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Antibiotic Susceptibility Testing, Plasmid Detection and Curing of Clinically Isolated *Enterococcus* Species

G. A. C. Ezeah[1*], M. C. Ugwu[2], A. O. Ekundayo[3], O. F. Odo[4], O. C. Ike[5] and R. A. Akpe[3]

[1]Department of Microbiology, Enugu State University Teaching Hospital Parklane, Enugu State, Nigeria.
[2]Department of Medical Laboratory Science, College of Health Sciences, Nnamdi Azikiwe University, P.M.B. 5001, Nnewi, Anambra State, Nigeria.
[3]Department of Microbiology, Ambrose Alli University, Ekpoma, Edo State, Nigeria.
[4]Department of Histopathology, Enugu State University Teaching Hospital Parklane, Enugu State, Nigeria.
[5]Department of Industrial Chemistry, Enugu State University of Science and Technology, Enugu State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author GACE designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors GACE and AOE managed the analyses of the study. Authors MCU, OFO, OCI and RAA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Vancomycin resistant enterococci (VRE) are a major medical concern globally. Their significantly greater prevalence and the ability to transfer resistance to vancomycin from other bacteria made them an object of interest and intense research. The isolates of *Enterococcus* sp. were subjected to

**Corresponding author: E-mail: ezeahg@yahoo.com, princeconsult@yahoo.com;*

antibiotic susceptibility testing before curing. The three *Enterococcus* species exhibited different antibiotic resistance profile. Pre-curing antibiotic resistance of nosocomial isolates compared with community acquired isolates revealed that high percentage of the nosocomial isolates were resistant to antibiotics compared to community isolate. Post-curing antibiograms of the isolates showed different resistant and susceptibility pattern. Also, DNA plasmid pre-curing and post curing analysis of the isolates showed different resistance pattern. Six of the 15 representative isolates selected on the basis of their high pre-curing antibiotic resistance for plasmid analysis with 0.8% agarose electrophoresis were positive for plasmid DNA. Four (4) of the positive isolates (*E. faecium, E. faecium, E. faecalis,* and *E. avium*) had plasmid fragment of greater than 1000 bp while two (2) of them (*E. faecalis* and *E. faecalis*) had fragments of between 100 and 500 bp. The remaining nine (9) had no plasmid DNA. The study revealed the pathogenicity factors demonstrated with the enterococcal isolates.

Keywords: Enterococci; antibiotic susceptibility; DNA; plasmid detection; pathogenicity.

1. INTRODUCTION

High level of intrinsic antibiotic resistance is one of the important features of the genus *Enterococcus*. Some of them are intrinsically resistant to some β-lactam-based antibiotics (some penicillins and virtually all cephalosporins) as well as to many aminoglycosides [1]. Between 1989 and 2009, strains of particularly virulent and vancomycin-resistant enterococci (vancomycin-resistant Enterococcus or ERV) emerged in hospital-acquired nosocomial infections, particularly in the United States of America [2].

Resistance to vancomycin occurs when a sensitive enterococcus acquires a plasmid that confers resistance to vancomycin.The new strain is called vancomycin resistant *Enterococcus* (VRE). Unrelated bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) can acquire vancomycin resistance from VRE to form new strains called VRSA. Furthermore, MRSA (VRE organisms) are usually resistant to more than one antibiotic [2]. VRE can be transmitted from person to person and are increasing problems in chronic hospitalized patients. The most prevalent *enterococcus* species in humans are *Enterococcus faecalis* and *Enterococcus faecium*; contributing to more than 90% of clinical isolates [3]. Other enterococcal species are: *Enterococcus avium, Enterococcus gallinarum, Enterococcus casselflavus, Enterococcus durans, Enterococcus raffinosus* and *Enterococcus mundtii*. The most vancomycin resistant strain is *E. faecium* [2].

Enterococci's acquisition of vancomycin resistance has seriously affected these organisms' treatment and infection control. Six phenotypes of vancomycin resistance termed vanA, vanB, vanC, vanD, vanE and vanG have been described. The vanA, vanB phenotypes are clinically significant and mediated by 1-2 acquired transferable operons that consist of 7genes in 2 clusters termed VANA VANB operons. First reported in enterococcal strains were these gene clusters in 1988. VanA is carried on a plasmid-mediated Transposon Tn 1546 [4].

However, DNA plasmid curing achieved by treatments with some reagents is most likely to promote the loss of resident plasmid DNA from a cell and to cause loss of resistance. Curing of plasmid is done to determine whether a plasmid encodes a trait or not. A trait is said to be plasmid-borne if plasmid encodes information about it. Curing of plasmid could be achieved using any of these: novobiocin, ethidium bromide (EtBr), acriflavin, acridine orange dye, plumbagin, sodium deodecyl sulphate (SDS) [5].

Enterococci virulence is lower than organisms such as *Staph. aureus* [6]. Risk factors for mortality associated with enterococcal bacteraemia include disease severity, the age of the patient and the use of broad-spectrum antibiotics [7]. Some enterococcal strains produce gelatinase, a proteolytic enzyme with an extracellular zinc containing metalloproteinase [8]. Gelatinase can hydrolyse gelatin, collagen, fibrinogen, casein, haemoglobin and other bioactive peptides [9]. It is also responsible in oral infection for inflamed pulp and periapical lesions [8]. Gelatinase has played a major role in most pathogenic bacteria's pathogenicity. Due to its cytotoxic and tissue destructive potential and inhibitory effects on phagocytes, the enzyme was associated with disease progression [10]. The production and activity of gelatinase in enterococcal infections in clinical isolates are higher than that of healthy volunteers [11].

1.1 Vancomycin - Dependent *Enterococcus* (VDE)

In 1993, the first documented strain of vancomycin-dependent enterococcus (VDE) was isolated from the urine of a 46-year-old woman at Thomas Jefferson University Hospital in Philadelphia, Pennsylvania [12]. Twenty additional cases of VDE have been reported worldwide since this first isolate was reported, including both *E. Fecalis* and *E. faecium* strains. Even though the clinical significance of VDE remains unclear, a 1999 outbreak of VDE was reported in a bone marrow transplant unit at Johns Hopkins University [13] and shows its potential to become a pathogen of clinical significance.

Another mechanism of enterococci resistance to antibiotics is the formation of biofilm. Biofilm is a population of cells in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids that are irreversibly attached to many biotic and abiotic surfaces [14]. Biofilm formation is a complex development process involving surface attachment and immobilization, cell-to-cell interaction, microcolony formation, confluent biofilm formation and the development of a three-dimensional biofilm structure [15]. In a biofilm, bacteria behave differently from their free-floating counterparts (planktonic). By producing extracellular signal molecules called autoinducers, the regulation of bacterial gene expression in response to cell population density, called quorum sensing, is accomplished [16]. Biofilm production is regulated in several bacterial pathogens by quorum sensing systems. Biofilms are known to be hard to eradicate and are a source of many chronic infections. Biofilms are medically important, according to the National Institutes of Health, accounting for more than 60% of microbial infections in the body [17]. A mature biofilm can tolerate antibiotics at concentrations of 10–1000 times more than are required to kill planktonic bacteria. Bacteria in biofilms are phagocytosis resistant, making it extremely difficult for biofilms to be eradicated from live hosts [17]. Bacteria in biofilms colonize a wide variety of medical devices, such as catheters, artificial cardiac pacemakers, heart valves and orthopedic devices, and are associated with a number of human diseases, including endocarditis of the valve, burning wound infections, chronic otitis media with effusion and cystic fibrosis [18].

2. MATERIALS AND METHODS

2.1 Study Area

Samples for this study were sourced from:

Enugu State University of Technology (ESUT) Teaching Hospital, Parklane and University of Nigeria Teaching Hospital (UNTH), Ituku/Ozalla in Enugu State, Nigeria.

Study design: This is a cross-sectional study. Three categories of patients were included in the study.

In-patients: 504 in-patients admitted in ESUT Teaching Hospital and University of Nigeria Teaching Hospital both in Enugu who submitted their samples of urine, wound swabs, aspirates, sputum, ear swabs, high vaginal swabs, urethral swabs, semen, CSF and blood to the Microbiology Departments for microscopy, culture and sensitivity.

