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Prevalence, antibiotic susceptibility pattern and risks of multiple drug resistant *Enterococcus* species in Ojo, Lagos, Nigeria.

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Abstract: Enterococci are part of the normal flora in human and animals, as well as occurring naturally in both treated and untreated water as an emerging pathogen of the urinary tract. A total of 300 urine samples were collected aseptically from outpatients in Ojo, Lagos, which were investigated using standard microbiological methods. Out of the 300 samples examined, 195(65%) had bacterial isolates. Of these, 165 (84.6%) were positive for Enterococci, 18 (9.2%) were *Streptococcus pyogenes* and 12 (6.2%) were *Staphylococcus aureus*. *Enterococcus faecalis* was the most prevalent with 117 (70.9%), followed by *Enterococcus faecium* 33 (20%) while *E. dispar* 9(5.5%) and *E. durans* (3.6%) were the least recovered. Antibiotic susceptibility pattern was determined by Kirby Bauer disk diffusion method. Seventy two (70.5%) *E. faecalis* were resistant to Erythromycin, 63 (61.7%) to Tetracycline, 18 (17.6%) to Ampicilin, 10 (9.8%) to Vancomycin, 8 (7.8%) to Ciprofloxacin and 3 (2.9%) to Norfloxacin respectively. A generally higher susceptibility was shown by the *E. faecium* as against *E. faecalis* in this study. Ten of the *E. faecalis* isolates were Vancomycin resistant Enterococci (VRE). Eighteen of the 135 Enterococci tested produced biofilm, 12 of which *E. faecalis* and 6 were *E. faecium*. Both VRE and biofilm producers showed high multiple resistance to most of the antibiotics tested. This study reveals that multiple antibiotic resistance among Enterocococci appears to be emerging in the study area, thus antimicrobial susceptibility testing of enterococcal isolates should be available before prescription of antibiotics in order to promote rational drug use.

Keywords: Enerococococci, *Enterococcus faecalis*, *Enterococcus faecium*, Urinary tract infection, Antibiotic susceptibility testing**.**

(1) Introduction

Enterococci are natural parts of the intestinal flora in humans which are ubiquitously present in the soil, plants, vegetables and in both treated and untreated water. Enterococci are gram positive bacteria, widely distributed among the natural and digestive tract of human and animal. They are important pathogens of urethral infection, soft tissue infection, sepsis and meningitis (Sun e*t al.*, 2019) Enterococci are constituents of the gut microflora in a number of animal species including humans although species of *Enterococcus* cause serious infections in immunocompromised and hospitalized patients (Lebreton *et al.*, 2014). The *Enterococcus* species of greatest importance to human health are *E.feacalis* and *E. faecium,* each of which causes a variety of infections including urinary, soft tissue and blood stream infections (Kang and Song, 2013) Enterococci exert dual functions both as commensals and as pathogens. When inside the body they are well adapted to an ecologically complex niche in the gut, genitourinary tract and oral cavity (Jett *et al.*, 1994).

Drug resistance is the main reason for the dramatic emergence of Enterococci as a cause of healthcare associated infections throughout the world. Antimicrobial resistance is one of the primary factors contributing to the morbidity or deaths with infections caused by *Enterococcus* species (Gilmore 2002). *Enterococcus* populations can increase relative to other bacteria because they are resistant to a number of commonly used antimicrobials such as cephalosporins in response to antimicrobial therapy (Adukhari *et al.,* 2018)

Treatment of enterococcal infections has been complicated by the emergence of strains possessing a high level of resistance to almost all the antibiotics used in clinical settings especially aminoglycosides, β-lactams and glycopeptides. Multidrug resistance by *Enterococcus* species to a wide spectrum of antibiotics in different parts of the world have been associated vancomycin resistance, biofilm formation and biofilm

production which has been rarely reported in Nigeria (Anvarijenad *et al.*,2017; Agegne *et al.,*2018; Angadi *et al.,* 2018; Sun *et al.*, 2019) Enterococci have gained resistance to almost the entire antimicrobial spectrum used against this organism (Assadollahi *et al* 2018). The study was designed to determine the prevalence, diversity, antimicrobial resistance, vancomycin resistance and biofilm formation of enterococci isolated from urine samples from patients in Ojo, Lagos, Nigeria.

