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Research Article

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Isolation and Characterization of Multidrug resistant *Enterococcus faecium* isolated from sputum specimen

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Abstract

Enterococcus faecium is a Gram-positive, gamma-hemolytic bacterium found in humans and animals' gastrointestinal tracts, potentially causing diseases like neonatal meningitis or endocarditis. *Enterococcus faecium* is a bacterium with multi-drug antibiotic resistance which uses colonization and virulence factors to form biofilms and combat pathogenic gut microbes like Escherichia coli. The study aimed at isolating and characterizing multidrug resistant *Enterococcus faecium* isolated from sputum specimen. A total of 300 isolates were used. The isolated bacteria were characterized by gram staining and other biochemical test such as, catalase, urease, oxidase, motility test etc. The antimicrobial susceptivity profile of these isolates to standard antibiotics using agar disk diffusion technique to obtain MDR pathogenic bacteria isolates was determined. Extraction of the genomic DNA was done using ZR Fungal/bacterial DNA mini prep extraction kit. The extracted DNA was quantified using the Nanodrop 1000 spectrophotometer and the 16srRNA genes of the isolates were amplified using 27f: s'-AGAGTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' Primers. Sequencing of the extracted DNA was done using the BigDye Terminator kit on 3510ABI sequencer by Inqab Biotechnological, Pretoria South Africa. Phylogenetic analysis of the obtained sequence was edited using the bioinformatics algorithm, trace edit similar sequence were downloaded from the National center for Biotechnology information (NCBI) data base using BLASTIN. Molecular detection of virulence and resistance genes were detected using PCR. Statistical analysis were carried out using SPSS and MS-Excel software package. Gram staining results of the isolates were observed under a microscopic oil immersion lens for the confirmation of cellular morphology and arrangement. The phylogenetic distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16s rRNA and revealed a closely relatedness to *Enterococcus faecium, Neisseria yangbaofengii*, and *Citrobacter freundii*. The antibiotic result revealed that MDR *Enterococcus Faecium* was resistance to five different antibiotic class. The highest resistance was

seen on ofloxacin. The result of the virulent and resistances genes revealed the virulent gene associated with *Enterococcus hyl* and two resistant genes CTX-M and TEM. Multidrug resistant is becoming a treat to public health. Enterococcus faecium is the main cause of hospital acquired infections.so health care personnel should and patient should be aware and practice good hygiene. Multidrug resistant *Enterococcus faecium* were isolated from sputum specimens and identified using 16S rRNA gene sequencing. Multidrug resistant is becoming a treat to public health.

Keywords: *Enterococcus faecium,* Multidrug-resistant, sputum specimen, Isolation.

1.0 Introduction

Enterococci are Gram-positive facultative anaerobic cocci in short and medium chains, first discovered in 1899 in the human gastrointestinal tract. They are the first of the ESKAPE organisms (*Enterococci* spp, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa, Enterobacter* spp.) highlighted by the WHO as rising causes of *infections*. Enterococcus is a diverse genus, it predominantly causes over 90% of infections by E. faecalis and E. faecium, with multidrug resistant enterococci genomes containing over 25% mobile elements.

Enterococci are found in the soil, water, food, sewage, plants, human skin, the oral cavity, and the large intestine, constituting less than 1% of the total microbiota. (Raza *et al.,* 2018) Nosocomial and antibiotic-resistant infections in the last few decades are threatening public health. (Zhen *et al.,* 2019). *Enterococci* are highly resilient and can survive various difficult conditions like common antiseptics and disinfectants, promoting their widespread persistence on ordinary hospital inanimate items (Fiore *et al.,* 2019). More importantly, they are found in the hands of healthcare workers (HCW), accounting for their easy transmission (Reyes *et al.,* 2016). Enterococcal colonization of the GI tract is the main predisposing factor for severe infections, which occur through gut translocation. *Enterococci* are phagocytosed and transported across the intestinal wall and resist killing by the lymph system. (Fernández *et al.,* 2019). Currently, in the USA, enterococci rank second only after staphylococci, in nosocomial infections, antibiotic-resistant pathogenic diseases, central line-associated bacteremia, and hospital-associated endocarditis. (Chiang *et al.,* 2017). Reports suggest that 60% of all infections by *enterococci* are healthcare-acquired, including the ICU setting. *Enterococci* are the third most common cause of community-acquired endocarditis in North America after *Staphylococcus aureus* and *Streptococcus viridans*, higher than anywhere else in the world (Fernández *et al.,* 2019). Multidrug resistance is a microorganism's capacity to resist three or more medication classes. It can also be viewed as the antimicrobial resistance exhibited by various microorganisms species to antimicrobial medications. The World Health Organization, WHO, claims that these resistant microbes can fend off an attack by antimicrobial medications, resulting in poor treatment that causes diseases to remain and spread.