Out-patients: 504 out-patients who visited ESUT Teaching Hospital, Parklane and University of Nigeria Teaching Hospital, Ituku/Ozalla and who submitted clinical samples to the Microbiology Departments for microscopy, culture and sensitivity.

Controls: 20 male and 20 female volunteers who did not have symptoms of any infection. They were selected from outside the hospital environment and were used as controls.

Sample collection: Sterile universal containers containing boric acid preservative were used for urine sample collection while sputum, stool, aspirates and CSF were collected with sterile plain universal bottles. Sterile swabs were used to collect high vaginal, urethral, wound, nasal, ear, anal sample. For blood culture, five milliliters of blood was collected with syringe and put aseptically into fifty milliliters of sterile brain heart infusion (BHI) broth contained in a bijou bottle.

2.2 Vancomycin Susceptibility Testing

The vancomycin antibiotic susceptibility patterns of isolates were determined using disk diffusion method as described by CLSI [19]. Reference type *E. faecalis* strain (ATCC 29212) was used as control.

2.3 Other Antibiotic Susceptibility Patterns of Isolates

The isolates were subjected to antibiotic screening by disk diffusion method as described by CLSI [19]. Reference type *E. faecalis* strain (ATCC 29212) was used as control. The antibiotics used, their classes and disc concentrations were as follows:

- **Fluoroquinolones**: ciprofloxacin (5 µg), pefloxacin (5 µg), levofloxacin (5 µg) and ofloxacin (5 µg)
- **Cephalosporins (cephems)**: cefuroxime (30 µg) and ceftriaxone (30 µg)
- **β- lactam -β- lactamase inhibitor combination**: augmentin (20/10 µg)
- **Penicillins (β- lactams)**: ampicillin (10 µg) and cloxacillin (5 µg)
- **Macrolides**: erythromycin (15 µg)
- **Glycopeptides**: vancomycin (5 µg)
- **Aminoglycosides**: gentamicin (10 µg)

The interpretative criteria were based on CLSI [19].

2.4 Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR index was determined by dividing the number of antibiotics to which the test isolate was resistant by the total number of antibiotics to which test isolate was evaluated for sensitivity using the formula MAR =X/Y, where X is the number of antibiotics to which test isolates displayed resistance and Y is the total number of antibiotics to which test organism was evaluated for sensitivity.

2.5 Plasmid Profile Analysis of Isolates Using 0.8% Agarose Gel Electrophoresis

The plasmid profile analysis of isolates using 0.8% agarose gel electrophoresis was carried out following the method described by Diana-Roxana et al. [20]. Fifteen isolates that were highly resistant to antibiotics were selected for plasmid analysis. These were six isolates of *E. faecium*; five isolates *E. faecalis* and four isolates of *E. avium*. The isolates were subjected to bacterial cultures for plasmid profile analysis.

2.6 Curing of Plasmid DNA

The curing (elimination) of the resistant plasmids of the enterococci isolated was done using sub-inhibitory concentration of 0.10 mg/ml of acridine orange as described by Akortha and Filgona [21]. Isolates were grown for 24 hours at 37ºC in Mueller-Hinton broth containing 0.1mg/ml acridine orange. The broth was agitated to homogenize the content and a loopful of the broth medium was cultured on Muller-Hinton agar (MHA) plates and antibiotic sensitivity testing was carried out as previously described. The resistant isolate that became sensitive after curing was regarded as having been cured of the plasmid DNA (plasmid-borne) while the isolate that remained resistant was not cured (chromosomal-borne).

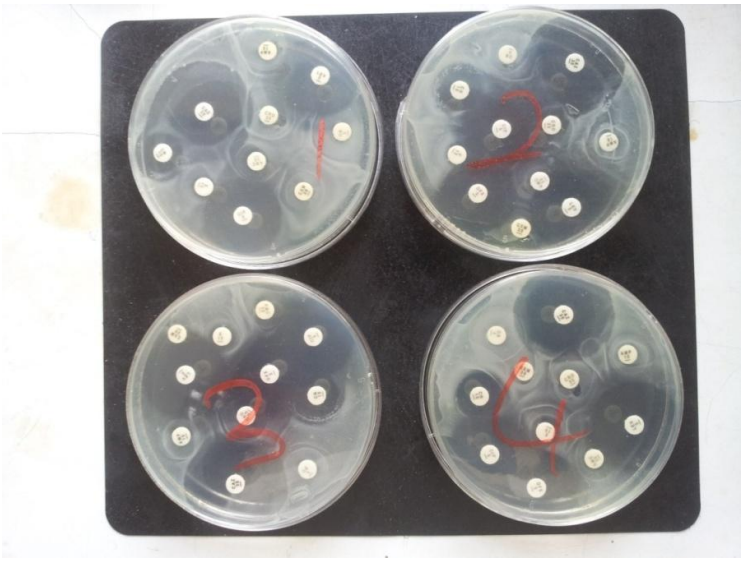

Plate 1. Antibiotic susceptibility patterns of *Enterococcus* sp isolated during this study

2.7 The Pathogenicity Factors of the Isolates

These were determined by monitoring virulent determinants such as;

Haemolysin: Haemolysin production by the enterococcal isolates was assessed by a method described by Giridhara et al. [22].

Gelatinase: Gelatinase production by the enterococcal isolates was assessed by the liquefaction of yeast extract agar containing gelatine plates as described by Giridhara et al. [22].

Caseinase production: Casein hydrolysis was assessed as described by Archimbaud et al. [23].

Lipase production: Lipase activity was determined as described by Gunn et al. [24].

MSCRAMM-Ace: A drop of distilled water was placed on an end of a slide. A colony of the test organism was emulsified in the drop. A loopful of the patient's serum was added to the suspension and mixed gently. Clumping within 30 seconds indicated a positive reaction [22].

2.8 Detection of β-Lactamase Production

Using sterile forceps, a nitrocef disc (Oxoid Ltd) was removed from the vial and placed on an empty petri dish. Immediately the remaining unused disks were placed into the freezer. Prior to inoculation, the nitrocef disc was allowed to equilibrate to room temperature. Each disc was moistened with one drop of sterile deionized water. The disc was not allowed to over-saturate, which could dilute the reagent. Water is critical to the development of the color reaction, if the disc begins to dry out it may be necessary to rehydrate the reaction area of the nitrocef disc with a small amount of water. With a sterilized loop, a well-isolated colony was removed and spread on the disc surface. The inoculated disc was observed for the development of an orange/red color.

A positive beta-lactamase result was recorded when the nitrocef disc changes color from its original yellow to orange or red. Most positive bacterial strains will produce a color change within 5-60 minutes. A positive beta-lactamase result predicts the following: Resistance to penicillin, ampicillin and amoxicillin as well as acylamino-, carboxy-, and ureido-penicillins. A negative beta-lactamase result was recorded when the Nitrocef Disc remains yellow in color. A negative result did not rule out resistance due to other mechanisms.

2.9 Statistical Analysis of Results

The results obtained from this work were analyzed statistically using Chi-square with computer program SPSS version 18 to show significant different.

3. RESULTS

3.1 Susceptibility Testing, Plasmid Detection and Curing

3.1.1 Summary of precuring antibiogram of the isolates

The 68 isolates of *Enterococcus* sp. were subjected to antibiotic susceptibility testing before curing using twelve (12) commonly used antibiotics in the study area as shown in Table 1.

68 (100%) of the isolates were resistant to the penicillins (β-lactams) in this work which were ampicillin and cloxacillin. The isolates exhibited high level of sensitivity to β-lactam-β-lactamase inhibitor which was represented by augmentin, 10 (14.7%) of the isolates were resistant to augmentin while 58 (85.3%) were sensitive to augmentin. 21 (30.9%) of the isolates were resistant to vancomycin while 14 (20.6%) were intermediate and 33 (48.5%) were susceptible. The isolates were highly resistant to the macrolides (erythromycin). 63 (92.6%) of the isolates were resistant to erythromycin 5 (7.4%) were intermediate while none was susceptible.

