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ENTEROCOCCUS ISOLATES IN CLINICAL SAMPLES FROM IN-PATIENTS IN UYO, NIGERIA

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Abstract*: In this study, the prevalence of *Enterococci* from 3 clinical samples (wound, urine and stool) of patients attending University of Uyo Teaching Hospital and Anua General Hospital and their virulence factors were investigated. A descriptive cross-sectional study design was adopted and data obtained were analyzed statistically. Two hundred samples, including Urine, Wound and Stool were obtained and cultured by standard methods. Isolates obtained were tested for their antimicrobial susceptibilities and the presence of virulence factors. Isolates obtained were *Enterococcus faecalis* (56.7%), *Enterococcus faecium* (26.5%), *Enterococcus durans*(10.5%) and *Enterococcus gallinarium*(5.3%). The prevalence of *Enterococci species* in the clinical samples from the two hospitals was 9.5% of which more isolates were obtained from University of Uyo Teaching Hospital (11.4%) than Anua General Hospital (6.5%). The difference in the prevalence of *Enterococci* isolates among the subjects in the two hospitals was statistically significant (9.5%). The isolates showed high resistance to Erythromycin (89.5%), Gentamycin (84.2%) and Vancomycin (68.4%) while the susceptibility to Ampicillin and Aztreonam was seen with increasing frequency. The virulence factors detected among *Enterococci* species were haemolysin and biofilm formation. The production of biofilm in *Enterococcus faecalis* (63.6%) were comparatively higher than *Enterococcus faecium* (60.0%), also the production of hemolysin were comparatively higher in *E. faecalis* (81.7%) than *E. faecium* (60.0%). The presence of these pathogens in patients and elaboration of virulence factors indicates a possible outbreak of nosocomial infections by these pathogens in hospital settings; hence, there is need for regular monitoring of these pathogens in hospital laboratories to curb the emergence of these infections.

Keywords*: *Enterococci, Antimicrobial suceptibility, Virulence factors.

1. INTRODUCTION

Enterococci are gram positive cocci bacteria that are normally present in the human intestine and in the female genital tract and are often found in the environment. Though they were believed to be harmless commensals for many years with no medical significance, they have emerged recently as one of the most common nosocomial pathogen (Lopez *et al.*, 2006). *Enterococci* are part of the normal flora or organisms found in the intestine of humans and animals. They have been long recognized as important human pathogen and are becoming increasingly so. The genus *Enterococcus* includes more than seventeen (17) species (Patel *et al*., 2000), although only a few cause clinical infection in humans (Lopez *et al*.,

2006). *Enterococci exist* as commensals, in harmony with gut flora, and the dynamics of the host commensals relationship like antibiotics treatment, host injury, diminished immunity; allow these intestinal bacteria to gain access to extra intestinal host sites to cause infection. The nosocomial *Enterococci* might have extra capacities to colonize, overgrow, and invade host tissue. This poses an emerging threat to patients' safety. Since the beginning of the antibiotic era, they have posed major therapeutic challenges, including the need for synergistic combination of antibiotics to successfully treat *Enterococcal* infective endocarditis (IE). In 2003, fifteen years after the first public report in 1988 of clinical strains of *Enterococci* in Nigeria as one of the major cause of hospital acquired infections. The organism in the last three years has become very resistant to many antimicrobial agents and is one of the major causes of hospital acquired infections. Recently, the National Nosocomial Surveillance System (NNSS) predicted that the percentage of *Enterococci* isolates exhibiting resistance in Nigeria will be on the increase. A study of the prevalence rate of Enterococciand the virulence expressed by this organism in hospital settings in Uyo, South-South Nigeria, is required especially as records of such studies are not available. This pathogen can cause important nosocomial epidemics and can increase morbidity, mortality and cost of management of the disease. Therefore, this study was carried out to determine the prevalence and virulence factors of *Enterococci* species isolates from urine, wound and stool samples of patients attending Secondary and Tertiary Hospitals in Uyo, South-South Nigeria, as such records of prevalence of *Enterococci* species are not available in the state in other to compare with the 23% prevalence rate of *Enterococci* species from urine, wounds and stool samples as obtained from Oshogbo, South -Western Nigeria,(Agarwal *et al* 2009). And also access its antimicrobial profile, so as to suggest possible antibiotic for its treatment in order to reduce the risk of nosocomial infections, such as urinary tract infection, Endocarditis and bacteremia caused by *enterococci* species.

