International Journal of Biological, Physical and Chemical Studies
ISSN: 2709-1554
DOI: 10.32996/ijbpcs
Journal Homepage: www.al-kindipublisher.com/index.php/ijbpcs

IJBPCS
AL-KINDI CENTER FOR RESEARCH AND DEVELOPMENT

| RESEARCH ARTICLE

Biofilm forming *Enterococci* and their Status as Emerging Multidrug Resistant Bacteria

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| ABSTRACT

Urine specimens were collected aseptically and inoculated directly onto CLED and MacConkey agar medium and incubated overnight. Single target colonies were sub cultured, and identification was based on culture, morphology, and biochemical characteristics. *Enterococci* isolates were subjected to biofilm assay (Tube method), and resistance pattern was determined for both biofilm-formers and non-biofilm-formers. Genomic DNA was extracted using the Bioneer kit, and the gene for virulence was detected by PCR and agarose gel electrophoresis. 148 Patients having one or more urinary symptoms were the study population. The aim of the study was to determine the multidrug resistant ability of biofilm-forming *Enterococci*. A number of growths were encountered; *Enterococci* accounted for only 9.46% (14), the highest prevalence was seen in the age group 21-30 (35.71%), and females (64.29%) were more prone to enterococcal infection than their males (35.71) counterpart. There is a relationship between biofilm production and antibiotic resistance because multidrug resistant *Enterococci* isolate produced bands against esp gene with an average of 510kbp. Biofilm forming strains showed the highest resistance to gentamicin and penicillin (83.3%), and vancomycin can be considered a good alternative therapy in enterococcal UTIs because of its lower resistance (75%).

| KEYWORDS

Enterococci, urine, esp gene, biofilm, polymerase chain reaction (PCR)

| ARTICLE DOI: 10.32996/ijbpcs.2022.4.1.1

1. Introduction

The important opportunistic pathogens and indigenous flora of the intestinal tract, oral cavity, and genitourinary tract of both humans and animals are *Enterococci* (Banwo *et al*., 2013). *Enterococci* are the most controversial group of lactic acid bacteria. They are common nosocomial agents that infect the urinary tract, bloodstream, intra-abdominal and pelvic regions, surgical sites, and central nervous system (Bharti *et al.,* 2016*). Enterococcus faecalis* and *Enteroccus faecium* are found to be the most frequent *Enterococci* species in human infections and represent the third most frequently isolated multidrug resistant nosocomial pathogens from Bacteremia (Soares *et al*., 2014; Van Tyne and Gilmore, 2017). *Enterococcus faecalis* is the most common *Enterococci* species, and it is responsible for 80–90% of human enterococcal infections, *Enterococcus faecium* accounts for the remainder of infections caused by *Enterococci spp* (Sreeja, 2012). *Enterococci* are once viewed as part of the normal gut flora with little clinical significance, and they are now recognized as the cause of several types of community and hospital-acquired infections (Upadhyaya, 2011).

Biofilm provides compatible conditions for horizontal gene transfer, such as high cell density, increased genetic competence, and accumulation of genetic elements or uptake of resistance genes (Fux *et al*., 2015). Conjugation is the only mechanism of horizontal gene transfer of resistance genes to several antibiotics. Few studies suggested that conjugation has been shown to be more efficient in biofilm as compared to planktonic ones (Krol, 2013).

Nowadays, enterococci take up a significant position among the bacteria causing nosocomial infections. One of the main factors of enterococci virulence is biofilm formation (Tendolkar *et al*. 2006). While in the biofilm, microbes can be protected from numerous

agents such as UV-light, antibiotics, and other antimicrobial agents. The matrix of the biofilm provides a mechanical and biochemical shield that provides the conditions needed to alternate the activity of drugs (e.g., low oxygen, low pH, high CO_2, and low water availability). Under these conditions, it is difficult to eliminate bacteria using conventional antibiotics. Bacterial antibiotic resistance is one of the consequences of the bacterial biofilm communities, which contribute to chronic infections.

2. Methodology

2.1 Collection of specimen

The research was conducted in Bauchi Metropolis, the capital city of Bauchi State, and North East of Nigeria. Bauchi State lies between latitude 09^0 52^1 and 09^0 86^1North of the Equator and longitude 10^045^1 and 10^0 45^1East of the prime meridian. This study is cross-sectional and was undertaken in two locations, namely; Abubakar Tafawa Balewa University Teaching Hospital and Yelwa Domiciliary Clinic, respectively. Urine was collected from patients via the clean catch midstream technique.