The fluoroquinolones were averagely active against the isolates 24 (35.3%) of the isolates were resistant to ciprofloxain, 7 (10.3%) were intermediate while 37 (54.4) were susceptible 22 (32.4%) of the isolates were resistant to Levofloxacin, 8 (11.8%) were intermediate while 38 (55.8%) were susceptible. 24 (35.3%) of the isolates were resistant to pefloxacin, 10 (14.7%) were intermediate while 34 (50.0%) were susceptible. 28 (41.3%) of the isolates were resistant to ofloxacin, 9 (13.2%) were susceptible. Aminoglycosides (Gentamicin) also exhibited average activity against the isolates. 25 (36.8%) of the isolate were resistant to the gentamicin, 4 (5.8%) were intermediate while 39 (57.4%) were susceptible.

Table 1. Summary of precuring antibiogram of the enterococcal isolates (n=68)

Antibiotics	No (%) of resistant isolates	Prevalence (%) n=632	No (%) of intermediate isolates	No (%) of susceptible isolates
AMP	68 (100)	10.8	-	0 (0)
CL	68 (100)	10.8	0 (0)	0 (0)
AMC	10 (14.7)	1.6	-	58 (85.3)
VAN	21 (30.9)	3.3	14 (20.6)	33 (48.5)
E	63 (92.6)	10.0	5 (7.4)	0 (0)
CIP	24 (35.3)	3.8	7 (10.3)	37 (54.4)
LEV	22 (32.4)	3.5	8 (11.8)	38 (55.8)
PEF	24 (35.3)	3.8	10 (14.7)	34 (50.0)
OFX	28 (41.3)	4.4	9 (13.2)	31 (45.6)
GN	25 (36.8)	4.0	4 (5.8)	39 (57.4)
CXM	49 (72.1)	7.8	12 (17.7)	7 (10.3)
CRO	49(70.)	7.8	19(27.9)	1(1.5)

Key: AMP= Ampicillin. CL= Cloxacillin. AMC= Augmentin. VAN= Vancomycin. E= Erythromycin. CIP= Ciprofloxacin. LEV= Levofloxacin. PEF= Pefloxacin. OFX= Ofloxacin. GN= Gentamicin. CXM= Cefuroxime. CRO= Ceftriaxone

Table 2. Comparison of pre-curing antibiotic resistance profile of the three *Enterococcus* species

Antibiotics	*E. faecium* N = 39	*E. faecalisi* N = 25	*E. avium* N = 4	Chi square	p-value
AMP	39 (100)	25 (100)	4 (100)	0	1.000
CL	39 (100)	25 (100)	4 (100)	0	1.000
AMC	6 (15.3)	3 (12.0)	1 (25.0)	5.24	0.07
VAN	16 (41.0)	4 (16.0)	1 (25.0)	11.73	0.002
E	37 (94.9)	23 (92.0)	3 (75.0)	2.51	0.26
CIP	14 (35.9)	8 (32.0)	2 (50.0)	4.56	0.102
LEV	17 (43.6)	5 (20.0)	0 (0)	41.48	0.000
PEF	15 (38.5)	7 (28.0)	2 (50.0)	6.24	0.04
OFX	21 (53.8)	6 (24.0)	1 (25.0)	16.72	0.000
GN	15 (38.5)	7 (28.0)	3 (75.0)	25,81	0.000
CXM	28 (71.7)	20 (80.0)	1 (25.0)	29.85	3.300
CRO	25 (64.1)	22 (88.0)	2 (50.0)	10.96	0.004

Key: Chi square. AMP= Ampicillin. CL= Cloxacillin. AMC= Augmentin. VAN= Vancomycin. E= Erythromycin. CIP= Ciprofloxacin. LEV= Levofloxacin. PEF= Pefloxacin. OFX= Ofloxacin. GN= Gentamicin. CXM= Cefuroxime. CRO= Ceftriaxone

The cephalosporins showed low level of activity against the isolates. 49 (72.5%) of the isolates were resistant to cefuroxime, 12 (17.7%) were intermediate while 7 (10.3%) were susceptible. 48 (70.6%) of the isolates were resistant to ceftriaxone 19 (27.9%) were intermediate while 1 (1.5%) was susceptible.

3.1.2 Antibiotic resistance profile of the three *Enterococcus* species

Penicillins (β-lactams): All the isolates that make up the three species were resistant to the β-lactam antibiotics used for susceptibility testing. They are 100% resistant to ampicillin and cloxacillin. The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi- square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to ampicillin and cloxacillin (Table 2).

β-lactam-β- lactamase inhibitor combination (Augmentin): Generally, the resistance of the isolates to augmentin was low. 6 (15.3%) of *E. faecium* were resistant to augmentin; 3 (12.0%) of *E. faecalis* were resistant to augmentin while 1 (25%) of *E. avium* was resistant to augmentin. The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi-

Table 3. Comparison of pre- curing antibiotic resistance of nosocomial isolates and community acquired enterococcal isolates

Antibiotics	Nosocomial isolates n = 41	Community acquired isolates n = 27	CHI-S	P-VAL
AMP	41 (100)	27 (100)	0	1.00
CL	41 (100)	27 (100)	0	1.00
AMC	8 (19.5)	2 (7)	5.53	0.01
VAN	15 (36.6)	6 (22)	3.64	0.05
E	41 (100)	22 (81.5)	1.98	0.17
CIP	16 (39.0)	8 (29.6)	1.28	0.25
LEV	17 (41.5)	5 (18.5)	8.82	0.002
PEF	18 (39.0)	8 (29.0)	1.47	0.22
OFX	20 (48.8)	8 (29.6)	4.70	0.03
GEN	15 (36.6)	10 (37.0)	0.002	0.96
CXM	39 (95.1)	10 (37.0)	2.55	0.04
CRO	33 (80.5)	16 (59.3)	3.22	0.07

Key: Chi-S= CHI-SQUARE. P-VAL= P-VALUE. AMP= Ampicillin. CL= Cloxacillin. AMC= Augmentin. VAN= Vancomycin. E= Erythromycin. CIP= Ciprofloxacin. LEV= Levofloxacin. PEF= Pefloxacin. OFX= Ofloxacin. GN= Gentamicin. CXM= Cefuroxime. CRO= Ceftriaxone

Table 4. Summary of post-curing antibiograms of the isolates

Antibiotics	No (%) of (post-curing) resistant isolates	No(%) of (post-curing) intermediate isolates	No(%) of (postcuring) susceptible isolates
AMP	58(85.3)	-	10(14.7)
CL	46(67.6)	0(0)	22(32.4)
AMC	4(5.9)	-	64(94.1)
VAN	16(23.5)	9(13.2)	43(63.3)
E	28(41.2)	2(2.9)	38(55.9)
CIP	16(23.5)	4(5.9)	48(70.6)
LEV	18(26.5)	2(2.9)	48(70.6)
PEF	20(29.4)	3(4.4)	45(66.2)
OFX	15(22.1)	5(7.4)	48(70.6)
GEN	5(7.4)	1(1.5)	62(91.2)
CXM	42(61.8)	3(4.4)	23(33.8)
CRO	45(66.2	5(7.4)	18(26.5)

Key: AMP= Ampicillin. CL= Cloxacillin. AMC= Augmentin. VAN= Vancomycin. E= Erythromycin. CIP= Ciprofloxacin. LEV= Levofloxacin. PEF= Pefloxacin. OFX= Ofloxacin. GN= Gentamicin. CXM= Cefuroxime. CRO= Ceftriaxone

square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to augmentin (Table 2).

Glycopeptides (vancomycin): 16 (41.0%) of *E. faecium* were resistant to vancomycin; 4 (16.0%) of *E. faecalis* were resistant to vancomycin while 1 (25%) was vancomycin resistant *Enterococcus* (VRE). The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi-square statistics and this revealed that there was significant difference ($p<0.05$) in their resistance to vancomycin (Table 2).

Macrolides (erythromycin): Generally, all the isolates were highly resistant to erythromycin 37 (94.9%) of *E. faecium* were resistant to erythromycin; 23 (92.0%) of *E. faecalis* were resistant to erythromycin and 3 (75.0%) of *E. avium* were resistant to erythromycin. The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi- square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to erythromycin (Table 2).

3.1.3 Fluoroquinolones

Ciprofloxacin: 14 (35%) of *E. faecium* were resistant to ciprofloxacin, 8 (32%) of *E. faecalis* were resistant to ciprofloxacin; 2 (50%) of *E. avium* were resistant to ciprofloxacin.