(2) Materials and Methods

Urine samples were obtained from patients attending the Lagos State University Health Center, Ojo and Ojo Primary Health Center. A total of 300 urine samples 150 each from males and females were collected in Ojo which constitutes one of the 20 Local Governments Areas in Lagos, located near Badagry in the Western flank of Lagos with a population of 941,523, an area of 182 km square, density of 6,866 km square. Demographic data which included age, sex, educational status, marital status, religion among others were collected and written on each sample bottle. A set of questionnaire was designed to effectively cover the required data. Samples were collected between June and December, 2019.

Institutional ethical clearance was obtained and samples were collected after informed consent of the subjects.

Three urine samples were collected in sterile urine bottles aseptically by qualified laboratory personnel from patients. The samples were kept in an iced park and taken to the laboratory, if sample was not immediately analyzed. The samples were kept in the refrigerator at 4°C and the sample was always analyzed within 24 hours of collection.

Three urine swabs were examined from each patient. Two swabs for isolation of bacteria and wet mount microscopy were obtained. The sterile cotton-tipped swab was moistened with normal saline before sample collection. For the isolation of bacteria from collected specimens, the microbiological media used were blood, chocolate, MacConkey, cysteine, lactose electronic deficient (**CLED**) agars which were incubated for 16-24 hours at 37°C. Representative bacteria colors recovered after incubation were sub-cultured on blood agar plates which were incubated at 35°C in the presence of 5% CO_2 for 25 hours (Semedo-Lemsaddek *et al.,* 2016).

The identification and characterization methods for the bacteria were carried out by standard procedures. Gram staining and cell morphology from air-dried heat fixed smears were performed. The motility of the isolates was carried out by hanging drop (**HD**) technique. Further characterization was carried out by different biochemical diagnostic tests including Gram positive, catalase negative positive bile esculin (bile insolubility) test growth in 6.5% NaCl broth. Final identification of different species of *Enterococcus* was conducted by fermentation of specific sugar, glucose, lactose, mannitol, arabinose, sorbitol, sucrose and raffinose (*Desai et al.,* 2001).

The bacterial isolates were subjected to antibiotic sensitivity testing in Mueller-HInton agar by Kirby-Bauer Standard disk diffusion method. The inoculated plates were incubated for 16-18 hours in ambient air incubators at 35°C and the results were recorded by mean zone of inhibition according to the CLSI, 2014. *Enterococcus* isolates were tested for susceptibility to vancomycin (30μg), erythromycin (15μg), ampicillin (10μg), linezolid (30μg), ciprofloxacin (5μg) nitrofurantoin (30μg) and gentamicin (120μg). *E. faecalis* ATCC 29212 was used as quality control.

All enterococcus isolates were examined for vancomycin susceptibility by agar incorporation Ten μl of a 0.05 McFarland bacterial suspension (final concentration=10^6 degree (CFU/Ml) was spotted on the brain heart infusion (BHI) agar (Merck, Germany) containing 6μg/Ml vancomycin allowed to air dry for about 5 minutes and incubated at 35^{0}C (CLSI, 2014). Culture plates were examined at 24 and 48 hours of incubation for any discernible growth.

All the enterococci isolates were checked for biofilm production by the procedures of Mabarez *et al* (2013). Freshly sub-cultured strains of *Enterococcus* on blood agar plates were inoculated in 1ml of brain heart infusion [BHI] broth with 1% glucose and incubated at 37^{0}C for 24 hours. To 180μl of fresh BHL medium, 20μl of 24 hours old bacterial growth was added which corresponded to a turbidity of 0.5 McFarland standard, 200 μl of the suspension of the bacterial isolates and the control strain (*E. faecalis* ATCC 29212) were inoculated into flat bottom microtiter plates in duplicates and incubated at 37 ^{0}C in 5% CO_2 for 24 hours. After incubation, the contents of the plates were removed, tapped and washed three times with phosphate buffer saline. The biofilm was fixed by adding 150μl of methanol for 20 minutes. It was air-dried for about 30 minutes in an inverted position and later stained with 0.1% crystal violet for 15 minutes. Excess stain was removed and plates were washed with distilled water.150μl of 33% acetic acid was added in each well and kept for 30 minutes without shaking. The optical density (OD) was measured at 570nm. Based on the OD values, the isolates were categorized as strong biofilm formers (OD_{570}) but <2, medium (OD_{570}>1 but <2) weak (OD_{570}>0.5<1), and non-biofilm formers (OD_{570} ≤0.5) (Mohammed *et al.,* 2007).