2.0 Materials and Methods

2.1 Ethical Clearance: Prior to sample collection, Ethical clearance for the study was obtained from Imo State Ministry of Health Owerri.

2.2 Sample collection

Three hundred sputum specimen were obtained from different patients from outpatient department (OPD) of Federal University Teaching Hospital Owerri, Imo State Nigeria., Sterile bottles were given to the patients to cough and pour their sputum in the container given to them. The containers were being labeled properly, covered very well and packed in a clean paper bag and transported to the laboratory for cultivation, identification and other analysis.

2.3 Cultivation and Identification of Isolates

The purulent part of the accepted sputum samples was inoculated on the chocolate agar and blood agar plates by using a sterile wire loop to collect the sputum and streak on the surface of the agar and incubated in CO2 enriched atmosphere at 37^0C for 18-24 hours. According to Cheesbrough, 2012.

2.4 Identification and Confirmation of the Test Isolates

Plates with visible colonies were counted and expressed as colony forming units per milliliter (CFU/ml) according to Cheesbrough, (2012). The bacterial isolates were identified using their growth morphological characteristics on different media used (such as size, edge, shape, elevation, colour, surface, consistency etc.), bacteriological identification tests (Gram staining and Motility test), biochemical identification test (Catalase, Urease, Oxidase, Sugar Fermentation test and IMVIC test- Indole production, Methyl red, Voges Proskeur, Citrate utilization) The results of the biochemical tests were matched with one obtained in Bergey's manual of determinative bacteriology for confirmation.

2.5 Antibiotics susceptibility testing AST

The disk diffusion method (Kirby–Bauer) were carried out using Muller Hinton agar (Oxoid, Hampshire, UK) as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2020). Similar pure colonies were sub-cultured into the nutrient broth, mixed, and incubated for 2–6 hours at 35–37^0C. The suspension was matched with the turbidity to that of (0.5MacFarlard standard solution). Then sterile swab sticks were dipped into the suspension to pick the isolates and then streak on the surface of Muller Hinton agar plates and ensure even distribution. This was done for all the isolates. With the petri-dishes lid in place, the inoculated plates were allowed to stand for 3 – 5 minutes for surface of the agar to dry. Using a sterile forcepts pick and place the disc antibiotics on the inoculated plates. Each disc was gently pressed down onto the agar to ensure complete contact with the agar surface. The plates were inverted and incubated at 37^0C. After 18 – 24 hours of incubation the plates were examined and the diameters of zones of inhibition were measured in millimeter and interpreted as susceptible, intermediate, or resistant. The zone of inhibitions was interpreted based on the clinical and Laboratory standards institute (CLSI) (2020). The following antibiotics with known concentrations recommended by the CLSI were examined: Amoxicillin/clavulanate (30 µg), ceftriaxone sulbactam (45 µg), cefotaxime (25µg), cefuroxime (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), imipenem/ cilastatin (10/10 µg), gentamicin (10 µg), nitrofurantoin (300µg), cefixime (5 µg), Erythromycin (15 µg), Levofloxacin (5 µg) and Azithromycin (15), Nalidixic Acid (30 µg), Ampiclox (10 µg), The final antibiotic susceptibility results of bacterial pathogens were interpreted using the 2017 CLSI breakpoints to categorize the isolates as susceptible or resistant. (CLSI, 2020). Isolates were defined as MDR when they were resistant to at least three antibiotics from different classes.