Table 5. Summary of DNA plasmid pre-curing and post-curing analysis of the isolates

Antibiotics	No (%) of (pre-curing) resistant isolates	No (%) of (pre-curing) intermediate isolates.	No (%) of (post-curing) resistant isolates	No (%) of (post-curing) intermediate isolates	No (%) of isolates cured of plasmids
AMP	68(100)	-	58(85.3)	-	10(14.7)
CL	68(100)	0(0)	46(67.6)	0(0)	22(32.4)
AMC	10(14.7)	-	4(5.9)	-	6(8.8)
VAN	21(30.9)	14(20.6)	16(23.5)	9(13.2)	10(14.7)
E	63(92.6)	5(7.4)	28(41.2)	2(2.9)	38(55.9)
CIP	24(35.3)	7(10.3)	16(23.5)	4(5.9)	11(16.2)
LEV	22(32.4)	8(11.8)	18(26.5)	2(2.9)	10(14.7)
PEF	24(35.3)	10(14.7)	20(29.4)	3(4.4)	11(16.2)
OFX	28(41.3)	9(13.2)	15(22.1)	5(7.4)	17(25)
GEN	25(36.8)	4(5.8)	5(7.4)	1(1.5)	23(33.8)
CXM	49(72.1)	12(17.7)	42(61.8)	3(4.4)	16(23.5)
CRO	49(72.1)	19(27.9)	45(66.2	5(7.4)	18(26.5)

Key: AMP= Ampicillin. CL= Cloxacillin. AMC= Augmentin. VAN= Vancomycin. E= Erythromycin. CIP= Ciprofloxacin. LEV= Levofloxacin. PEF= Pefloxacin. OFX= Ofloxacin. GN= Gentamicin. CXM= Cefuroxime. CRO= Ceftriaxone

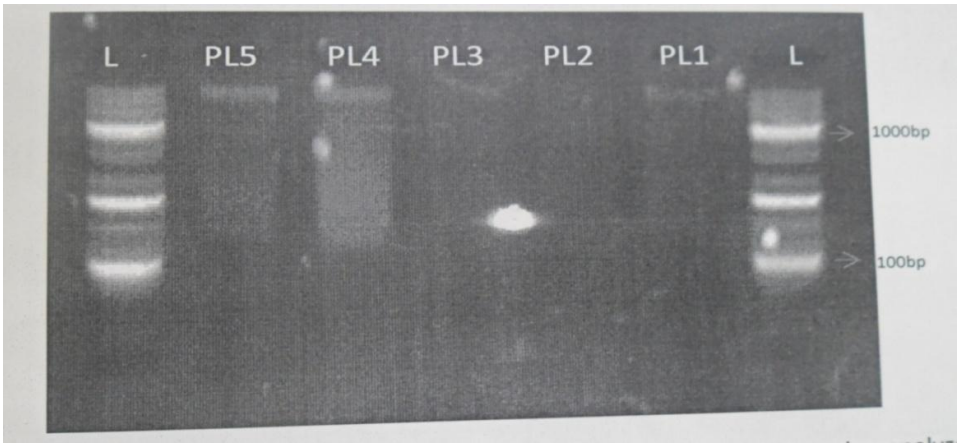

Plate 2. DNA plasmid profile of the first 5 of the isolates

Plasmid profiles of five multiple drug resistance Enterococcus species analyzed with 0.8% agarose gel electrophoresis. L is 100bp-1kbp ladder (molecular marker). Samples PL1, PL3, PL4 and PL5 are positive for plasmid genes with bands greater than 1000bp while sample PL2 is negative for plasmid genes. Keys: PL1 =Enterococcus faecium; PL2 = Enterococcus faecium; PL3 = Enterococcus faecium; PL4 = Enterococcus faecalis; PL5 = Enterococcus avium

The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi-square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to ciprofloxacin (Table 2).

Levofloxacin: 17 (43.6%) of *E faecium* were resistant to levofloxacin; 5 (20%) of *E. faecalis* to levofloxcin while 0 (0%) (None) of *E. avium* was resistant to Levofloxacin. The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi- square statistics and this revealed that there was significant difference ($p<0.05$) in their resistance to levofloxacin (Table 2).

Pefloxacin: 15 (38.5%) of *E. faecium* were resistant to pefloxacin; 7 (28.0%) of *E. faecalis* were resistant to pefloxacin; 2 (50%) of *E. avium* were resistant to Pefloxacin. The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi- square statistics and this revealed that there was significant difference ($p<0.05$) in their resistance to pefloxacin (Table 2).

Table 6. Pre-curing and post-curing antibiograms of the plasmid positive enterococcal isolates

ID no	Specie		CIP	PEF	LEV	OFX	CXM	AMC	CRO	CL	AMP	E	VAN	GN	MAR Index
PL 1	*E. faecium*	Pre	0^{R}	0^{R}	0^{R}	6^{R}	0^{R}	30^{S}	0^{R}	0^{R}	0^{R}	0^{R}	13^{R}	10^{R}	0.9
		Post	22^{S}	26^{S}	30^{S}	25^{S}	0^{R}	31^{S}	0^{R}	0^{R}	0^{R}	0^{R}	25^{S}	10^{R}	0.5
PL3	*E. faecium*	Pre	26^{S}	0^{R}	0^{R}	25^{S}	14^{R}	29^{S}	15^{I}	0^{R}	0^{R}	0^{R}	10^{R}	0^{R}	0.7
		Post	26^{S}	29^{S}	31^{S}	26^{S}	13^{R}	30^{S}	16^{I}	0^{R}	0^{R}	0^{R}	21^{S}	0^{R}	0.4
PL 4	*E. faecalis*	Pre	0^{R}	10^{R}	14^{R}	0^{R}	0^{R}	30^{S}	0^{R}	0^{R}	0^{R}	0^{R}	14^{R}	16^{S}	0.8
		Post	20^{I}	31^{S}	32^{S}	21^{S}	0^{R}	25^{S}	0^{R}	0^{R}	0^{R}	0^{R}	14^{R}	17^{R}	0.6
PL5	*E. avium*	Pre	20^{I}	0^{R}	0^{R}	0^{R}	15^{I}	25^{S}	0^{R}	0^{R}	0^{R}	0^{R}	20^{S}	10^{R}	0.7
		Post	21^{S}	26^{S}	19^{S}	7^{R}	16^{I}	34^{S}	0^{R}	0^{R}	0^{R}	0^{R}	21^{S}	9^{R}	0.5
PL 9	*E. faecalis*	Pre	0^{R}	20^{S}	0^{R}	20^{S}	0^{R}	30^{S}	10^{R}	0^{R}	0^{R}	0^{R}	13^{R}	0^{S}	0.8
		Post	32^{S}	22^{S}	25^{S}	25^{S}	0^{R}	28^{S}	10^{R}	0^{R}	0^{R}	0^{R}	15^{I}	0^{R}	0.5
PL 10	*E. faecalis*	Pre	0^{R}	0^{R}	0^{R}	0^{R}	0^{R}	28^{S}	0^{R}	0^{R}	0^{R}	0^{R}	15^{I}	10^{R}	0.8
		Post	22^{S}	31^{S}	22^{S}	27^{S}	0^{R}	35^{S}	0^{R}	0^{R}	0^{R}	0^{R}	22^{S}	11^{R}	0.5
CTL	ATCC 29212		25^{S}	20^{S}	20^{S}	19^{S}	20^{S}	25^{S}	24^{S}	18^{S}	18^{S}	26^{S}	20^{S}	18^{S}	0

Key: S = Sensitisve; R = Resistant; I = Intermediate; CTL = Control; MAR = Multiple antbiotic resistance. AMP= Ampicillin. CL= Cloxacillin. AMC= Augmentin. VAN= Vancomycin. E= Erythromycin. CIP= Ciprofloxacin. LEV= Levofloxacin. PEF= Pefloxacin. OFX= Ofloxacin. GN= Gentamicin. CXM= Cefuroxime. CRO= Ceftriaxone

Ofloxacin: 21 (53.8%) of *E. faecium* were resistant to Ofloxacin; 6 (24%) of *E. faecalis* were resistant to ofloxacin; 1 (25%) of *E. avium* were resistant to Ofloxacin. The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi- square statistics and this revealed that there was significant difference ($p<0.05$) in their resistance to ofloxacin (Table 2).