Microsoft Excel, Microsoft word (version 8.1) and SPSS 16 package program were used for statistical analysis. Chi square test was used for

categorical variables and P values < was considered as significance.

(3) Results

The total bacterial isolates was 195, Enterococci were the most prevalent bacteria and constituted 165 (84.6%), 18 (9.2%) were *Streptococcus pyogenes* and 12 (6.2%) were *Staphylococcus aureus* (Figure 1)

Various *Enterococcus* species isolated were *E. faecalis* (117*), E faecium* (33), *E.dispar* (9) and *E. durans* (6) (Figure 2).

About 71.1% of the Enterococci were recovered from mates. The highest prevalence of Enterococci was from age group 21-30, the illiterates and those with primary education and the single participants had high prevalence of enterococci (Table 1). In the same vein, the males within age group 30-40, the illiterates and the married participants harbour red more Enterococci (Table 2).

Of the 102, *E. faecalis* and 33 *E. faecium* isolates tested against the commonly available antimicrobial agents, 60 (58.8%) *E. faecalis* and 20 (19.6%) *E. faecium* were found apparently resistant to 2 or more antimicrobial agents. Specifically, 72 (70.5%) *E. faecalis* were resistant to erythromycin, 63 (61.7%), 18 (17.6%), 10 (9.8%), 8 (7.8%) and 3 (2.9%) to tetracycline, vancomycin, ciprofloxacin, ampicillin and norfloxacin respectively. A generally higher susceptibility was shown by the *E. faecium* as against *E. faecalis* to the antibiotics tested (Table 3).

Ten (9.8%) of the *E. faecalis* isolates were vancomycin resistant Enterococci (VRE) from patients in the 31-40 age group. Seven vancomycin resistance patterns were observed (Table 4).

Among the 135 Enterococci constituting 102 *E. faecalis* and 33 *E. faecium* tested for biofilm production, 18 (13.3%) were biofilm producers, 12 (11.8%) were E. faecalis and 6 (18.2%) were *E. faecium*. Ten (55.6%) of the biofilm formers (6 E. faecium and *4 E. faecalis*) were strong biofilm producers. Six of the *E. faecium* were resistant to cotrimoxazole, ampicillin, vancomycin, gentamycin and streptomycin while only three *E. faecalis* showed multidrug resistance to cotrimoxazole and ampicillin

among other antibiotics tested. *E. faecium* isolates showed higher multiple resistance than the *E. faecalis* However, 117 (86.7%) isolates were not biofilm formers and they were both sensitive and resistant strains of *E. faecalis* (n=90) and *E. faecium* (n=29).
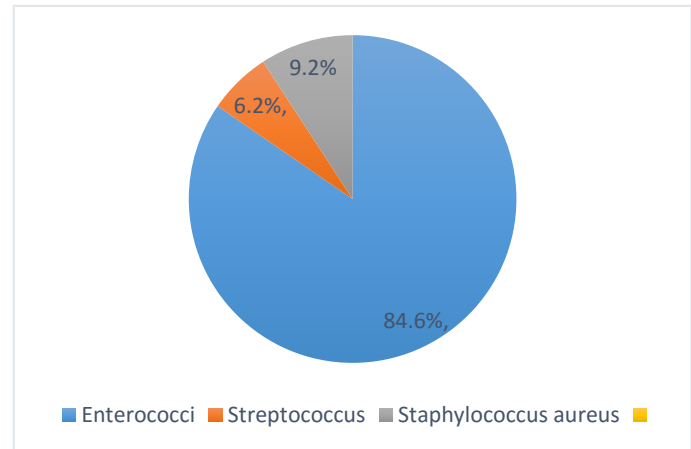

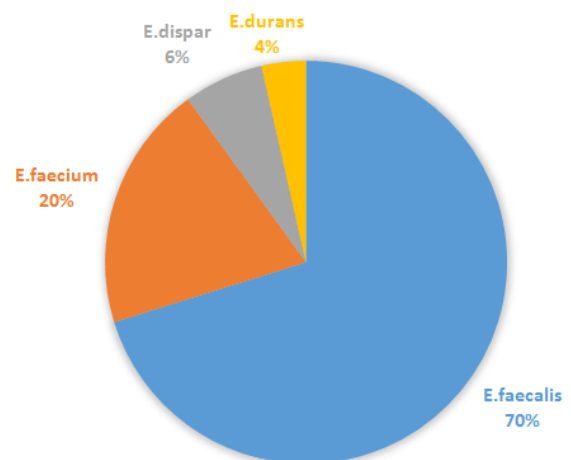**Figure 1:** Distribution of bacterial isolates from urine samples.