2. MATERIALS AND METHOD

Study Area

This research is a descriptive cross sectional study that was carried out over a period of six months in two health-care hospitals located in Uyo, which has latitude of 5.038963 and a longitude of 7.909470 in AkwaIbom State, Nigeria. The two highly patronized hospitals by patients offer general medical, surgical as well as gynecological and obstetrics services.Informed consent was obtained from each subject and ethical approval of the two hospital management was gotten before the conduct of the study.

Study Population

The study population comprise of patients on admission due to various infections including urinary tract infection, wound infection and post-operative surgical infections.

Sample Collection

Patients were given a sterile universal container and instructed on how to aseptically collect clean catch mid-stream urine and stool samples, while the wound samples was collected with the use of sterile swab stick. The collected samples were transported using a cold bag to the Microbiology laboratory of the University of Uyo Teaching Hospital within 2 hours of collection for processing (culture, isolation and biochemical identification of *Enterococci)* following recommended techniques and procedures.

Data Collection

All inpatients were interviewed using a standard structured questionnaire to obtain relevant information.

Laboratory Analysis

Isolation and Identification of *Enterococci* Isolate

Samples were inoculated on Cystein Lactose Deficiency Agar, and Bile-Esculin agar prepared according to the manufacturers instruction and incubated at temperature of 37^0C. Characteristics based on Grams staining, Catalase test, Salt tolerance test, AtimicrobialSuceptibility test, Bile-Esculin test,Virulence factor detection and conventional biochemical test were determined using standard microbiological techniques (Collins 1989; 6 Facklam and Teixeira, 1998).

Salt Tolerance Test for Identification of *Enterococci*

Brain heart infusion broth was prepared according to manufacturer's prescription, supplementedwith 6.5%Nacl and 0.2g of phenol red; it was sterilized at 1150C for 15mins. Ten mls of sterile water was transferred in to bijou's bottle and sterilized at 1150C for 15 mins, after sterilization, 0.1g of dextrose was inoculated into it and steamed for 30minutes. Five mls of the BHI broth was transferred into five different bijou's bottle properly labeled with five different suspected Enterococci isolates from five different clinical samples comprising of wound, urine and stool. 0.2mls of the sugar solution was transferred into the broth. About 2-3 colonies from an overnight culture were aseptically inoculated into the salt tolerance broth. It was inoculated aerobically for 24hours at 370C. There was the presence of an obvious bacterial growth (turbidity in the liquid medium) with a change in color from purple to yellow.

Bile-Esculin Test for the Identification of *Enterococci* Species

With an inoculating wire or loop, about 2-3, morphologically similar *enterococci* colonies was touched and inoculated and streaked on the surface of a bile-esculin plate for isolation.The inoculated plates were incubated at 370C for 24hours and the results were determined.

Black haloes were observed around isolated colonies and any blackening is considered positive for *enterococci* in bile esculin test. Two drops of 3% of freshly prepared hydrogen peroxide (H202) was placed on a grease free slide with the aid of a sterile glass rod, The Test Organism in 24hours old was transferred onto the slide and observed immediately for gas bubles, which indicates a positive reaction and non-production of gas bubbles, which indicates a negative result. This procedure was repeated for some of the samples that produce growth when comprise of urie, wound and stool, and since *enterococci* is catalase negative.

