcus* isolates was performed and interpreted according to the recommendations of the Clinical and Laboratory Standards Institute,[17] using the modified Kirby–Bauer disk diffusion method, on Mueller–Hinton agar (Oxoid, Basingstoke, UK) for all the antibiotics tested except glycopeptides (vancomycin and teicoplanin) which were tested using the epsilometer test (E-test) method.[18]

Antibiotics tested were β-lactam (penicillin – 10 units), quinolone (ciprofloxacin – 5 μg), aminoglycosides (gentamicin – 120 μg and streptomycin – 300 μg), tetracyclines (tetracycline – 30 μg), nitrofurantoins (nitrofurantoin – 300 μg), and oxazolidinones (linezolid – 30 μg). Susceptibility of each isolate to vancomycin and teicoplanin was tested using E-test strips for minimum inhibitory concentration (MIC) determination (Liofilchem, Roseto Degli Abruzzi, Italy). Plates were incubated for a full 24 h for accurate detection of resistance. *Enterococcus faecalis* ATCC® 29212 was used as the quality control strain.

High-level aminoglycoside resistance (HLAR) screening was done using disk diffusion method (high content gentamicin –120 μg and streptomycin – 300 μg disks).[17,18] Resistance was indicated by absence of zone of inhibition and susceptibility by a zone diameter of ≥10 mm for both gentamicin and streptomycin [Figure 1].

In this study, HLAR is defined as resistance to high-level gentamicin alone (HLGR), or high-level streptomycin alone (HLSR) or resistance to both.

E. faecalis ATCC® 29212 was used as the quality control strain (sensitive).

Chromogenic screening for vancomycin resistance was carried out on all confirmed enterococcal isolates using a chromogenic screening agar, CHROMagar™ VRE (CHROMagar Co, Paris, France). Pure colonies on nutrient agar were inoculated onto CHROMagar VRE and incubated for 24–48 h at 37°C in ambient air. Pink colonies were presumptively identified as VRE. *faecalis*/VR *E. faecium* [Figure 2], while blue colonies were identified as VR *E. gallinarum*/VR *E. casseliflavus* according to manufacturer's specifications. Agar plates showing no growth or growth with other colors were regarded as negative for VRE.[19]

Presumptively identified VRE were inoculated into the nutrient broth and stored at −20°C for subsequent molecular analysis by polymerase chain reaction (PCR) for *vanA* and *vanB* genes detection. The purified DNA of all presumptive VRE isolates was obtained using the phenol-chloroform DNA extraction method. The specific vancomycin-resistant genotype *(vanA, vanB)* was determined by genomic DNA amplification with real-time PCR analysis using specific primers (Bio-Rad Laboratories Inc., Marnes-la-Coquette, France) selected from published gene sequences.[20]

The extracted DNA and the forward and reverse primers for the resistance genes were added to a PCR master mix, GreenStar™ qPCR PreMix (Bioneer; Alameda, CA, US) for each test according to the manufacturer's instructions.

PCR amplifications were done in 1.5 mL reaction tubes each with reaction mixtures composed of 6.5μl of sample DNA (extracted enterococcal genomic DNA), 0.5μl (20 pmol) of each primer and 12.5μl of GreenStar™ qPCR PreMix were prepared and aliquoted in 20μl quantities in individual PCR capillary tubes.

Real-time PCR was used for amplification of fragments representing *vanA*/*vanB* genes, using Roche LightCycler, a quantitative real-time PCR thermal cycler (Roche Life Science, Mannheim, Germany).

The thermal cycling conditions were initial denaturation at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 15 s, annealing (50°C–52°C), depending on the primer pairs, for 15 s and extension at 72°C for 30 s.

Enterococcus faecium ATCC®51559 was used as *vanA*-positive control strain. *E. faecalis* ATCC®51299 was *vanB*-positive control. Negative control for each test consisted of PCR reagent master mix and 6.5μl of sterile molecular grade water.

A real-time PCR standard curve was generated for each test using the LightCycler software version 3.5 (Roche Life Science, Mannheim, Germany).