Figure 2: Occurrence of *Enterococcus* spp. in urine

Table 1: Socio-demographic characteristics of subjects and prevalence of Enterococcal infection Socio-demographic characteristics

Sex	Frequency	Enterococci present (%)	Enterococci absent (%)
Male	150	111(71.1)	54(32.7)
Female	150	54(28.8)	111(67.2)
Age in years			
10-20	63	56(88.9)	07(11)
21-30	198	88(44)	110(56)
31-40	39	21(15.5)	18(13.3)
Educational status			
Illiterate	80	70(87.5)	10(12.5)
Primary school	55	50(90.9)	5(9.1)
Secondary school	120	30(25)	90(75)
Above high school	45	15(33.3)	30(66.7)
Marital status			
Single	70	65(92.9)	5(7.1)
Married	130	52(40)	78(60)
Divorced	70	27(38.6)	43(61.4)
Widowed	30	21(70)	9(30)

Table 2: Socio-demographic characteristics of the study participant with other bacterial isolates.

Socio-demographic frequency	*Streptococcus pyogenes* (%)	*Staphylococcus aureus* (%)
Sex		
Male 15	12(66.7)	3(25)
Female 15	6(33.3)	9(75)
Age in years	n=18	n=12
10-20 0	-	-
21-30 12	6(50)	6(50)
31-40 18	12(667)	6(33.3)
Educational status		
Illiterate 12	8(66.7)	4(33.3)
Primary school 8	5(62.5)	3(37.5)
Secondary school 6	3(50)	3(50)
Above high school 4	2(50)	2(50)
Marital status		
Single	4(66.7)	2(33.3)
Married 14	8(57.1)	6(42.9)
Divorced 7	5(71.4)	2(28.6)
Widowed 3	1(33.3)	2(66.7)

Table 3: Antibiotic susceptibility pattern of bacterial isolates in urine

Antibiotics	*Enterococcus faecalis* n=102 R (%) I% S (%)	*Enterococcus faecum* n=33 R% I% S%
Erythromycin	72(70.5) 9(8.8) 21 (20.5)	24(72.2) 0(0) 9(27.2)
Tetracycline	63(61.7) 0(0) 39(38.2)	21(63.6) 0(0) 12(36.3)
Ciprofloxacin	18(17.6) 6(5.9) 78(76.5)	18(54.5) 0(0) 15(45.5)
Norfloxacin	3(2.9) 0(0) 99(97.0)	0(0) 0(0) 33(100)
Ampicillin	8(7.8) 0(0) 94(92.2)	0(0) 0(0) 33(100)
Gentamycin	0(0) 0(0) 102(100}	0(0) 0(0) 33(100)
Nitrofurantoin	0(0) 0(0) 102(100)	0(0) 3(9.0) 30(90.9)
Vancomycin	10(9.8) 0(0) 92(90.2)	0(0) 0(0) 33(100)
Teicoplanin	0(0) 0(0) 102(100)	0(0) 0(0) 33(100)
Linezolid	0(0) 0(0) 102(100)	0(0) 0(0) 33(100)

Key: R=Resistant, I= Intermediate, S=Sensitivity

Table 4: Profile of multidrug resistant pattern of VRE (n=10) among patient studied

Resistant rate	Combination of antibiotics	Numbers of isolates tested
R_1	VAN,TET,CIP	2
R_2	VAN,AMP,ERY	1
R_3	VAN,TET,ERY,CIP	2
R_4	VAN,ERY,NOR,CIP	2
R_5	VAN,ERY,TET,AMP,CIP	1
R_6	VAN,CIP,AMP,TET,NOR,ERY	2
Total		10

ERY, Erythromycin, TET, Tetracycline, CIP, Ciprofloxacin, NOR, Norfloxacin, AMP, Ampicillin, VAN, Vancomycin, R, Resistance, R_1- R_7 = Number of antibiotic resistant isolate from 1-7 respectively, MDR=organisms resistant to ≥ 2 antibiotics.