2.6 Molecular identification:

Bacterial genomic DNA extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa.

2.6.1 DNA Quantification: The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

2.6.2 16S RRNA Amplification

The 16s RRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

2.6.3 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

2.6.4 Phylogenetic Analysis

The obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Telles *et al.,*2018)). The bootstrap consensus tree inferred from 500 replicates (Eder, 2016) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method.

2.7 Molecular detection of virulence and resistance genes

Genes encoding resistance and virulence factors were detected using PCR. The virulence gene detected was *hyl* while the resistant genes were TEM and CTX-M genes

2.7.1 Amplification of *hyl* genes (hyaluronidase)

hyl genes from the isolates were amplified using the hylF: 5' CGCCTGTGTATTATCTCCCT-3' and hylR: 5'-CGAGTAGTCCACCAGATCCT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 56ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV transilluminator.

2.7.2 Amplification of resistant genes

TEM and CTX-M genes from the isolates were amplified using the TEMF: 5'-ATGAGTATTCAACATTTCCGTG-3' and TEMR: 5'-TTACCAATGCTTAATCAGTGAG-3' primers and CTX-MF: 5'-CGCTTTGCGATGTGCAG-3' and CTX-MR:5'-ACCGCGATATCGTTGGT-3' primers respectively on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 58ºC for 30 seconds; extension, 72ºC for

30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV transilluminator.

Statistical analysis was carried out using SPSS and MS-Excel software package.

3.0 Results

Out of the 500 sputum samples collected from out patients from Federal University Teaching Hospital Owerri Imo State, a total of 300 isolates of different species were recovered representing (60%). 135(45%) were Male patients of different ages while 165 (55%) isolates were from female of different age brackets. See Figure 1 and 2. Based on cultural characteristics and Gram results, colonies were purified for further processing. Following biochemical identification of pure cultures, it was observed that one hundred and eighty (180) 60% out of three hundred (300) isolates were confirmed *to be Enterococcus* sp. Twenty-five (25), 8.3% isolates were confirmed to be *Citrobacter* Sp fifteen (15)5.0% isolates were *Neisseria* sp while forty-seven (47),16.9% isolates were confirmed to be *Neisseria* Sp, thirty-five (35)11.7% isolates were *klebsiella* sp while forty-five (45)15% isolates were *Pseudomonas* sp see figure 3. Table 2 shows the antibiotic resistance profile (%) of *Enterococcus faecium.* Amoxicillin clavulanate recorded the highest resistance of 45.6% while Azithromycin recorded the least resistance. Plate 1 shows the Agarose gel electrophoresis of the amplified 16srRNA, Lanes 1-10 represent the amplified 16srRNA at 1500bp while lane L represents the 100bp DNA ladder. Fig 4 represents the Phylogenetic tree of the evolutionary distance between the bacterial isolates. The obtained 16s rRNA sequence from the isolates produced an exact match during the mega blast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolates showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Enterococcus, Neisseria and*

Citrobacter sp and revealed a closely relatedness to *Enterococcus feacium, Neisseria yangbaofengii and Citrobacter freundii.* Plate 2: shows the agarose gel electrophoresis of the amplified *hyl*. Lanes 1, 3, 4 6 and 7 represent the *hyl* gene bands at 600 bp while lane L represents the 100bp ladder. Plate 3: represents agarose gel electrophoresis of the amplified TEM. Lanes 1, 5, and 6 represent the TEM gene bands at 700 bp while lane L represents the 100bp ladder. Plate 4 represents agarose gel electrophoresis of the amplified CTX-M. Lanes 1-3, and 5 represent the CTX-M gene bands at 500 bp while lane L represents the 100bp ladder.

