Original Research Article

Antibiotic susceptibility testing, plasmid detection and curing of clinically isolated *Enterococcal species*

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ABSTRACT

Vancomycin resistant enterococci (VRE) are becoming a major concern in medical practice worldwide. Their increased prevalence and their ability to transfer vancomycin resistance to other bacteria have made them a subject of close scrutiny and intense investigation. The isolates of *Enterococcus* sp. were subjected to antibiotic susceptibility testing before curing. The three *Enterococcus* species exhibited different ~~A~~ntibiotic 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.

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Keywords: *Enterococci,* antibiotic susceptibility, DNA, plasmid detection, pathogenicity.

INTRODUCTION

An important feature of the genus *Enterococcus* is the high level of intrinsic antibiotic resistance. Some enterococci are intrinsically resistant to some β-lactam-based antibiotics (some penicillins and virtually all cephalosporins) as well as many aminoglycosides [1]. Between 1989 and 2009, particularly virulent strains of *Enterococcus* that are resistant to vancomycin (vancomycin resistant *Enterococcus* or VRE) have emerged in nosocomial infections of hospitalized patients especially in the United States of America [2].

Vancomycin resistance is acquired when a sensitive *Enterococcus* acquires a plasmid that confers resistance to vancomycin. The new strain is called vancomycin resistant *Enterococcus* (VRE). One concern is that VRE appears able to transfer vancomycin resistance to unrelated bacterial species such as methicillin resistant *Staphylococcus aureus*. In addition, VRE organisms are usually resistant to more than one antibiotic [2]. VRE can also spread from person to person and are increasing problems in hospitals and chronic health care facilities. Approximately 30% of all enterococcal infections are caused by vancomycin resistant strains ~~[3].~~ *Enterococcus faecalis* and *Enterococcus faecium* are the most prevalent species cultured from humans; accounting for more than 90% of clinical isolates [3]. Other enterococcal species known to cause human infections include: *Enterococcus avium, Enterococcus gallinarum, Enterococcus casselflavus, Enterococcus durans, Enterococcus raffinosus* and *Enterococcus mundtii* ~~[2].~~ *E. faecium* represents most vancomycin resistant *Enterococcus* (VRE) [2].

The acquisition of vancomycin resistance by enterococci has seriously affected the treatment and infection control of these organisms. 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 ~~[4].~~ In 1988, these gene clusters first were reported in enterococcal strains. vanA is carried on a transposon Tn 1546 that is almost always plasmid mediated [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].

The virulence of enterococci is lower than that of organisms such as *Staph. Aureus* [6]. The risk factors for mortality associated with enterococcal bacteraemia include severity of illness, patient's age and use of broad spectrum antibiotics [7]. 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 in enterococcal infections are higher in clinical than faecal isolates from healthy volunteers [11].

Vancomycin-dependent *enterococcus* (VDE)

The first documented strain of vancomycin-dependent *Enterococcus* (VDE) was isolated in 1993 from the urine of a 46-year-old woman at Thomas Jefferson University Hospital in Philadelphia, Pennsylvania [12]. Since this first isolate was reported, twenty additional cases of VDE have been reported worldwide, including both *E. faecalis* and *E. faecium* strains. Although the clinical significance of VDE remains unclear, an outbreak of VDE in a bone marrow transplant unit at Johns Hopkins University in 1999 was reported [13] and demonstrates its potential for becoming a clinically significant pathogen.

Numerous studies have shown, using pulsed-field gel electrophoresis, that these strains exclusively evolved from vancomycin-resistant strains [14]. This continued global spread of resistant organisms and the creation of new highly virulent pathogens from transfer of

resistance genes underscore the importance of infection control and prevention, active surveillance and use of appropriate antibiotics.

Another mechanism of resistance to antibiotics by enterococci is biofilm formation. Biofilm is a population of cells attached irreversibly on various biotic and abiotic surfaces, and encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids [14]. Biofilm formation is a complex developmental process involving attachment and immobilization on a surface, cell-to-cell interaction, microcolony formation, formation of a confluent biofilm, and development of a three-dimensional biofilm structure [15]. Bacteria in a biofilm behave differently from their free-floating (planktonic) counterparts. The regulation of bacterial gene expression in response to cell population density, called quorum sensing, is accomplished through the production of extracellular signal molecules called autoinducers [16]. Biofilm production is regulated by quorum sensing systems in several bacterial pathogens. Biofilms are notoriously difficult to eradicate and are a source of many chronic infections. According to the National Institutes of Health, biofilms are medically important, accounting for over 80% 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 resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts [17]. Bacteria in biofilms colonize a wide variety of medical devices, such as catheters, artificial cardiac pacemakers, prosthetic heart valves and orthopaedic appliances, and are associated with several human diseases, such as valve endocarditis, burn wound infections, chronic otitis media with effusion and cystic fibrosis [18].