DNA extraction and PCR were done at DNA laboratories, a diagnostic and research laboratory with facilities for molecular biology located at Kaduna, Nigeria.

Statistical analysis of data was performed using statistical package for social sciences (SPSS) software version 20 (IBM Corp., Armonk, NY, United States).

Ethical approval for the study was obtained from the institution's Health Research Ethics Committee.

Results

Five-hundred and sixty-one enterococci were isolated from stool samples. Of these, 258 (46.0%) were identified as *E. faecium*, 121 (21.6%) were *E. faecalis*, 104 (18.5%) were *Enterococcus gallinarum*, and 29 (5.2%) were *Enterococcus casseliflavus*. The remaining 8.7% comprised *Enterococcus hirae*, *Enterococcus durans*, *Enterococcus mundtii*, *Enterococcus raffinosus, Enterococcus dispar,* and unidentified species.

Antimicrobial resistance of enterococci

Of the 561 isolates, 92 (16.4%) were resistant to linezolid, 149 (26.6%) resistant to penicillin, 241 (43.0%) to nitrofurantoin, 367 (65.4%) to tetracycline, and 399 (71.1%) to ciprofloxacin [Table 1].

A total of 116 of 561 isolates (20.7%) were HLAR. Of these 116 HLAR isolates, 4 (3.4%) were resistant to high-level gentamicin alone, 74 (63.8%) were resistant to high-level streptomycin alone, and 38 (32.8%) demonstrated resistance to both. Forty-two isolates in total (7.5%) had high-level gentamicin resistance (HLGR), while a total of 112 (20.0%) were high-level streptomycin resistant (HLSR) (*P* 0.02) [Table 1].

Linezolid resistance was observed in 43 (16.7%) *E. faecium*, 15 (12.4%) *E. faecalis*, 16 (15.4%) *E. gallinarum,* and 9 (31.0%) of *E. casseliflavus* [Table 2].

Table 1: Antimicrobial resistance profile of *Enterococcus* isolates (disk diffusion method)

Antibiotic (concentration)	Resistance, *n* (%)
Linezolid (30 µg)	92 (16.4)
Penicillin (10 units)	149 (26.6)
Nitrofurantoin (300 µg)	241 (43.0)
Gentamicin (120 µg)	42 (7.5)
Streptomycin (300 µg)	112 (20.0)
Tetracycline (30 µg)	367 (65.4)
Ciprofloxacin (5 µg)	399 (71.1)

Vancomycin-resistant enterococci

With the E-test method, all isolates in the study were sensitive to both vancomycin and teicoplanin (MIC for both glycopeptides ranged between 0.38 and 4.0 µg/mL) [Figure 1], with the exception of one *E. gallinarum* isolate which was intermediately sensitive to vancomycin (MIC of 8.0 µg/mL).

However, chromogenic agar screening identified 6 (1.1%) presumptive VRE (including the *E. gallinarum* which showed intermediate sensitivity with the E-test method). All the six presumptively identified VRE (100.0%) had *vanA* genotype detected by real-time PCR, while none had *vanB* genotype.

Of the six VRE, 3 (50.0%) were E. f*aecium,* 2 (33.3%) E. g*allinarum,* and 1 (16.7%) E. c*asseliflavus*.

Vancomycin-resistant enterococci antibiotic resistance pattern

All (100.0%) the VRE were resistant to linezolid, tetracycline, and ciprofloxacin; 4 (66.7%) to penicillin, 3 (50.0%) to nitrofurantoin, 1 (16.7%) to high-level gentamicin, and 1 (16.7%) to high-level streptomycin. All the *E. faecium* were resistant to penicillin and nitrofurantoin.