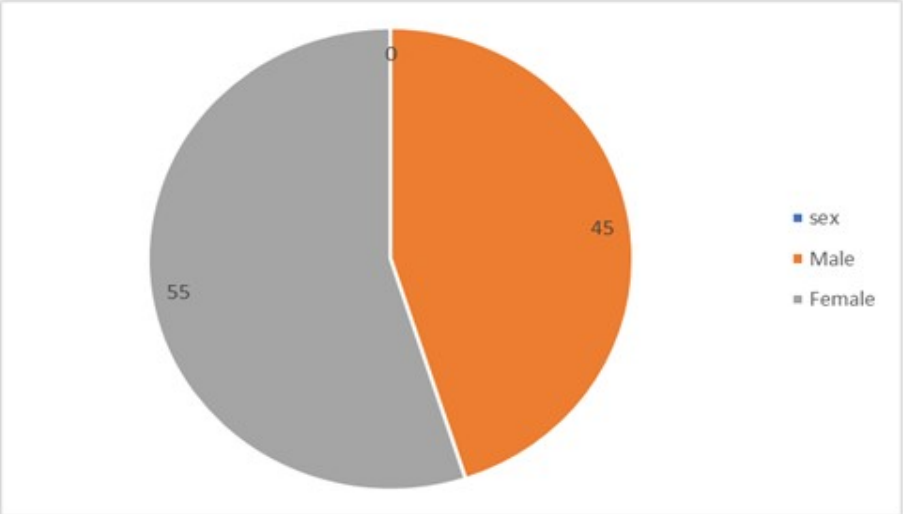

Figure 1 Distribution of *Enterococcus* spp and other isolates by gender.