2.2 Identification of Enterococci

Urine Specimen was inoculated directly onto MacConkey agar and CLED agar using a sterile calibrated loop. Sub culture of single target colonies was done on MacConkey agar at 37°C for further tests. Identification of the genus was done based on Gram staining, biochemical tests such as catalase test, motility test, esculin hydrolysis, heat/salt tolerance, gelatinase production, and biofilom assay.

2.3 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test against *E. faecalis* and *E. faecium* was carried out using Kirby-Bauer disc diffusion techniques described by Arora (2011). A loopful of growth of each isolate on agar medium was suspended in sterile water and then was diluted in steps of 1:10 to give turbidity equivalent to the 0.5 McFarland standards (a density of $1x10^8$ cells/ml) before inoculation. The Muller-Hinton agar medium was prepared according to manufacturer's instructions and was poured (about 25ml of the media) into each of sterile petri-plates, the plates were allowed to solidify, and a sterile swab stick was used to pick 0.5 ml suspension of each to isolate adjusted to $1x10^8$cells/ml and was spread uniformly on the agar. A sterile cotton swab was dipped in inoculums, removed the excess fluid by pressing and rotating the swabs against the wall of the tubes, and then streaked on the surface of Muller Hinton plates. The inoculated plates were allowed to dry for about 5minutes. Using disc dispenser, single disc antimicrobial agents (Oxoid, Basingstoke United Kingdom): Cotrimoxazole (25µg), Tetracycline (30µg), Ciprofloxacin (5µg), Trimethoprim (5µg), Vancomycin (30µg), Gentamycin (10µg), Streptomycin (10µg), Penicillin (10µg), Chloramphenicol (30µg), Cefotaxime (30µg), Erythromycin (5µg), Ampicillin (10µg), and Amoxicillin (10µg) were dispensed onto the surface of the agar plates. After 30minutes of applying the discs, the plates were incubated aerobically at 37^0C for 18hours. The results were interpreted as susceptible, intermediate, or resistant according to Clinical and Laboratory Standard Institute (CLSI) guidelines for 2015 (CLSI, 2015).

2.4 DNA Extraction

A 200µl of phosphate buffer saline (PBS) was added to the slant to wash the cultured cells, it was centrifuged, and the supernatant was discarded carefully. 20µl of proteinase K and 200µl of lysis buffer was added and mixed immediately by vortex mixer and incubated at 37°C for 30 minutes. A 400µl of absolute ethanol was added and mixed well by pipetting; this was transferred carefully into the collection tube and was centrifuged at 8000rpm for 1 minute. The solution was discarded from the collection tube into a disposable bottle, and the collection tube was reused again. 500µl of wash buffer 1 (W1) was added and centrifuge at 8000rpm for 1 minute. The solution was discarded from the collection tube into a disposable bottle, and the collection tube was reused again. 500µl of wash buffer 2 (W2) was added and centrifuge at 8000rpm for 1 minute. The solution was discarded from the collection tube into a disposable bottle, and the collection tube was reused again. The tube was centrifuged once more at 13,000rpm for 1 minute to completely remove ethanol. The collection tube was transferred to a new 1.5ml tube for elution. 50µl of elution buffer (EA buffer) was added and allowed to stand for 1 minute at room temperature (15-25°C) and centrifuge at 8000rpm for 1 minute. The DNA was stored at -20°C (Safari *et al.,* 2017).

2.5 PCR Amplification of Enterococcal Surface Protein (esp) Gene

The oligonucleotide primer was used to amplify 2 isolates of *Enterococci*. The templates (2µl), specific primers (1µl each), and water (16µl) were added to the premix. The amplification program consisted of pre-Denaturation with an initial cycle of 5 min at 94°C, followed by denaturation of 30 seconds at 94°C (30 cycles), annealing for 30 seconds at 52°C, extension for 1 min at 72°C (35cycles), and final extension of 5 min at 72°C.

***Enterococci* specific primers used and their characteristics**

Target gene	Primer pair sequence $5^1 \rightarrow 3^1$	PCR product (bp)	Reference
esp (Enterococcal surface protein)	F(AGATTTCATCTTTTGATTCTTGG) R(AATTGATTCTTTAGCATCTGG)	510	Safari *et al.*, 2017.