MATERIALS AND METHODS

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.

Ethical approval: Ethical approval was obtained from ESUT Teaching Hospital, Parklane and University of Nigeria Teaching Hospital, Ituku/Ozalla. Only patients who gave their informed consent were recruited for the study.

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.

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.

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 (5µg), pefloxacin (5 µg), levofloxacin (5µg) and ofloxacin (5µg)
- **Cephalosporins (cephems)**: cefuroxime (30 µg) and ceftriaxone (30µg)
- **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].

Plate 3: Antibiotic susceptibility patterns of Enterococcus sp isolated during this study

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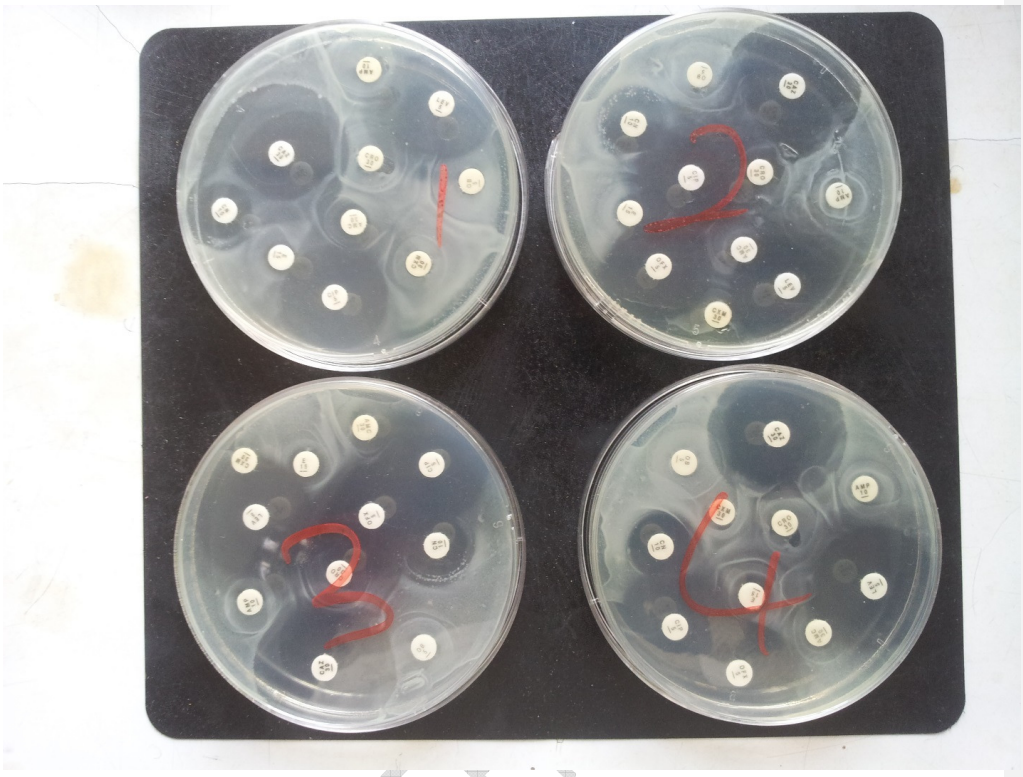

Determination of Multiple Antibiotic Resistance (MAR) Index

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.

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 6 isolates of *E. faecium*; 5 isolates *E. faecalis* and 4 isolates of *E. avium*. The isolates were subjected to bacterial cultures for plasmid profile analysis.

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Curing of plasmid DNA

The curing (elimination) of the resistant plasmids of the enterococci isolated was done using sub-inhibitory concentration of 0.10mg/ml of acridine orange as described by Akortha and Filgona [21]. Isolates were grown for 24hrs at 37°C 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).

Comment [N11]: 0.10 mg/ml

Comment [N12]: 24 hrs

The pathogenicity factors of the isolates

Comment [N13]:

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].

Detection of β-Lactamase Production

Comment [N14]:

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.

Statistical analysis of results

The results obtained from this work were analyzed statistically using Student t-test and Chi-square of computer program SPSS version 18 to show significant different.

Results

Susceptibility testing, plasmid detection and curing

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.

Comment [N15]: 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.

Comment [N16]: 68 (100%)

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.