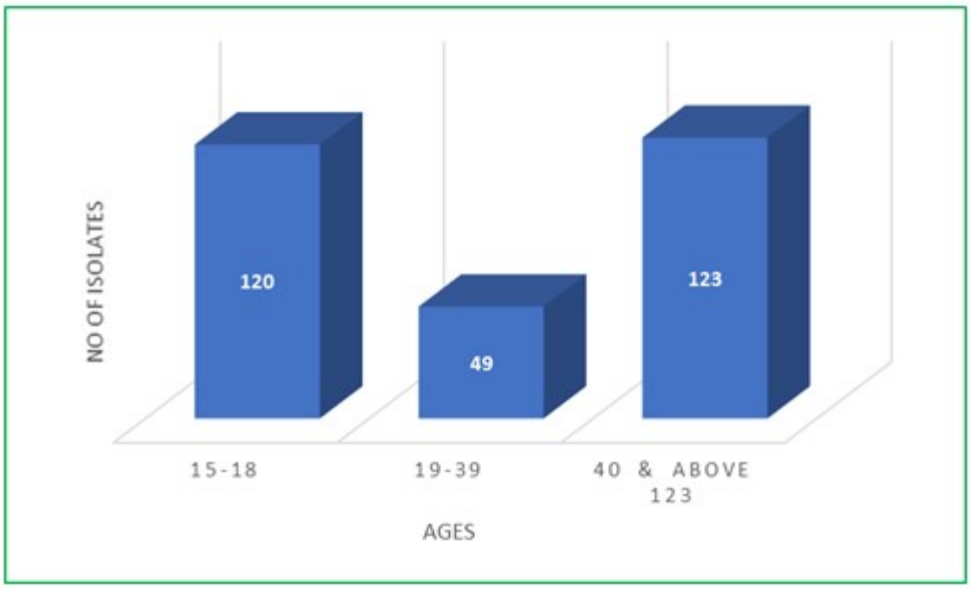

Figure .2. Distribution of *Enterococcus sp* and other isolates by Age

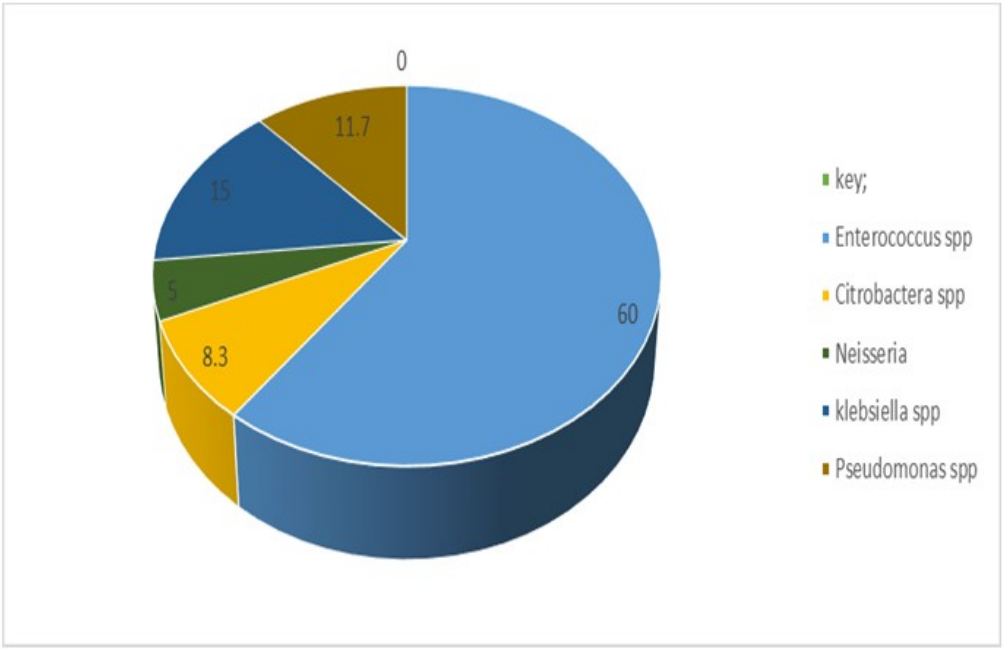

Figure 3 Percentage of biochemically confirmed bacteria isolates.

Table 2: Antibiotic Resistance Profile (%) of *Enterococcus faecium*

Names of antibiotics	Resistance(n=180)
Ceftriaxone sulbactam (CRO)	77(42.8%)
Gentamicin (GN)	47(26.1%)
Levofloxacin (LBC)	74(41.1%)
Erythromycin (ERY)	71(39.4%)
Azithromycin (AZN)	54(30%)
Amoxicillin Clavulanate (AUG)	82(45.6%)