Antimicrobial Susceptibility Test

Colonies of similar appearance to the test were touched with a sterile wire loop and emulsified in 5mls of sterile physiological saline in a bijou bottle. The innoculum in the bottles was incubated for 3-4hours and the turbidity adjusted to match that of McFarland standard. A swab against the slide of the tube above the level of the suspension. This was used to inoculate already prepared Mueller Hinton medium by streaking the swab evenly over the surface of the medium in three directions, rotating the plate approximately 600c to ensure even distribution. The petri dish was allowed for 3-5minutes for the petri dish to dry. The petri dish was allowed for 3-5 minutes for the petri dish to dry. Appropriate antimicrobial disc was placed on the inoculated plate 25mm apart using a sterile forceps. The plate was inverted and incubated aerobically overnight at 370C . After overnight incubation the plate was examined by measuring the diameter of zone sizes of each antimicrobial agent using a ruler and the result interpretated following the CLSI guidelines and interpretative criteria (CLSI, 2014). The same procedure was used to carry out antimicrobial susceptibility testing of all the bacteria isolates.

Detection of Virulence Factors

Haemolysin Detection

Hundred (100) mls of brain heart infusion agar was prepared according to manufacturer's prescription and sterilizes at1210C for 15mins.. After sterilization, the agar was allowed to cool for about 450C, and then it was supplemented with 5% sheep blood and was properly mixed together. Then it was poured and allowed to set.. An overnight culture of *Enterococci* was inoculated onto the agar using the streaking method.. It was incubated at 370C and observed for hemolysis after 24 and 48 hours respectively.

Biofilm Detection using the Tube Method

100mls of the trypticase soy broth was prepared according to the manufacturer's prescription and sterilized at 1210C for 15 minutes. After sterilization, 1g of glucose was added to the 100ml of the broth and was properly mixed together.. Using a 10ml sterile syringe, 10ml of the broth was collected into ten (10) test tubes. An overnight culture was aseptically picked using a sterile wire loop and inoculated into each of the test tubes.. The tubes were incubated for 24hours at 370C. After 24hours, the supernatant in each tube were decanted and the tubes were washed with phosphate buffer saline with a pH of 7.2 and allow drying in inverted position.. After decanting, the tubes were stained with crystal violet (0.1%) for 30minutes.. Excess stain was washed with washed. The tubes were dried in inverted position and observed for biofilm formation.

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3. RESULT

A total of 200 clinical samples(Urine, Wound and Stool) were collected from two study areas(University of Uyo Teaching Hospital and Anua General Hospital) with more of the samples coming from UUTH, because of the high level of patronage.Enterococccci species were more prevalent in UUTH(11.5%) than AGH(6.5%).Pearson Chi-square statistical analysis showed that the prevalence of enterococci obtained from patients admitted into different hospital wards was statistically significant(x^2=9.47 at 4df, p=0.001)

Table I: Prevalence of *Enterococci* isolates from patients in UUTH and AGH

Hospital Wards	No. of patients in UUTH	No. of isolates (%)	No. of patients in AGH	No. of isolates (%)
Orthopedic	24	4(16.7)	18	0(0.0)
Medical	63	5(7.9)	35	1(2.9)
Surgical	26	2(7.7)	19	2(10.5)
Postnatal	6	1(16.7)	3	1(33.3)
Burns	4	2(50.0)	2	1(50.0)
Total	123	14(11.4)	77	5(6.5)

Zero (0) denotes, no isolates was obtained from Orthopedic ward of Anua General Hospital.