Note: PCR= Polymerase Chain Reaction, bp=base pair

2.6 Identification of PCR Products using Agarose Gel Electrophoresis

Electrophoresis of the PCR amplicons was performed with 2% agarose gel; the solution was placed in a water bath until agarose was completely dissolved. It was allowed to cool in a water bath set at 50-55^{o}C. The ends of the gel were sealed, and appropriate numbers of combs were placed in the gel tray. 5µl of ethidium bromide was added to cool the gel for 15-20 minutes at room temperature. The combs were removed and placed in an electrophoresis chamber, and covered with buffer (TBE). DNA and ladder were loaded onto gel and electrophoreses for 1 hour at a given voltage. The bands were visualized using a UV light box.

3. Results

Table 1. Distribution of *Enterococci* Isolates According to Patients Demographic Characteristics

Demographic characteristics	No of samples collected	No of Enterococci isolates. N=14	Percentage of isolates (%)
Age (years)			
1-10	09	00	00.00
11-20	24	02	14.28
21-30	51	05	35.71
31-40	33	04	28.57
41-50	19	02	14.28
51-60	10	01	07.14
61-70	02	00	00.00
Sex			
Male	45	05	35.71
Female	103	09	64.29
Location			
ATBUTH	101	10	68.24
YDC	47	04	31.76

1. Key: ATBUTH=Abubakar Tafawa Balewa University Teaching Hospital, YDC= Yelwa Domiciliary Clinic

Table 2. Multidrug Resistant Pattern of Biofilm Producing *Enterococci*

Antibiotics (µg)	No. of biofilm producers (n=12) and susceptibility pattern	
	Sensitive	**Resistant**
Ampicillin (10)	04(33.3)	08(66.7)
Amoxicillin (10)	04(33.3)	08(66.7)
Cefotaxime (30)	05(41.7)	07(58.3)
Chloramphenicol(30)	03(25.0)	09(75.0)
Ciprofloxacin (5)	04(33.3)	08(66.7)
Cotrimoxazole (25)	03(25.0)	09(75.0)
Erythromycin (5)	06(50.0)	06(50.0)
Gentamicin (10)	02(16.7)	10(83.3)
Penicillin (10)	02(16.7)	10(83.3)
Streptomycin (10)	08(66.7)	04(33.3)
Tetracycline (35)	06(50.0)	06(50.0)
Trimethoprim(5)	05(41.7)	07(58.3)
Vancomycin (30)	09(75.0)	03(25.0)

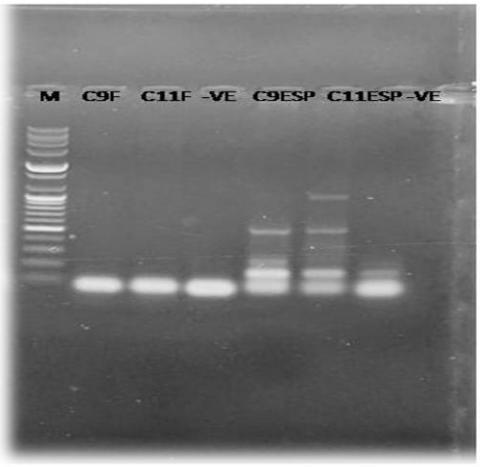

Plate 1. DNA bands of enterococci isolate against **esp** gene. Lane M is the molecular marker, C9esp and C11esp are amplicons in specific 510kbps, (-ve) is the negative control for esp gene.

4. Discussion

4.1 characteristics of Enterococci isolates according to demography

The study indicates that patients suspected of UTI with one or more urinary symptoms were diagnosed with culture and microscopy. *Enterococci* were isolated along with other organisms. *Enterococci* have evolved from normal commensals to leading pathogens that cause human infections. In this study, the overall prevalence of *Enterococci* was found to be 9.46%. This was in line with the report of *Enterococci* from Enugu 10.8% (Ekundayo *et al.* 2019). However, the prevalence was lower in the study conducted in Egypt, which was 6.2% (Yilema *et al*., 2017). The lower prevalence might be due to the variation in the study participants and the methods employed for the detection of *Enterococci.* In this study, the highest prevalence of *Enterococci* infections was seen in the age group 21-30years 51(34.5%), similar findings of higher infection rate in the age group 21-30years, 36(33.96%) were reported by Bose *et al.,* 2013. Females 9(64.29%) were more prone to *Enterococci* infection as compared to males (35.71%), this is in accordance with the study of Bharti *et al.,* 2016 who reported that females (66.04%) have a higher prevalence of *Enterococci* infection than their male counterpart (33.96%). This is also comparable to the work of Shrivastav *et al*., 2013 in which the number of females infected with *Enterococci* infection was more (72%) than a number of males (28%).