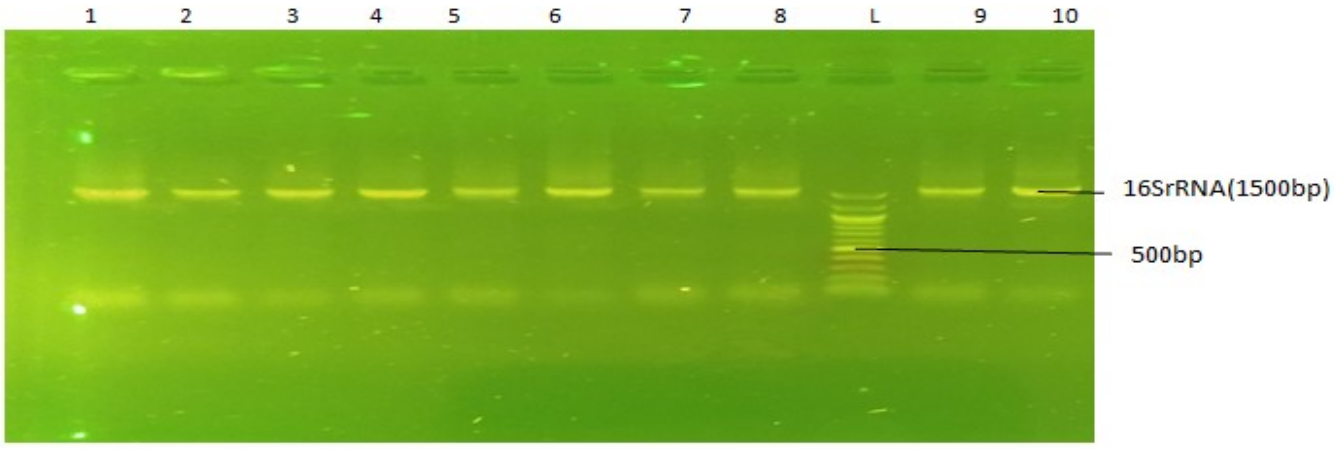

Plate 1: Agarose gel electrophoresis showing the amplified 16srRNA

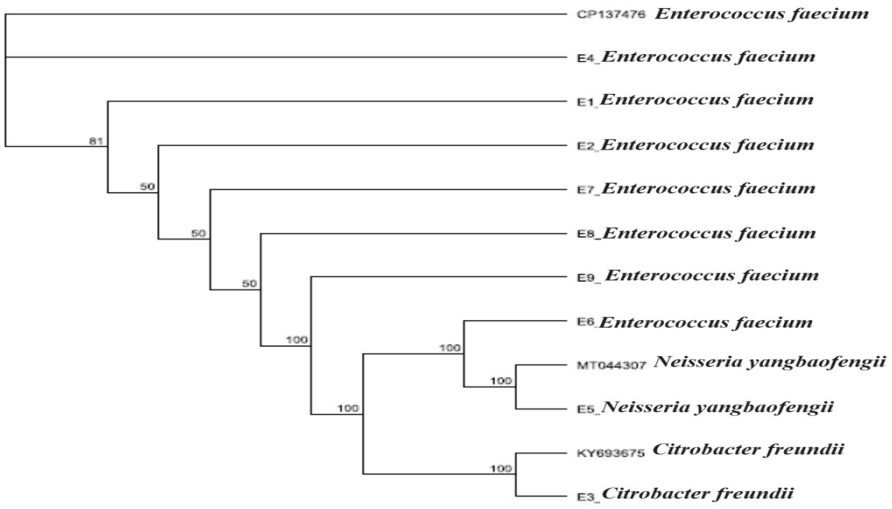

Figure 4 The Phylogenetic tree of the evolutionary distance between the bacterial isolates.