(4) Discussions

Enterococci are part of the human and animal intestinal flora which has emerged as community acquired pathogens and a leading cause of hospital acquired infections. Enterococcus is one of the significant pathogen affecting all age groups. Therefore, nosocomial infections with enterococci are a major concern of many hospitals in the world (Shridhar and Dhanashree, 2019). Speciation and antibiotic susceptibility testing are necessary to detect the emergence and changing pattern of drug resistance among pathogens including *Enterococcus*.

In the present study, Enterococci were the most common microorganism isolated of the UTI constituting 84.6% of the total bacterial isolates. *Streptococcus pyogenes* and *Staphylococcus aureus* accounted for 9.2% and 6.2% respective which were also gram positive. The overall prevalence of colonization by Enterococci was at 65%. These findings disagree with studies by Amin *et al* (2009) and Setu *et al* (2016) that gram negative bacteria were the most commonly recovered microorganisms in urinary tract infections Ndubuisi *et al* (2017), however, recovered several enterococcal species from patients and hospital environment in Abuja. The similarities and differences in the type and distribution of uropathogens may be due to different environmental conditions and host. Others reasons may include healthcare and education programmes, socioeconomic standards as well as hygiene practices in the different countries. The media and procedures used in the studies can also lead to variation and differences in the bacterial isolates. Of the 165 Enterococci recovered, 117(70.9%) of which were *E. faecalis* while 33(20 %) were *E faecium*. Males accounted for 71.1% of the Enterococci. These findings are in line with 63% prevalence reported in Ethiopia by Agagne *et al* (2018) and Jahansepas *et al* (2018). A study in India reported 66% prevalence of Enterococci in urine samples while Adukhari *et al* (2018) reported 61.6 % prevalence in Nepal. A lower prevalence of Enterococci causing UTIs has also been reported in by Angadi *et al* (2018).

Generally, Enterococci have been implicated in approximately 10% of all UTIs. Ndubuisi *et al* (2017) reported a high prevalence of *Entrococcus* spp. from stool and urine from hospital environment in Abuja, Nigeria. Enterococci are the second most common cause of nosocomial urinary tract and wound infections and third most common nosocomial bacteremia. A high prevalence of *E faecalis* (57.8%) and *E faecium* (23.5%) was reported by Ndubuisi *et al* (2017). This result is comparable to previous work on *Enterococcus* spp. in other parts of the world where both prevalences of *E. faecium* and *E. faecalis* were 62 % (Olawale *et al* (2011) reported 5.9% prevalence of Enterococci in Osun State, Nigeria. Azza *et al* (2013), however, reported more *E faecium* than *E faecalis.* Mendiratta *et al* (2008) isolated more *E faecalis* than *E faecium* in India. The variation in prevalence of Enterococci in these studies may be due to differences in sample size, isolation techniques, types of patient used or non-use of selective enterococcal media. The finding in this study was lower than 82.83% reported in Egypt. According to Pawar and Malik (2019) in India and abroad, *E faecials* and *E faecium* are the two common species causing infections in human being as other enterococci are infrequently isolated even human infections (caporyszewka *et al*., 2018).

The occurrence of Enterococci was more frequent in the 10-20, 21-30, age groups while *Streptococcus pyogenes* and *S. aureus* had higher prevalence within 21-30 and 31-40 age ranges. The illiterates and those with primary education had more enterococcal infections and people within the same age group and educational status had non-enterococal bacteria. The single and married had more enterococcal infections as well as staphylococcal and streptococcal infections. The findings on age, educational status and marital status is in line with reports by Anvarijenad *et al* (2017) in their study on diabetics with 58.8% males and 44.2% with lower