Aminoglycosides (Gentamicim): 15 (38.5%) of *E. faecium* were resistant to Gentamicin; 7 (28.0%) of *E. faecalis;* 3 (75%) of *E. avium* were resistant to Gentamicin. The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi- square statistics and this revealed that there was significant difference ($p<0.05$) in their resistance to Gentamicin (Table 2).

3.1.4 Cephalosporins (cephems)

Cefuroxime: 28 (71.7%) of *E. facium* were resistant to Cefuroxime, 20 (80.0%) of *E .faecalis* were resistant to Cefuroxime 2 (50%) of *E. avium* were resistant to Cefuroxime. The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi- square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to Cefuroxime (Table 2).

Ceftriaxone: 25 (64.1%) of *E. faecium* were resistant to ceftriaxone; 22 (88.0%) of *E. faecalis* were resistant to ceftriaxone while 2 (50%) of *E. avium* were resistant to ceftriaxone. The pre-curing antibiogram of the three *Enterococcus* species were compared using Chi- square statistics and this revealed that there was significant difference ($p<0.05$) in their resistance to ceftriaxone (Table 2).

3.1.5 Pre-curing antibiotic resistance of nosocomial isolates compared with community acquired isolates

β-lactams: (**Ampicillin and cloxacillin):** 41 (100%) of the nosocomial isolates were resistant to Ampicillin and Cloxacillin while 27 (100%) of the community acquired isolates were resistant to Ampicillin and Cloxacillin. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to ampicillin and cloxacillin (Table 3).

β-lactam-β-lactamase inhibitor combination (Augmentin): The nosocomial isolates registered low resistance to Augmentin. Only 8 (19.5%) of the nosocomial isolates were resistant to Augmentin. 2 (7%) of the community acquired isolates were resistant to Augmentin. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to augmentin (Table 3).

Glycopeptides (Vancomycin): 15 (36.6%) of the nosocomial isolates were resistant to vancomycinwhile 6 (22%) of the community acquired group were resistant to vancomycin. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to vancomycin (Table 3).

Macrolides (Erythromycin): 41 (100%) of the nosocomial isolates were resistant to Erythromycin while 22 (81.5%) of the community acquired isolates were resistant to Erythromycin. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to erythromycin (Table 3).

3.1.6 Fluoroquinolones

Ciprofloxacin: 16 (39.0%) of the nosocomial isolates were resistant to Ciprofloxacin while 8 (29.6%) of the community acquired group were resistant. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to ciprofloxacin (Table 3).

Levofloxacin: 17 (41.5%) of the nosocomial group were resistant to levofloxacin while 5 (18.5%) of the community acquired isolated were resistant. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was significant difference ($p<0.05$) in their resistance to levofloxacin (Table 3).

Pefloxacin: 16 (39.0%) of the nosocomial isolates were resistant to pefloxacin while 8 (29.6%) of the acquired isolates were resistant to

pefloxacin. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to pefloxacin (Table 3).

Ofloxacin: 20 (48.8%) of the nosocomial isolates were resistant to ofloxacin while 8 (29.6%) of the community acquired isolates were resistant to ofloxacin. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was significant difference ($p<0.05$) in their resistance to ofloxacin (Table 3).

Aminoglycosides (Gentamicin): 15 (36.6%) of the nosocomial isolates were resistant to Gentamicin while 10 (37.0%) of the community acquired isolates were resistant to Gentamicin. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to gentamicin (Table 3).

3.1.7 Cephalosporins

Cefuroxime: 39 (95.1%) of nosocomial isolates were resistant to Cefuroxime while 10 (37.0%) of the community acquired group were resistant to Cefuroxime. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was significant difference ($p<0.05$) in their resistance to cefuroxime (Table 3).

Ceftriaxone: 33 (80.5%) of the nosocomial group were resistant to Cefriaxone while 16 (59.3%) of the community acquired group were resistant to Ceftriaxone. The pre-curing antibiogram of nosocomial isolates was compared with community acquired isolates using Chi-square statistics and this revealed that there was no significant difference ($p>0.05$) in their resistance to ceftriaxone (Table 3).

3.1.8 Summary of post-curing antibiograms of the isolates

Penicillins (β-lactams): 58 (85.3%) of the isolates were resistant to ampicillin after curing of the isolates as shown in Table 4 while 10 (14.7%) were susceptible. 46 (67.6%) of the isolates were resistant to cloxacillin while 22 (32.4%) were susceptible.

β-lactam β-lactamase inhibitor: 4 (5.9%) of the isolates were resistant to Augmentin while 64 (94.1%) were susceptible.

Glycopeptides: 16 (23.5%) of the isolates were resistant to vancomycin 9 (13.2%) intermediate whereas 43 (63.3%) were susceptible

Macrolides: 28 (41.2%) of the isolates were resistant to Erythromycin, 2 (2.9%) intermediate and 38 (55.9%) susceptible.

Fluoroquinolones: 16 (23.5%) of the isolates were resistant to ciprofloxacin 4 (5.9%) were intermediate, whereas 48 (70.6%) of the isolates were susceptible. 20 (29.4%) of the isolates were resistant to pefloxacin 3 (4.4%) intermediate and 45 (66.2%) were susceptible. 15 (22.1%) of the isolates were resistant to pefloxacin, 5 (7.4) were intermediate, 48 (70.6%) were susceptible.

Aminoglycosides: 5 (7.4%) of the isolates were resistant to Gentamicin, 1 (1.5%) was intermediate whereas 62 (91.2%) were susceptible.

Cephalosporins: 42 (61.8%) of the isolates were resistant to cefuroxime, 3 (4.4%) intermediate and 23 (33.8%) susceptible 45 (61.2%) of the isolates were resistant to ceftriaxone, 5 (7.4%) intermediate and 18 (26.5%) susceptible.

3.1.9 Summary of DNA plasmid pre-curing and post curing analysis of the isolates as shown in Table 5

Penicillins (β-lactams): 10 (14.7%) of the Isolate were cured of the plasmid DNA. This meant that 14.7% of ampicillin resistance was plasmid mediated. 22 (32.4%) of the isolates were cured of cloxacillin resistance plasmid DNA.

β-lactam β-lactamase inhibitor: 6 (8.5%) of the isolates were cured of augmentin resistant plasmid DNA

Glycopetide: 10 (14.7%) of the isolates were cured of vancomycin resistance plasmid DNA.

Macrolides: 38 (55.9%) of the isolates were cured of erythromycin resistance plasmid DNA.

Fluoroquinolones: 11 (16.2%) of the isolates were cured of ciprofloxacin resistant plasmid

DNA. 10 (14.7%) of the isolates were cured of levofloxacin resistance plasmid DNA. 11 (16.2%) of the isolates were cured of pefloxacin resistant plasmid DNA. 17 (25%) of the isolates were cured of ofloxacin resistance plasmid DNA

Aminoglycoside: 23 (33.8%) of the isolates were cured of gentamicin resistance plasmid DNA.

Cephalosporins: 16 (23.5%) of the isolates were cured of cefuroxime resistance plasmid DNA while 18 (26.5%) were cured of ceftriaxone resistance plasmid DNA.

3.1.10 Pre-curing and post-curing antibiograms of the six plasmid positive enterococal isolates

The pre-curing and post-curing antibiograms of the six plasmid positive isolates were shown on Table 6. The identification numbers are PI1, PI3, PI4, PI5, PI9 and PI10 with CTL as control. These represent *E. faecium, E. faecalis, E. avium, E. faecalis* and *E. faecalis* respectively. The pre-curing multiple antibiotic resistance (MAR) index for the first isolate PI1 (*E. faecium*) was 0.9 while the post-curing MAR index was 0.5. The pre-curing multiple antibiotic (MAR) index for second isolate (*E. faecalis*) was 0.7 while the post-curing MAR index was 0.4. Generally the pre- curing MAR index ranged from 0.7 to 0.9 while the post-curing MAR index ranged from 0.4 to 0.6.