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.

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.

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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- 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)

Comment [N18]: *E. faecalis* (italic)

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)

Comment [N19]: .

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. 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)

Comment [N20]: .

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)

Comment [N21]: .

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)

Comment [N22]: .

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)

Comment [N23]: .

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)

Comment [N24]: .

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)

Comment [N25]: .

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)

Comment [N26]: .

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.

Comment [N27]: In explanation, CRO: ceftriaxone must be written

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).

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).

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).

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.

Comment [N28]: In explanation, CRO: ceftriaxone must be written

Summary of post-curing antibiograms of the isolates

Penicillins (β-Iactams): 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. 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.

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.

Comment [N29]: In explanation, CRO: ceftriaxone must be written

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.

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 Pl1, Pl3, Pl4, Pl5, Pl9 and Pl10 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 Pl1 (*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.

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

Comment [N30]: .

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.

Comment [N31]: In explanation, CRO: ceftriaxone must be written

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

Comment [N32]: .

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.

DNA plasmid profile of the representative isolates

Six of the fifteen (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 4 shows five isolates analysed with 0.8% agarose gel electrophoresis. Samples PL1, PL 3, PL 4 and Pl5 were positive for plasmid genes with bands greater than 1000 bp while sample Pl l2 was negative.

Comment [N33]: ???

Plate 5 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 6 shows five isolates analysed with 0.8 % agarose gel electrophoresis. Samples PL 11, PL 12, PL13, PL 19 and PL 24 were negative for enterococcal plasmid genes.

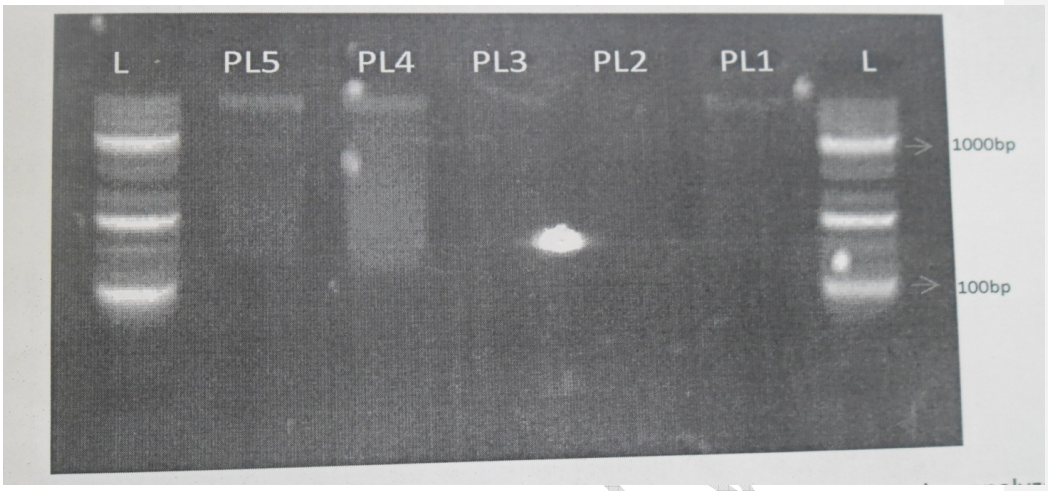
Table 7: DNA plasmid profile of the representative enterococcal isolates

Comment [N34]: .

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 4: DNA plasmid profile of the first 5 of the isolates

Comment [N35]: .



Plasmid profiles of five multiple drug resistance *Enterococcusspecies* 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 1200bp while sample PL2 is negative for plasmid genes.