education as having enterococcus infections. This suggests that patients in the above category with other associated infections are at high risk of enterococcal infections. Arias and Murray (2015) reported that VRE is fast becoming a major cause of health care-associated UTIS, accounts for 15% of all catheter-associated urinary tract infections, (CAUTIs) and are more common in men in association with recurrent UTIs, previous antibiotic treatments, indwelling catheters, instrumentation and abnormalities of the genitourinary tract. The results of antimicrobial susceptibility testing showed that most of the antibiotics tested were effective against the *Enterococcus* species. All enterococci were sensitive to gentamycin, nitrofurantoin, teicophanin and linezolid. Both *E faecalis* and *E faecium* showed very high resistance to erythromycin and tetracycline but mild resistance to ciprofloxacin, ampicillin, vancomycin and norfloxacin. Similar findings have been reported by Angadi *et al* (2018) but a contrasting prevalence of antibiotic resistance by Anvarijenad *et al* (2017). The finding in our study may be due to the fact that the *Enterococcus* spp. had been rarely treated with the antibiotics tested as most of the antibiotics are not available over the counter, so are likely not to be abused. Knowledge of the causative microorganisms and their antimicrobial susceptibility profiles is essential for appropriate treatment and infection eradication. Knowledge of the characteristics of infection can help in choosing an appropriate antibiotic, even if the culture report is being awaited at the time of initiation of antibiotic therapy (Poorabbas *et al.,* 2015; Anvarijenad *et al*., 2017). It can as well be due to differences in treatment regimens used for patients in different healthcare settings. The vancomycin resistance pattern of the enterococcal isolates showed low prevalence of multi-drug resistant enterococcal infection among the emergence of VRE. This had been attributed to excessive and indiscriminate use of broad spectrum antibiotics, imprudent use of antibiotics. Agegne *et al* (2018) reported that vancomycin resistance can be due to colonization pressure and non-compliance with the infection control measures.

The 7.4 % VRE encountered in this study is lower than that 81.8%, 50% and 16.9% of *E mendtu, E. faecium* and *E. faecalis* respectively in Lagos reported by Iregbu *et al*., 2002. Agegne *et al* (2018) reported an overall prevalence of 7.7% of VRE in Ethiopia which is consistent with our finding. From this study*, E. faecium* was susceptible but mildly resistant to most antibiotics including vancomycin as it had been reported to be responsible for most VRE infections (Ndubusi *et al*., 2017). In a multicenter study conducted in United States, vancomycin resistance was detected in 10% of *E. faecalis* and

vancomycin resistance was detected, in 76.9%, linezolid resistance was found in 15% *E. faecium* strains. The ten VRE showed *Enterococcus* 6 resistant patterns. In our study, 100% susceptibility to linezolid was observed in all the *Enterococcus* spp. tested (gentamycin, nitrofurantoin and teicoplanin). The low prevalence of multidrug resistant enterococcal infection among the subjects may result from regulated and careful use of broad spectrum antibiotics. Most of these antimicrobials are not sold over the counter so are not readily available to patients in Nigeria. The emergence of VRE may be due to imprudent use of vancomycin by pharmacists and patient medicine sellers, the colonization pressure and non-compliance with standard infection control measures (Mukherjee *et al* 2016). The divergence in VRE prevalence might be due to variation in the study population and personal habits like animal contact which was supported by Bekele and Ashenah (2009) that reported 100% VRE from feces of chicken and cattle in Ethiopia.

In this study, 13.3% of enterococcal isolates produced biofilm which included 18.2% *E. faecium* and 11.8% of *E. faecalis*. Shridar and Dhanashree (2019) reported 21.9% biofilm producers which included 27.5% *E. faecium* and 17.7% *E faecalis,* and stated that the method used for the detection of biofilm and the origin of the isolate will influence biofilm formation. It was shown from the study that all biofilm producing *Enterococcus* species were multidrug resistant. Shridar and Danashree (2019) reported an association of biofilm and presence of *esp* gene which could not be done in our study as it was part of the limitations of this study. The high prevalence of biofilm production by *E. faecium* and *E. fae calis* suggests that biofilm production enhances the pathogenicity and virulence of enterococci. This finding agrees with Kafil and Mobarez (2015) that *E. faecalis* have genetic determinants mediating antibiotic resistance within biofilms and *E. faecalis* employs biofilm specific mechanisms and not the simple extracellular matrix diffusion barrier to keep antibiotics away from their targets. The same mechanisms may as well be applied by *E faecium,* which has been reported to show more resistance to antimicrobial agents (Shridhar and Dhanashree, 2019). Attempts should be made by health authorities to determine the prevalence of colonization with VRE, biofilm formers, biofilm producers and identify the associated risk factors in healthcare setting as a mandatory preventive measure.

Conclusion:
A high prevalence of Enterococci in urine was found in this study. The results of the antibiotic susceptibility test showed that both *E. faecium* and *E.*

faecalis were generally susceptible to the antibiotics tested while a few were resistant. Vancomycin resistant Enterococci (VRE) as well as biofilm producing and biofilm formers which were resistant to the most commonly used broad spectrum antibiotics were encountered which occurrence has been rarely reported in Nigeria. The changing pattern of *Enterococcus* species as a causative agent of clinical infection should be considered especially with the unusual emergence with high level of resistance particularly in the developing countries.

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