3.1.11 DNA plasmid profile of the representative isolates

Six of the 15 representative isolates selected on the basis of their high pre-curing antibiotic resistance for plasmid analysis with 0.8 agarose electrophoresis were positive for plasmid DNA (Table 7). Four (4) of the positive isolates (*E. faecium, E. faecium, E. faecalis,* and *E. avium*) had plasmid fragment of greater than 1000 bp while two (2) of them (*E. faecalis* and *E. faecalis*) had fragments of between 100 and 500 bp. The remaining nine (9) had no plasmid DNA. Plate 2 shows five isolates analysed with 0.8% agarose gel electrophoresis. Samples PL1, PL3, PL4 and PL5 were positive for plasmid genes with bands greater than 1000 bp while sample PLI2 was negative.

Plate 3 shows five isolates analysed with 0.8 agarose gel electrophoresis. Samples PL9 and PL10 were positive for plasmid genes with bands between 100 and 500bp while samples PL6, PL7 and PL8 were negative for plasmid genes. Plate 4 shows five isolates analysed with 0.8 % agarose gel electrophoresis. Samples PL11, PL12, PL13, PL19 and PL24 were negative for enterococcal plasmid genes.

Pathogenicity factors: Virulent determinants demonstrated with the enterococcal isolates during the study are displayed on Table 8.

Haemolysin: Of the thirty nine (39) *E. faecium* isolates, twenty six (26) were positive for haemolysin while thirteen (13) were negative. Of the twenty five (25) *E. faecalis* isolates, seventeen (17) were positive for haemolysin while eight (8) were negative. Of the four (4) *E. avium* isolates, one (1) was positive while three (3) were negative. In total, 44 (64.7%) of the isolates were positive for haemolysin while 34 (35.3%) were negative.

Table 7. DNA plasmid profile of the representative enterococcal isolates

Isolate id	Names of isolates	Plasmid fragment
PL1	*E. faecium*	>1000bp
PL2	*E. faecium*	Nil
PL3	*E. faecium*	>1000bp
PL4	*E. faecalis*	>1000bp
PL5	*E. avium*	>1000bp
PL6	*E. faecium*	Nil
PL7	*E. faecium*	Nil
PL8	*E. faecium*	Nil
PL9	*E. faecalis*	Between 100 and 500bp
PL10	*E. faecalis*	Between 100 and 500bp
PL11	*E. faecalis*	Nil
PL12	*E. faecalis*	Nil
PL13	*E. avium*	Nil
PL19	*E. avium*	Nil
PL24	*E. avium*	Nil

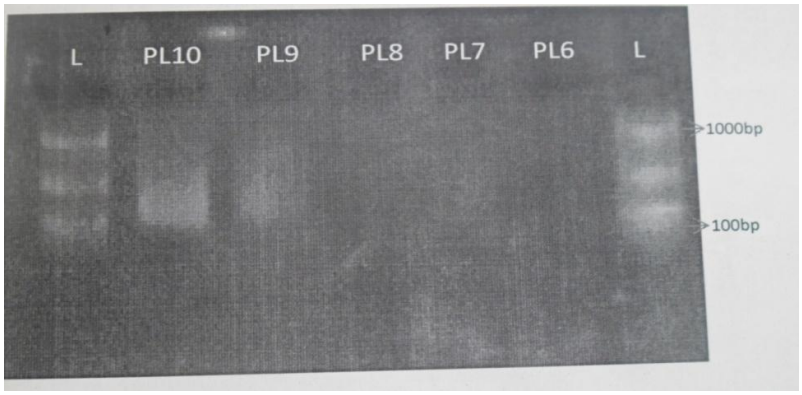

Plate 3. DNA plasmid profile of the second 5 of the enterococcal isolates

Plasmid profiles of five multiple drug resistance Enterococcus isolates analyzed with 0.8% agarose gel electrophoresis. L is 100bp-1kbp ladder (molecular marker). Samples PL9 and PL10 are positive for plasmid genes with bands greater than 200bp while samples PL6, PL7 and PL8 are negative for plasmid genes. Key: PL6 =Enterococcus faecium; PL7 = Enterococcus faecium; PL8 = Enterococcus faecium; PL9 = Enterococcus faecalis; PL10 = Enterococcus faecalis

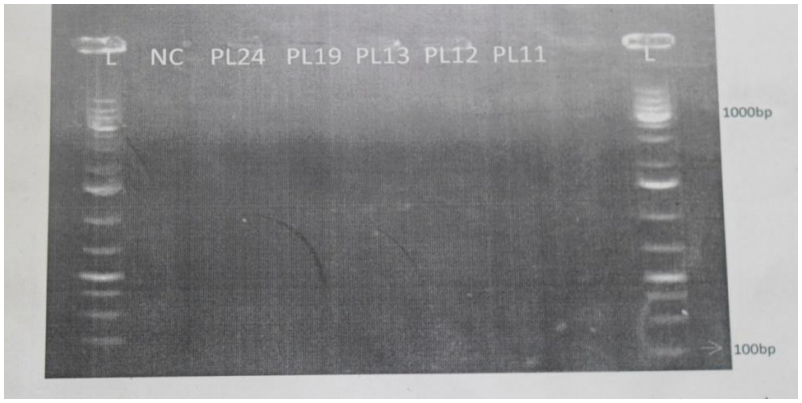

Plate 4. DNA plasmid profiles of 5 of the enterococcal isolates

Plasmid profiles of five multiple drug resistant Enterococcus isolates analysed with 0.8% agarose gel electrophoresis. L is 100 bp-1 kbp ladders (molecular marker). Samples PL11, PL12, PL13, PL19 and pl24 were negative for enterococcal plasmid genes. Keys: PL11 =Enterococcus faecalis; PL12 = Enterococcus faecium; PL13 = Enterococcus avium; PL19 = Enterococcus avium; PL24 = Enterococcus avium

Table 8. Virulent determinants of the enterococcal isolates

Virulent factors	*E. faecium* (n=39)		*E. faecalis* (n=25)		*E. avium* (n=4)		Total (%)
Haemolysin	Positive	26	Positive	17	Positive	1	44 (64.7)
	Negative	13	Negative	8	Negative	3	24 (35.3)
Gelatinase	Positive	2	Positive	25	Positive	0	27 (39.7)
	Negative	37	Negative	0	Negative	4	41 (60.3)
Caseinase	Positive	25	Positive	10	Positive	2	37 (54.4)
	Negative	14	Negative	15	Negative	2	31 (45.6)
Lipase	Positive	20	Positive	21	Positive	1	42 (61.8)
	Negative	19	Negative	4	Negative	3	26 (38.2)
MSCRAMM-ACE	Positive	2	Positive	1	Positive	1	4 (5.9)
	Negative	37	Negative		Negative	3	64 (94.1)
β-lactamase	Positive	19	Positive	14	Positive	2	35 (51.5)
	Negative	20	Negative	11	Negative	2	33 (48.5)

Gelatinase: Of the thirty nine (39) *E. faecium* isolates, two (2) were positive while thirty seven (37) were negative. The twenty five (25) isolates of *E. faecalis* were positive for gelatinase. The four (4) *E. avium* isolates were negative for gelatinase. In total, 27 (39.7%) of the isolates were positive for gelatinase while 41 (60.3%) were negative.

Caseinase: Of the 39 *E. faecium* isolates, 25 were positive for caseinase while 14 were negative. Of the 25 isolates of *E. faecali*s, 10 were positive for caseinase while 15 were negative. Of the 4 *E. avium* isolates, 2 were positive while 2 were negative. In total, 37 (54.4%) of the isolates were positive for caseinase while 31 (45.6%) were negative.

Lipase: Of the 39 *E. faecium* isolates 20 were positive for lipase while 19 were negative. Of the 25 isolates of *E. faecalis*, 21 were positive while 4 were negative. Of the 4 *E. avium* isolates, 1 was positive while 3 were negative. In total, 42 (61.2%) were positive for lipase while 26 (38.8%) were negative.

Microbial surface component recognizing adhesive matrix molecule adhesin of collagen from enterococci MSCRAMM ACE: Of the thirty nine (39) *E. faecium* isolates, two (2) were positive for MSCRAMM ACE while thirty seven (37) were negative. Of the twenty five (25) *E. faecalis* isolates, one (1) was positive while twenty four (24) were negative. Of the four (4) *E. avium* isolates, one (1) was positive for MSCRAMM ACE while three (3) were negative. In total, 4 (5.9%) of the enterococcal isolates were positive for MSCRAMM-ACE while 64 (94.1%) were negative.