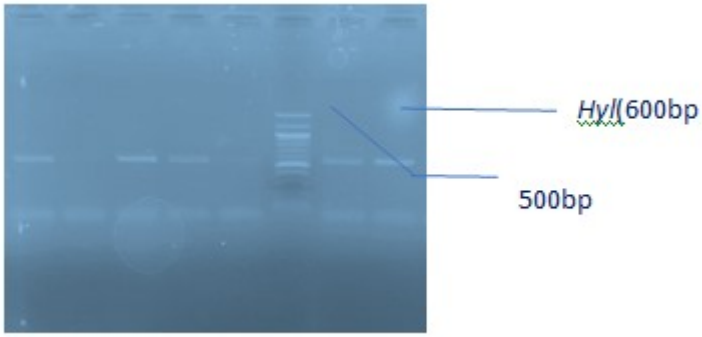

Plate 2: Agarose gel electrophoresis of *hyl* gene

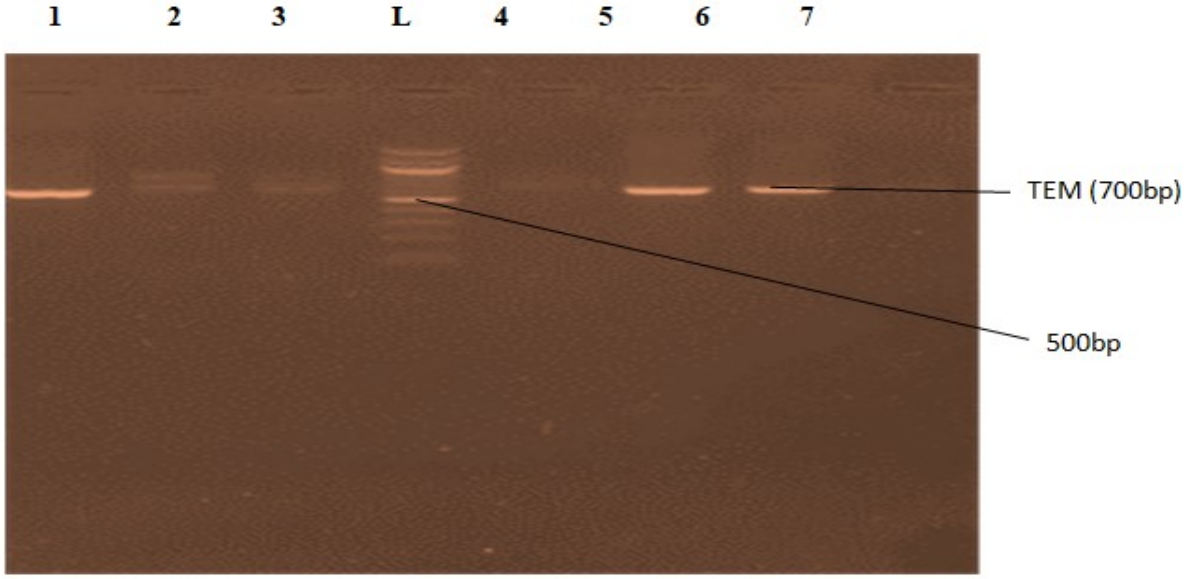

Plate 3: Agarose gel electrophoresis of *TEM* gene

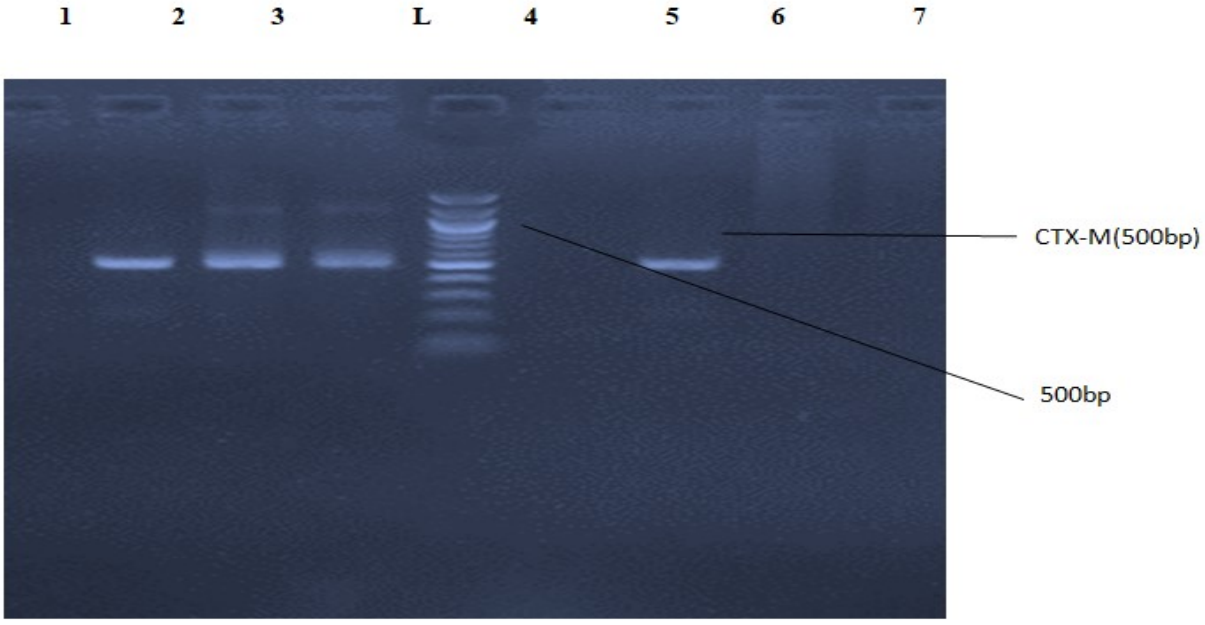

Plate 4. Agarose gel electrophoresis of *CTX-M* gene

4.0 Discussion of findings

Since antimicrobial resistance poses a concern to international health security, biomedical science has looked to plants as possible sources of medications for the prevention and treatment of human illnesses. (Van and Doi, 2017) Public health are declining and socioeconomic crises are being brought on by the growing prevalence of multidrug resistant microbes, or MDR microbes, which are harmful bacteria and fungi. (Van and Doi, 2017) This research mainly concentrates on the isolation and characterization of multidrug-resistant human pathogenic *Enterococcus faecium.* Minimum inhibitory concentration or high zone of inhibition elaborates the bacterial susceptibility or bacterial reaction to the antibiotic used. (Mohammed *et al.,*2021).

In this study, multi-drug resistant *Enterococcus faecium* was isolated from sputum specimen this was contrary to Gorica *et al.*, 2019 that isolated ESBL *Escherichia coli*, Methicillin resistant *Staphylococcus aureus* and *Streptococcus pneumoniae* from sputum culture. (Garica *et al.,* 2019). The isolation of MDR-organisms from sputum promotes unnecessary use of antibiotics, which should be overcome by combined efforts of clinicians and the patients. The agarose gel electrophoresis of the amplified 16srRNA of the isolates were found at Lanes 1-10 at 1500bp while lane L represents the 100bp DNA ladder see plate 1 and the 16srRNA genes confirms the isolates to be *Enterococcus faecium,* with GenBank accession number PP998343. Isolating *Enterococcus faecium* from sputum agrees with Chong et al, (2019), which says that these organisms are harmless in their natural habitat but in the presence of hospital environment they become pathogenic. Isolation of MDR *Enterococcus faecium* with production of extended-spectrum β-lactamase (ESBL) is similar with other reports by several international surveillance programs (Liu *et al.,* 2018b). In the present study, *Enterococcus faecium* was found to be resistance to five (5) different classes of antibiotics. This confers multidrug resistance, this may be because of the antibiotics abuse and misuse (ventol, 2015) or an unsuitable prescription of antibiotics. (Strachan and Davies, 2017).