Comment [N36]: *Enterococcus* species

Keys

PL1 =*Enterococcus faecium.*

PL2 = *Enterococcus faecium.*

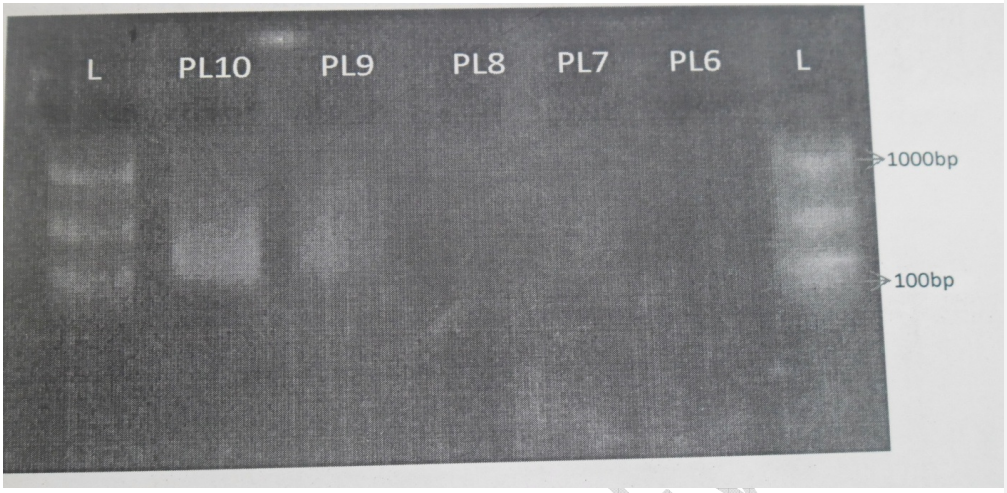
PL3 = *Enterococcus faecium.*

PL4 = *Enterococcus faecalis.*

PL5 = *Enterococcus avium.*

Plate 5: DNA Plasmid profile of the second 5 of the enterococcal isolates

Comment [N37]: .



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.*

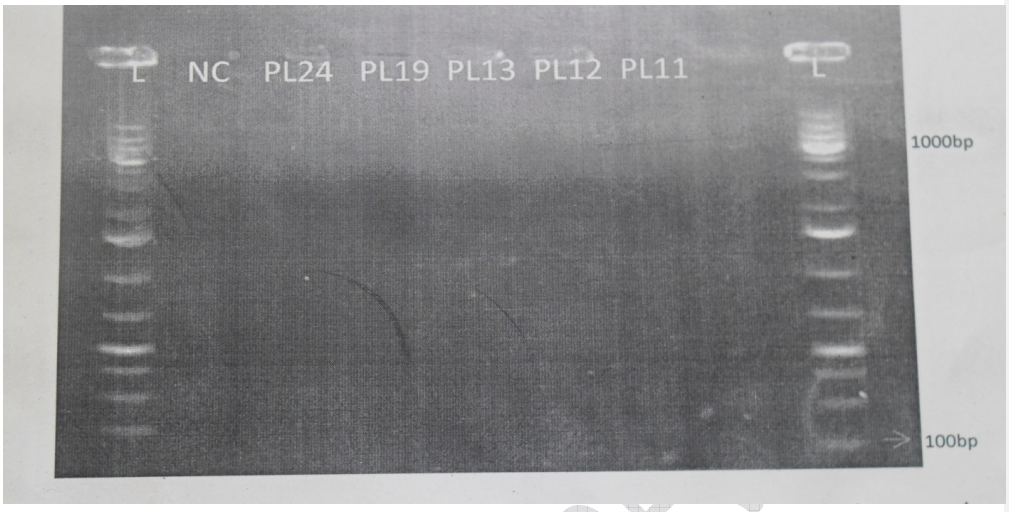
PL8 = *Enterococcus faecium.*

PL9 = *Enterococcus faecalis.*

PL10 = *Enterococcus faecalis.*

Plate 6: DNA Plasmid profiles of 5 of the enterococcal isolates

Comment [N38]: .



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.

Comment [N39]: PL24

Keys

PL11 =*Enterococcus faecalis.*

PL12 = *Enterococcus faecium.*

PL13 = *Enterococcus avium.*

PL19 = *Enterococcus avium.*

PL24 = *Enterococcus avium.*

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

Comment [N40]: E

Comment [N41]: .

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

Comment [N42]: .

Caseinase: Of the 39 *E. faecium* isolates, 25 were positive for caseinase while 14 were negative. Of the 25 isolates of *E. faecalis*, 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.

Table 8: Virulent determinants of the enterococcal isolates

Comment [N43]: .

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)

DISCUSSION

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.

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].

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.

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.

Comment [N44]: p

Comment [N45]: p

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.

Comment [N46]: o

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

Comment [N47]: g

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*. (2003).

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].

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].

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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.

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 [32].

Gelatinase: Two (5.1%) of the thirty nine (39) *E. faecium* isolateswere 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 [33]. Verges *et al.* [34] 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].

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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 [35].

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 [36]. 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 [37].

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*. [38] 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.

Comment [N51]: There is no **Marcia** in the reference section.

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 [39]. Its presence among both commensal and pathogenic isolates of *E. faecalis* is apparently expressed during infections in humans [40]. Employing anti Ace antibodies, Ace was detected in 90% of enterococcal endocarditis patients' sera samples suggesting that Ace is expressed in vivo [39].

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*. [38] 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.* [41] 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.

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.

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