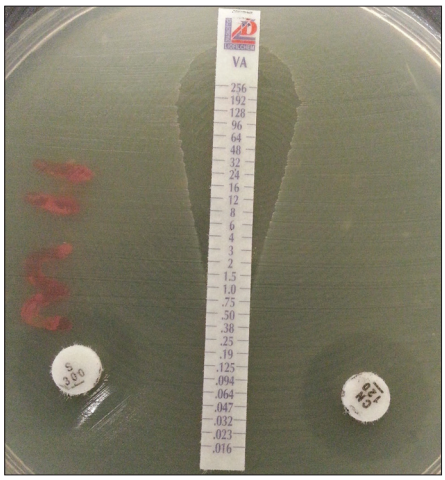

Figure 1: Vancomycin epsilometer test strip on Mueller–Hinton agar showing sensitivity with minimum inhibitory concentration of 1.0 µg/ml. High content gentamicin and streptomycin antibiotic disks showing zone of inhibition

Table 2: Antimicrobial resistance pattern of four most common *Enterococcus* species isolated in the study

Antibiotic (concentration)	*Enterococcus faecium* (*n*=258), *n* (%)	*Enterococcus faecalis* (*n*=121), *n* (%)	*Enterococcus gallinarum* (*n*=104), *n* (%)	*Enterococcus casseliflavus* (*n*=29), *n* (%)
Linezolid (30 µg)	43 (16.7)	15 (12.4)	16 (15.4)	9 (31.0)
Gentamicin (120 µg)	22 (8.5)	5 (4.1)	9 (8.7)	4 (13.8)
Streptomycin (300 µg)	50 (19.4)	26 (21.5)	19 (18.3)	4 (13.8)
Penicillin (10 units)	71 (27.5)	29 (24.0)	30 (28.8)	6 (20.7)
Nitrofurantoin (300 µg)	109 (42.2)	53 (43.8)	43 (41.3)	13 (44.8)
Tetracycline (30 µg)	176 (68.2)	84 (69.4)	58 (55.8)	22 (75.9)
Ciprofloxacin (5 µg)	193 (74.8)	83 (68.6)	63 (60.6)	23 (79.3)

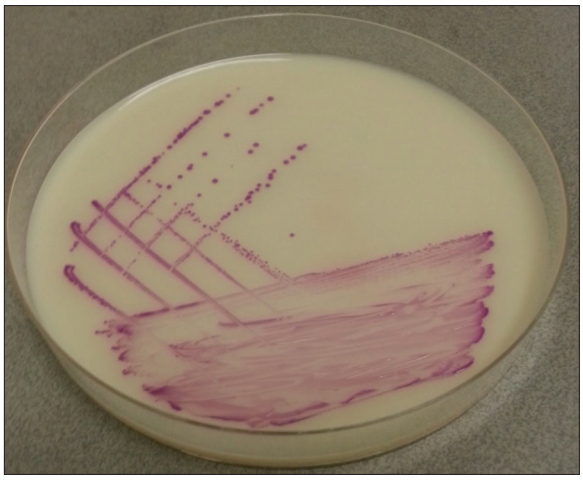

Figure 2: Vancomycin-resistant enterococci colonies on CHROMagar plate. Vancomycin-resistant enterococci are revealed in pink color (*Enterococcus faecium*)

Three isolates, one *E. durans*, one *E. casseliflavus,* and one *E. raffinosus*, were resistant to all classes of antibiotics tested except glycopeptides (vancomycin and teicoplanin).

Discussion

E. faecium and *E. faecalis* were the two most commonly isolated enterococcal species from stool samples in our study. Worldwide, the same pattern is seen both from clinical samples and from gastrointestinal commensals, although other species are increasingly being isolated as well.[2,9,21-24]

Although the high linezolid sensitivity seen in this study is similar to findings elsewhere across the world,[23,25-27] the resistance level observed is worrisome, considering that this drug is rarely used in our environment and mechanisms of resistance to other ribosomal protein synthesis inhibitors do not confer cross resistance to it.[28] Previous studies have similarly reported linezolid resistance in the absence of selective drug pressure.[1,29] This was attributed to horizontal transfer of plasmid-mediated resistance to linezolid due to *cfr* gene, which encodes a 23S rRNA methyltransferase.[29,30] The same mechanism may apply in our environment as linezolid resistance may have been imported by many of our patients who go on medical tourism to other countries where clinical use of the drug is more common.