Table II: Prevalence of *Enterococci* Isolates from Patients in different Hospital Wards

Hospital Wards Total	No. of Patients	Total No. of Isolates (%)	X^2 at 4df	P-value
Orthopaedic	42	4(9.5)		
Medical	98	6(14.3)		*0.001
Surgical	45	4(8.8)	9.47	
Postnatal	9	2(22.2)		
Burns	6	3(50.0)		
Total	200	19(9.5)		

*Statistically significant (X^2 at 4df = 9.47; P<0.05); df = degree of freedom

Table III: Distribution of *Enterococci* isolates according to clinical samples

Clinical sample	No. of Samples	No. of Isolates	Clinical sample	No. of Samples	Different Isolates (%)			
					E.faecalis	*E.durans*	*E. faecium*	*E.gallinarium*
Urine	133 (56.5)	9(47.4)	Urine	133 (56.5)	7(77.8)	0	2(22.2)	0
Stool	54 (27.0)	6(31.6)	Stool	54 (27.0)	2(33.3)	0	3(50.0)	1(16.7)
Wound	65(16.5)	4(21.1)	Wound	65(16.5)	2(50.0)	2(50.0)	0	0
Total	200(100)	19(9.5)	Total	200(100)	11(57.9)	2(10.5)	5(26.5)	1(5.3)

Table IV: Antimicrobial susceptibility pattern of *Enterococci* isolates

Isolates	Cpx S .I. R	Gen. S .I .R	Van. S .I. R	Azt. S .I .R	Ery. S .I. R	Amp S .I .R
E. faecalis	0 2 8	0 0 10	0 0 8	4 0 7	0 1 10	5 0 7
E. faecium	0 1 4	0 0 5	0 2 3	0 0 5	0 1 4	5 0 7
E. durans	0 1 1	0 2 0	1 0 1	1 0 1	0 0 2	1 0 1
E.gallinarium	0 0 1	0 0 1	0 0 1	0 0 1	0 0 1	1 0 0

Cpx= Ciproflaxin Gen=Gentamycin Van= vancomycin Azt= Aztreonam Ery=Erythromycin Amp=Ampicillin S=Suspectibilty I=Intermediate and R=Resistance

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Table V: Virulence factors production by various isolates

Isolates	Total No. of Isolates (%)	No. with Biofilm Production (%) Strong, Moderate, Weak			No. with Heamolysis production (%) Gamma, Alpha Beta		
Enterococcus faecalis	11(57.9)	6(54.5)	1(9.1)	4(36.4)	2(18.2)	6(54.5)	3(27.2)
Enterococcus faecium	5(26.5)	2(40.0)	1(20.0)	2(40)	2(40)	2(40.0)	1(20.0)
Enterococcus duran	2(10.5)	0	0	2(10.5)	0	0	2(100.0)
Enterococcus gallinarium	1(5.3)	0	0	1(5.2)	0	0	1(100.0)

4. DISCUSSION

In this study, the prevalence rate, antimicrobial susceptibility profile and the expression of various virulence factors were determined from 200 clinical samples comprising of urine, wound and stool samples. A total of 19(9.5%) of Enterococci isolates was obtained from hospitalized patients from the two study areas with more of the isolates obtained from patients with urinary tract infection (47.4%) even when compared with patients having wound infection (21.1%) (Table 4.5). This was also the case in a survey done by Center for Disease Control(CDC) on nosocomial infections in which *Enterococcus* accounted for 63.9% of urinary tract infections being next to *Escherichia coli* as a causative agent of hospital acquired urinary tract infection (Desai *et al*.,2001).

Although recent studies stated that there is an increase in the isolation of *E. faecium* and other Enterococcal species (6 Jains S. *et al*., 2011), in this study, *E.faecalis* (57.8%) constitute the major isolates followed by *E.faecium* (26.5%), *E.durans*(10.5%) and *E. gallinarium* (5.3%).Similar findings were reported by 7 Facklam*et al*.,1999. From the two hospitals in the study, it was discovered that *Enterococci* isolates were more prevalent in University of Uyo Teaching Hospital (73.7%) compared to Anua General Hospital (26.3%). In this study the prevalence rate of *Enterococci* isolates in the observed number of patients from the various wards of the two hospitals was obtained as 9.47 which is approximately 9.5%, and is statistically significant, which indicate the possibility of *Enterococci* infection occurring among patients in the hospitals. Although, the p-value is statistically significant, the occurrence rate of *Enterococci* species in this study is low compared to a similar work done in Oshogbo, South Western Nigeria where Enterococci had a total of 23% prevalence rate from the entire clinical samples used in the study. From the two hospitals in this study, it was discovered that *Enterococci species* had a higher prevalence rate in UUTH (11.4%) than that obtained in AGH (6.5%), which may have been as a result of high level of patronage and more samples been collected from patients in UUTH than AGH.