4.2 Characteristics of Biofilm Producing Enterococci based on Multidrug Resistant Pattern

The resistance pattern of each isolate was determined; almost all the isolates were multidrug resistant. *Enterococci* were best sensitive to vancomycin (75%), followed by streptomycin (55.6%) (Table 2).

In this study, many MDR *Enterococci* isolates produced biofilms. Out of the fourteen isolates, seven showed resistance to cotrimoxazole and gentamicin; Biofilm exhibits more resistance to broad spectrum antibiotics. This agrees with the findings of (Mathur *et al.,* 2006). A significant relationship was found between biofilm formation and antibiotic resistance to ciprofloxacin, cotrimoxazole, tetracycline, gentamycin, penicillin, amoxicillin, and erythromycin. Almost all the resistant isolates tested positive for biofilm production.

Biofilm exhibits more resistance to broad spectrum antibiotics. This supports that biofilm adds to the virulence profile of microorganisms. Resistance to several antimicrobial agents was prevalent among the Enterococci isolates recovered in the hospital.

4.3 Genotypic Characteristics of Biofilm Producing Enterococci

Virulence gene (enterococcal surface protein) was amplified via PCR using specific primers. Two multidrug resistant *Enterococci* isolates were used, and the DNA bands with an average of 510kbps showed a considerate relationship between biofilm production and antimicrobial drug resistance to esp gene (plate 1).

In this study, PCR was used to detect the presence or absence of genes for virulence determinants and is the most sensitive of the existing rapid methods to detect microbial pathogens in clinical specimens. The DNA bands showed a considerate relationship with an average of 510kbp genomic DNA profile using agarose gel electrophoresis. The resistant and sensitive isolates that produced biofilm revealed bands against the enterococcal surface protein (esp) gene. Esp is a surface protein involved in colonizing ability and immune invasion. The presence of esp increases cell hydrophobicity, adherence to antibiotic surfaces, and biofilm formation capacity of all strains (Shankar *et al.,* 2006). Clinical isolates of *Enterococci* show the presence of esp which shows their ability to form biofilms; this suggests a role for esp in the process of biofilm development. Consistent with the findings of Toledo-Arana *et al*., esp made a significant contribution to biofilm formation.

Biofilms and a number of biochemical features can contribute to the virulence of antibiotic resistance of many bacteria, including *Enterococci*. However, numerous genes involved in the pathogenicity of these bacteria have been identified. The virulence factors most frequently tested in human clinical isolates of enterococci include aggregation substance (asa1), collagen-binding protein (ace), enterococcal surface protein (esp), gelatine (gelE), and hyaluronidase (hyl) (Golinska *et al.,* 2013).

4.4 Summary

A total of 148 urine specimens were collected from patients of different sex and age with one or more urinary symptoms from ATBUTH and Yelwa domiciliary clinic in Bauchi metropolis. The specimens were cultured directly onto CLED and MacConkey agar. Sub cultures of single target colonies were made, and isolates were identified based on culture, morphology, and biochemical characterization. A number of bacterial growths were encountered; enterococci accounted for only 9.46%. The study revealed that there is a considerate relationship between biofilm formation and multidrug resistance of enterococci isolates.

5. Conclusion

The positive urine culture with antibiotic sensitivity of the isolates is very important to antimicrobial therapy, as antibiotic resistance is a global problem that causes ineffectiveness of treatment. Early and proper treatment can decrease antibiotic resistance. According to the findings of this study, it was concluded that biofilm forming enterococci showed resistance to multiple antibiotics, and vancomycin can be considered a good alternative therapy because of the lower resistance.

5.1 Recommendations

1. An elaborate molecular study is further needed to understand various gene expressions during biofilm formation. These various molecular mechanisms can be targeted to control biofilm formation.
2. Species identification of *Enterococci* may be useful as an epidemiologic tool in the investigation of outbreaks of nosocomial infection and for clinical decisions about therapy because antimicrobial susceptibility may vary by species.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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