E. casseliflavus demonstrated the highest resistance to linezolid of the four most common species isolated. Although there have been reports of linezolid-resistant *E. casseliflavus* from both human and animal origin, high rates of resistance to this drug among this species have rarely been reported.[31,32]

Resistance of majority of the isolates to ciprofloxacin is similar to results obtained from previous studies reported by other authors.[11,33,34] This high rate of resistance is likely due to selective drug pressure from intense use in the hospital and the community.[35] In the hospital setting, fluoroquinolones such as ciprofloxacin are a common choice for empiric treatment of UTI, of which enterococci are a common cause.[18,34,36] A study in Japan demonstrated that fluoroquinolone resistance was significantly associated with previous use of fluoroquinolones, which in turn was significantly related with amino acid mutations in the quinolone resistance-determining regions which ultimately results in resistance.[37]

Overall, sensitivity to glycopeptides, high-level aminoglycosides, and linezolid is high. This finding is reassuring because it is an indication that these agents can be used as empiric therapy for life-threatening enterococcal infections in our environment.

The relatively high prevalence of HLAR, in this study, agrees with findings of a study conducted in Bangladesh.[10] The higher level of resistance of enterococci to streptomycin compared to gentamicin is similar to the pattern observed in previous studies conducted in Nigeria and Iran.[3,4] The higher rate of streptomycin resistance may be explained by the fact that enterococci can develop resistance to this agent through multiple mechanisms which include enzymatic mechanisms associated with production of aminoglycoside-modifying enzymes and high-level resistance to streptomycin arising from just a single-step mutation in the 30S ribosomal subunit.[1,7]

The finding of three commensal isolates (*E. durans, E. casseliflavus,* and *E. raffinosus*) with resistance to all antibiotics, except glycopeptides, is not surprising as enterococci in the guts of humans and animals easily acquire resistance genes from other gut flora under selective pressure from ongoing use of antimicrobials.[38,39]

CHROMagar VRE demonstrated 100% sensitivity in detecting VRE, and this finding agrees with the reports from previously conducted studies on chromogenic screening for VRE.[19,40,41] The PCR method also identified the *vanA* gene in the same isolates, while the E-test method did not detect any VRE. It is a known fact that diffusion methods are usually unreliable for detection of vancomycin resistance, especially in cases of low-level inducible resistance,[42] and this may be attributed to the large size of the vancomycin molecule which has difficulty diffusing through agar media.[43]

The high concordance between CHROMagar VRE and PCR in the detection of VRE is of clinical interest because an accurate, rapid, and cost-effective screening method are desirable for prompt institution of treatment and control measures.[19] The implication of the finding in this study is that CHROMagar VRE can be reliably used to screen and detect VRE in the absence of expensive molecular systems in low- and middle-income countries such as Nigeria.[44]

In this study, only *vanA* gene was found, similar to the finding of Akpaka *et al*. in Bermuda.[27] *vanA* gene has been reported to be the most common vancomycin resistance gene.[1,18]

E. faecium is known to be a major reservoir of acquired vancomycin resistance,[45] and this was also seen in our work as it constituted majority of the VRE. The low prevalence of VRE in this study, when compared to reports from the United

States as well as countries in Asia,[46-48] may be a reflection of the low use of vancomycin in this locality. A previous study in Lagos, Nigeria, by Iregbu *et al*. found no VRE among enterococci isolates.[3]

The relatively lower resistance of VRE to the high-level aminoglycosides compared to the other antibiotics tested means that these agents could still be used for the treatment of VRE infections in our environment in combination with a cell wall active agent if the isolate is susceptible.

The 100% resistance of VRE to linezolid in our study is worrisome. This is in view of the fact that it is one of the few drugs used for management of VRE infections.[49] Similar finding of linezolid resistance among VRE has been reported from different regions.[50-52]

Conclusion

Isolates colonizing the gastrointestinal tract of patients seen in our study were sensitive to glycopeptides, linezolid, and high-level aminoglycosides. These drugs should be used for empiric treatment of enterococcal infections in our environment. Although vancomycin resistance was very low and limited to a few species, there is need for surveillance among hospitalized patients using rapid and accurate techniques like chromogenic agar screening to control the spread of VRE.

Acknowledgment

National Hospital, Abuja provided financial assistance.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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