According to the study, resistance genes and virulent genes were found in all the isolates. hyl gene was isolated from *Enterococcus faecium.* see plate 2. This agrees with Arias, *et al.,* 2009 who also isolated hyl_{Efm} gene in three *E. faecium* strains obtained from the United States. The presence of *hyl* genes was significantly associated with the phenotypic resistance to AUG, CRO, LBC, CIP, ERY, GEN, and AZN. Resistance genes CTX-M was also isolated in this study, see plate 4, TEM gene was also isolated from *enterococcus faecium* see plate 3. CTX-M and TEM are beta-lactam genes. The acquisition of virulent and resistance genes in these isolates is believed to increase the pathogenicity of *enterococcus faecium* and the intensity of the infection. Furthermore, in this study the occurrence of more than one beta-lactamase within the same isolate has been detected. This identifies their wider dissemination probably due to involvement of genetic elements in mobilization of these genes. (Trupt *et al.,* 2017) Bacteria are known for their ability to adapt changes in their environment. Mutation has an important role to play in the evolution of antibiotic resistance, the predominant factor for escalation of antibiotics resistance is the acquisition of antibiotic resistance genes. The microbial resistance of gram-positive organisms has built up gradually during the last few years, leading to increased incidence of out breaks of infections due to existence of multi-drug resistant bacteria. Resistant bacterial strains are spreading speedly where they have space to come closely to other bacteria, sharing resistance genetic material and thus spreading the antimicrobial resistance phenotypes.

4.1 Conclusion and Recommendations

Multidrug resistant *Enterococcus faecium* were isolated from sputum specimens and identified using 16S rRNA gene sequencing. Multidrug resistant is becoming a treat to public health. Enterococcus faecium is the main cause of

hospital acquired infections. so health care personnel, patient and persons visiting hospitals should be aware and practice good hygiene.

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