Antibiotics resistance by Enterococci is a global problem. In this study, the highest resistance was seen against Erythromycin (89.5%), Gentamycin (84.2%) followed by Vancomycin (68.4%). Other recent studies stated that antibiotic resistance is high in *Enterococci faecium* than *Enterococci faecalis* (Jains *et al*.,2011) while in this study among the Enterococci species obtained, antibiotics resistance was high in *E.faecalis* (44.5%) than *E. faecium* (33.3%), which is in agreement with other studies carried out in india (Agarwal, *et al*.,2009). It was also observed that *Enterococci* species were subceptible to Ampicillin and Aztreonam in an increasing frequency while only *Enterococcus durans* was susceptible to vancomycin. The occurrence of high level Gentamycin resistance (HLGR) among the Enterococci isolates in this study was seen to be 84.2% with a high resistance seen in *Enterococcus faecalis* (44.5%) than *Enterococcus faecium* (33.3%). Mendiratta *et al*., (2008) reported greater resistance to HLG in *E.faecium* as compared to *E.faecalis* isolates.

The Virulence factors detected among Enterococci species were hemolysin, and biofilm as (Table 4.8). The Haemolysin producing stains were found to be more than those producing Biofilm, although the activity of biofilm formation was not demonstrated in *E.gallinarium* and *E.durans*. *Enterococcus faecalis* (57.9%) was the commonest species seen to express more of the virulence factors when compared with *E. faecium* (26.3%). The production of biofilm in *Enterococcus faecalis* (63.6%) were comparatively higher than *Enterococcus faecium* (60.0%), while the production of hemolysis were comparatively higher in *E.faecalis*(81.7%) than *E.faecium* (60.0%). over 141.7% of *E.faecalis* possessed both biofilm and hemolysin,while production of both factors was substantially lesser in the case of *E.faecium* isolates (123.6%). This may possibly be one of the reasons why the species of *E.faecalis* is responsible for a greater number of infections than *E.faeciu*m

5. CONCLUSION

Enterococci species isolated in the course of this study were *E. faecalis*, *faecium*, *gallinarium*and *durans*with *E. faecalis* having the highest occurrence rate (56.7%) followed by *E .faecium* (26.5%). From the number of isolates obtained from patients in the two study areas, the prevalence rate of Enterococci isolates was obtained as 9.5%. In this study, resistant pattern differed among the *Enterococci species.* Overall, *E. faecalis* have a higher occurrence of resistance among the antibiotics, particularly erythromycin (89.5%), gentamycin (84.2%) and vancomycin (68.4%), while *E. faecium* had a relatively low prevalence rate. Also a total of 81.9% of *E. faecalis* was susceptible to Aztreonam and Ampicillin while 80.0% of *E. faecium* was susceptible to Ampicillin. Among the virulence factor, it was discovered that the production of biofilm in *E. faecalis* (63.6%) were comparatively higher than *E. faecium* (60.0%), which were both strong and moderate respectively while the production of hemolysis were comparatively higher in *E. faecalis* (81.7%) than *E. faecium* (60.0%). The rising prevalence of antimicrobial resistance trait among Enterococci species has critical outcome on health care system due to increase in mortality as a result of existence of severe infection such as endocarditis without any effective antimicrobial therapeutic agents(Bhardway.,2006).Hence, emergence of antimicrobial resistance, particularly multi-antibiotic resistance bacteria strains and shortage antimicrobial agents with different mechanism of action from current antibiotic would be a serious problem in the future and consequently development of novel alternative to conventional antibiotics is a necessity.

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