β-lactamase production: β-lactamase enzyme was detected in 19 out of the 39 isolates of *E, faecium* while 20 were negative. Of the 25 isolates of *E. faecalis,* 14 were positive for β-lactamase while 11 were negative. Of the 4 isolates of *E. avium* 2 were positive for β-lactamase while 2 were negative. In total, 35 (51.5%) were positive for β-lactamase production while 33 (48.5%) were negative.

4. DISCUSSION

4.1 Prevalence of Vancomycin Resistant Enterococci

The prevalence of vancomycin resistant *Enterococcus* (VRE) in this study was 3.3% whereas its percentage among the isolates was 30.9%. This is corroborated by the report of Fisher and Philips [2] that in the last three decades, particularly virulent strains of *Enterococcus* that were resistant to vancomycin (vancomycin resistant *Enterococcus* or VRE) have emerged in nosocomial infections of hospitalized patients. The seriousness of this situation will be clearer with the work of Bearman and Winzel, [25] in United Kingdom which demonstrated that the risk of death from vancomycin-resistant enterococci (VRE) is 75%, compared with 45% for those infected with a susceptible strain.

4.2 Antibiotic Susceptibility Patterns of the Isolates

68(100%) of the isolates were resistant to the penicillins (β-lactams) in this work which were ampicillin and cloxacillin. The cephalosporins showed low level of activity against the isolates. 49 (72.5%) of the isolates were resistant to cefuroxime, 12 (17.7%) were intermediate while 7 (10.3%) were susceptible. 48 (70.6%) of the isolates were resistant to ceftriaxone 19 (27.9%) were intermediate while 1 (1.5%) was susceptible. The isolates were highly resistant to the macrolides (erythromycin). 63 (92.6%) of the isolates were resistant to erythromycin 5 (7.4%) were intermediate while none was susceptible. This agreed with the work of David et al. [26] who reported resistance to erythromycin to be 73.8% and cloxacillin 84.5%. These findings also agreed with the report of Calva et al. [27] who observed the resistance of enterococci to erythromycin. In summary, the pre-curing antibiogram showed that the isolates were completely resistant to ampicillin and cloxacillin (β-lactams), almost completely resistant to erythromycin (aminoglycoside), cefuroxime and ceftriaxone (cephalosporins). This is in accordance with the report of Ryan and Ray [1] which stated that some enterococci are intrinsically resistant to some β-lactam-based antibiotics (some penicillin and virtually all cephalosporins) as well as many aminoglycosides. β-lactam-β-lactamase inhibitor which was represented by augmentin, exhibited a high level of activity on the isolates. 10 (14.7%) of the isolates were resistant to augmentin while 56 (85.3%) were sensitive to augmentin. 21 (30.9%) of the isolates were resistant to vancomycin while 14 (20.6%) were intermediate and 33 (48.5%) were susceptible.

The fluoroquinolones were averagely active against the isolates 24 (35.3%) of the isolates

were resistant to ciprofloxain, 7 (10.3%) were intermediate while 37 (54.4) were susceptible 22 (32.4%) of the isolates were resistant to levofloxacin, 8 (11.8%) were intermediate while 38 (55.8%) were susceptible. 24 (35.3%) of the isolates were resistant to pefloxacin, 10 (14.7%) were intermediate while 34 (50.0%) were susceptible. 28 (41.3%) of the isolates were resistant to ofloxacin, 9 (13.2%) were susceptible. This agreed with the work of David et al. [26] who reported that resistance to fluoroquinolones ranged between ofloxacin (33.3%), pefloxacin (36.3%), norfloxacin (31.9%), ciprofloxacin (35.6%), levofloxacin (44.7%) and sparfloxacin (39.3%). Aminoglycosides (Gentamicin) also exhibited average activity against the isolates. 25 (36.8%) of the isolate were resistant to the gentamicin, 4 (5.8%) were intermediate while 39 (57.4%) were susceptible. David et al. [26] also reported that out of 568 *E. faecalis* strains isolated and tested for susceptibility 445 (78.3%) showed resistance to tetracycline, 420 (73.9%) to erythromycin, 457 (80.5%) to amoxicillin and 254 (44.7%) to gentamicin and that the highest and the least resistances were observed against cloxacillin and vancomycin with 84.5% and 17.43% respectively. He concluded that isolates were resistant to most antibiotics commonly used in clinical practice. Resistance to most antibiotics is very likely because the genes encoding resistance to these antimicrobials may be located on the same plasmid [28].

4.3 Antibiotic Resistant Profile of the Three *Enterococcus* Species

Penicillins: It is noted that all the three *Enterococcus* species isolated in this study were resistant to the penicillins evaluated. This has probably got to do with the presence of β-lactamase enzyme in the isolates and other resistance mechanisms. β-lactamase enzyme is an enzyme that breaks the β-lactam ring of the Pencillins (β-Lactams), thus rendering them ineffective against the organisms.

β-lactam-β-lactamase inhibitor combination (augmentin). The isolates in this study were found to register low resistance against augmentin. This is a result of the presence of β-lactamase inhibitor which prevents the β-lactamase produced by the isolates to break the β-lactam ring of the antibiotic.

Vancomycin: *E. faecium* was found to be averagely resistant to vancomycin (41%). *E. faecalis* has low resistance (16%) while *E. avium* also has low resistance (25%). The vancomycin resistance of *E. faecalis* (16%) and *E. avium* (25%) was in line with the report of David et al. [26] which recorded a low average vancomycin resistance of 17.4%

Erythromycin: The resistance of the isolates to Erythomycin was marked; *E. faecium* (94.9%), *E. faecalis* (92%); *E. avium* (75%). This is in accordance with the report of David et al. [26] which recorded 73.9% resistance to erythromycin.

4.4 Fluoroquinolones

Ciprofloxain: this study showed an average resistance of the isolates to Ciprofloxacin; *E. faecium* (35%); *E. faecalis* (32%); *E. avium* (50%). This is in line with the report of David et al. [26] which recorded 35.6% resistance to ciprofloxacin.

Levofloxacin: *E. faecium* had an average resistance of 43.6%; *E. faecalis* had 20% resistance to levofloxacin and isolates of *E. avium* were not resistant to levofloxacin. **Pefloxacin:** *E. faecium* had 38.5% resistance to pefloxacin. *E. faecalis* had 28% resistance to pefloxacin and *E. avium* had 50% resistance to pefloxacin.

Ofloxacin: The resistance of *E. faecium* to ofloxacin was high (53.8%) but *E. faecalis* and *E. avium* registered low resistance 24% and 25% respectively.

Gentamacim: Resistance to gentamicin by *E. faecium* was 38.5%. *E. faecalis* 18% and *E. avium* 75%.

Cephalosporins:

Cefuroxime: *E. faecium* and *E. faecalis* registered high resistance of 71.7% and 80.0% respectively while *E. avium* registered low resistance of 25% to cefuroxime.

Ceftriaxone: The resistance of the three species to Ceftriaxone was high; *E. faecium* (64.0%); *E. faecalis* (88.1%); *E. avium* (50%). This is in line with the report of Oni *et.al*. [29].

4.5 Precuring Antibiotic Resistance of Nosocomial Isolates and Community Acquired Isolates

The degree of resistance to some routine antibiotics used in this study by the enterococcal

isolates from hospital acquired group was significantly higher than that shown by the community group. Such routine antibiotics include augmentin, levofloxacin, ofloxacin and cefuroxime. Others that showed no significant differences were ampicillin, cloxacillin, vancomycin, erythromycin, ciprofloxacin, pefloxacin, gentamicin and ceftriaxone. However, a high sensitivity of 60% and above was observed in augmentin, vancomycin, ciprofloxacin, levofloxacin peloxacin ofloxacin and gentamicin. The antibiotics sensitivity profile in this study goes a long way to describe the degree of drug abuse and misuse of common routine antibiotics in our society. In addition, continuous exposure of bacteria to routine antibiotics used in the hospital consequently leads to development of resistant strains [29].

4.6 Multiple Antibiotic Resistance (MAR) Index

This is a measure of the response of isolates to an array of antibiotics. This is calculated as the ratio of the number of antibiotics to which the isolate is resistant to the total number of antibiotics to which the isolate is evaluated for susceptibility [30]. The higher the MAR index, the more multiple antibiotic resistant the isolate is.

The pre-curing multiple antibiotic resistance (MAR) index for *E. faecium* was 0.9 while the post-curing MAR index was 0.5. The pre-curing multiple antibiotic (MAR) index for *E. faecalis* was 0.7 while the post-curing MAR index was 0.4. Generally the pre- curing MAR index ranged from 0.7 to 0.9 while the post- curing MAR index ranged from 0.4 to 0.6. The precuring MAR index in this study is outrageous compared with the work of Osundiya et al. [30] whose MAR index of *Pseudomonas* and *Klebsiella* was 0.4. This has confirmed the fears that have been expressed as regards the intrinsic resistance and acquisition of resistance factors by bacteria that may result to the emergence of super bugs which may resist all available antibiotics. Of great concern is the ability of vancomycin resistant enterococci to transfer vancomycin resistance to other bacteria (including methycillin resistant *Staphylococcus aureus*) [1].

4.7 Plasmid Detection

DNA plasmids were detected in 40% of the representative isolates with DNA fragments (molecular sizes) ranging from >100bp to 1000bp. This is in line with the report of Marcinek et al. [31] that enterococci are known to acquire antibiotic resistance plasmids with relative ease and are able to spread these resistance genes (plasmids) to other species.

4.8 Pathogenicity Factors

Given the importance of *Enterococcus* as a pathogen and increasing prevalence of multiple drug resistant *Enterococcus* as shown by this study, the identification of virulent factors associated with invasiveness and disease severity has become an important subject for research. Five pathogenicity factors (virulent determinants) and β-lactamase were demonstrated with the enterococcal isolates during the study.

Haemolysin: Twenty six (66.7%) of the thirty nine (39) *E. faecium* isolates were positive for haemolysin while thirteen (33.3%) were negative. Seventeen (68%) of the twenty five (25) *E. faecalis* isolates were positive for haemolysin while eight (32%) were negative. One (25%) out of the four (4) *E. avium* isolates was positive while three (75%) were negative. In total, 44 (64.7%) were positive for haemolysin while 24 (35.3%) were negative. Haemolysin is a cytotoxic protein capable of lysing human, horse and rabbit erythrocytes and haemolysin producing stains are found to be associated with increased severity of infection [22].

Gelatinase: Two (5.1%) of the thirty nine (39) *E. faecium* isolates were positive for gelatinase while thirty seven (94.9%) were negative. The twenty five (100%) isolates of *E. faecalis* were positive for gelatinase. The four (100%) *E. avium* isolates were negative for gelatinase. Totally, 27 (39.7%) were positive for gelatinase while 41 (60.3%) were negative. Gelatin producing strains of enterococci have been shown to contribute to the virulence of endocarditis in an animal model [32]. Vergis et al. [33] showed that 64% of *E. faecalis* isolates from patients with bacteraemia produced gelatinase. Some enterococcal strains (45-68%) produce gelatinase which is an extracellular zinc containing metalloproteinase [8]. Gelatinase can hydrolyse gelatin, collagen. Fibrinogen, casein, haemoglobin and other bioactive peptides [9]. It is also responsible for inflamed pulps and periapical lesions in oral infection [8]. Gelatinase has played an important role in the pathogenicity of most pathogenic bacteria. The enzyme has been associated with disease progression due to

its cytotoxic and tissue destructive potential and inhibitory effects on phagocytes [10]. Gelatinase production and activity are higher in clinical than faecal isolates from healthy volunteers [11].

Caseinase: This is extracellular enzyme that catalyzes the hydrolysis of casein, a protein found in milk. Aside supporting the multiplication of the infecting bacteria, caseinase acts as an effective activator of haemolysin which in turn causes the haemolysis of erythrocytes of infected man and other animals [34].

Twenty five (64.1%) of the thirty nine (39) *E. faecium* isolates were positive for caseinase while fourteen (35.9%) were negative. Ten (40%) of the twenty five 25 isolates of *E. faecalis* were positive for caseinase while 15 (60%) were negative. Two (50%) of the four (4) *E. avium* isolates were positive while two (50%) were negative. Totally, 37 (54.4%) of the isolates were positive for caseinase while 31 (45.6%) were negative.

Lipase: This is an exoenzyme that hydrolyzes the lipid triacylglycerol. The most prominent role of this enzyme is digestion of host extracellular lipids for nutrient acquisition which results in sticking to the host tissue and neighbouring cells [35]. This enhances adhesion by degrading host surface molecules thereby liberating new receptors. Additionally, released free fatty acids (FFA) increases unspecific hydrophobic interactions. The biological role of lipase in infection by many organisms is considered the most important step in bacterial infections [36].

Twenty (51.3%) of the thirty nine (39) *E. faecium* isolates were positive for lipase while 19 (48.7%) were negative. Twenty one (84%) of the twenty five (25) isolates of *E. faecalis* were positive for lipase while 4 (16%) were negative. This agrees with the work of Marcia et al. [37] who demonstrated that 71.8% of *E. faecalis* presented lipolytic activity. One (25%) of the four (4) *E. avium* isolates was positive while three (75%) were negative. Totally, 42 (61.2%) were positive for lipase while 26 (38.8%) were negative.

Microbial surface component recognizing adhesive matrix molecule adhesin of collagen from enterococci (MSCRAMM ACE): Ace is a collagen binding MSCRAMM on enterococci and is structurally and functionally related to staphylococcal Cna adhesion [38]. Its presence among both commensal and pathogenic isolates of *E. faecalis* is apparently expressed during infections in humans [39]. Employing anti Ace antibodies, Ace was detected in 90% of enterococcal endocarditis patients' sera samples suggesting that Ace is expressed in vivo [38].

Two (5.1%) of the thirty nine (39) *E. faecium* isolates were positive for MSCRAMM ACE while thirty seven (94.9%) were negative. One (4%) of the twenty five (25) *E. faecalis* isolates was positive while twenty four (96%) were negative. This is not in consonance with the report of Marcia et al. [37] who showed that 40.6% of *E. faecalis* caused agglutination of rabbit erythrocyte. One (25%) of the four (4) *E. avium* isolates was positive for MSCRAMM ACE while three (75%) were negative. In total, 4 (5.9%) of the enterococcal isolates were positive for MSCRAMM-ACE while 64 (94.1%) were negative.

β-lactamase production: β-lactamase (also known as penicillinase) is an enzyme produced by some bacteria which has the ability to break the β-lactam ring of β-lactam antibiotics such as penicillins and cephalosporins, deactivating the molecule's antibacterial property. β-lactamase enzyme was detected in 19 out of the 39 isolates of *E. faecium* while 20 were negative. Of the 25 isolates of *E. faecalis,* 14 were positive for β-lactamase while 11 were negative. Of the 4 isolates of *E. avium* 2 were positive for β-lactamase while 2 were negative. In total β-lactamase was detected in 35 (51.5%) of the isolates. This result is not in line with the finding of Rahangdale et al. [40] who reported that strains of enterococci that produce β-lactamase are rare. The implication is that more of the enterococci now produce β-lactamase enzyme which helps them to resist penicillins and cephalosporins.

Development of some mechanisms like inhibition of action of virulence factors and β-lactamase or plasmid curing (removal) may provide an alternate method of therapy in the face of antimicrobial resistance.

5. CONCLUSION

It was observed that the prevalence of *Enterococcus* sp. was high and showed multiple drug resistance. It is therefore, advised that more attention should be given to this organism especially VRE.

Adequate antibiotic policy should be articulated and enforced to forestall the emergence of resistant strains and outbreak of the infection. It is recommended that antibiotic sensitivity be obtained before initiation of most antibiotic treatments. The benefits of antibiotic prophylaxis should be thoroughly weighed against the impending resistance to be encountered in the long run. This policy will not only encourage proper treatment of patients but will discourage the indiscriminate use of antibiotics and prevent further development of resistant strains of the bacteria.

CONSENT

Only patients who gave their written consent were recruited for the study.

ETHICAL APPROVAL

Ethical approval was obtained from ESUT Teaching Hospital, Parklane and University of Nigeria Teaching Hospital, Ituku/Ozalla.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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