Plasmid Profile Analysis and Curing of Multidrug-resistant Bacteria Isolated from Two Hospital Environments in Calabar Metropolis, Nigeria

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Authors’ contributions
This work was carried out in collaboration between all authors. Authors ENM, CIM and UOE designed the study, wrote the protocol and interpreted the data. Authors ENM, CIM and UOE anchored the field study, gathered the initial data and performed preliminary data analysis. While author ENM managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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ABSTRACT

Objective: The objective of this study was to determine the plasmid profile and curing of environmental isolates from General and Infectious Disease hospitals showing multi-drug resistance.

Methods: Isolates were obtained from air and surfaces of two hospitals using standard microbiological techniques. The isolates were then subjected to sensitivity using ofloxacin, riflacin, ciprofloxacain, augmentin, gentamycin, streptomycin, ceporex, nalidixic acid, septrin, ampicin, norfloroxacin, amoxyl, erythromycin, chloramphenicol, ampiclox, levofloxacin, ampicillon, cefoxitin, amoxicillin and cefazidime. Multi-drug resistant isolates were then selected for plasmid DNA analysis, quantification, electrophoresis and curing.

Results: The isolates that showed resistance to more than two antibiotics were S. aureus.
E. aerogenes, C. freundii, K. pneumoniae, P. aeruginosa, Salmonella species, S. marcescens, Proteus species, S. marcescens, E. coli, and coagulase-negative Staphylococcus. The extracted plasmid DNA ranged from 1.2 - 80.5 ng/µl in quantity and showed the presence of SHV, CTX-M and MecA genes in the isolates with sizes of 154, 300 and 600-1 000 bp, respectively. All isolates had SHV while a few had CTX-M genes except S. aureus and coagulase-negative S. aureus which had mecA gene in addition. Chi-square and Fisher exact test for SHV gene was significant while CTX-M was not. Treatment with ethidium bromide showed that at a concentration of 0-20 µl, all isolates exhibited very heavy growth (+++), at 50-100 µl, only P. aeruginosa showed very heavy growth (+++) while other organisms showed heavy to moderate growth. However, at 400 to 1,000 µl, no growth was observed.

Conclusion: The presence of plasmid-borne multiple resistant genes is of great public health concern and this highlights the need for antibiogram and molecular typing in the control of nosocomial infections.

Keywords: Plasmid analysis; nosocomial infections; mecA; SHV; CTX-M genes.

1. INTRODUCTION

Healthcare associated infections are a problem globally [1]. The widespread use of antibiotics continues to influence this menace giving rise to antibiotic-resistant bacteria in the hospital setting and in the environment [2]. The environment of the hospital is an obvious important focus for the selection and spread of multi-resistant bacteria and a possible direct source of nosocomial infections [1,3,4]. Studies have shown that β-lactam agents including penicillin and cephalosporins are among the most frequently prescribed antibiotics globally. This has led to the development of resistance by bacteria species against these agents via various mechanisms including the acquisition of extra-chromosomal elements called plasmids which induces the production of extra-cellular enzymes called β-lactamases. There are speculations that resistance to β-lactam agents has influenced resistance of bacteria to other classes of antibiotics such as the quinolones and aminoglycosides with its attendant effect more devastating most especially on immune compromised patients [5]. Therefore, this study is aimed at evaluating the plasmid profile and curing of selected multidrug resistant bacteria isolates recovered from hospital settings.

2. MATERIALS AND METHODS

2.1 Ethical Approval and Source of Isolates

Following ethical approval (RP/REC/2015/338) given by the Cross River State Health Research Ethics Committee, a total of 240 swabs were obtained from the hospital environment of two hospitals (General and Infectious Disease Hospitals) in Calabar, Cross River State, Nigeria and were aseptically manipulated using standard microbiological techniques as previously reported [6,7,8,9].

2.2 Sensitivity Testing (Disc Diffusion Method)

Bacteria isolates were subjected to antibiotics sensitivity test using the Kirby Bauer disc diffusion method on Muller Hinton agar (Oxoid, UK) plates following procedures already described [10]. The antibiotics used were OFX-Ofloxacin, PEF-Reflacine, CPX-Ciprofloxacin, AU-Augmentin, CN-Gentamycin, S-Streptomycin, CEP-Ceporex, NA-Nalidixic acid, SXT-Septrin, PN-Amoxicillin, NB-Norfloxacin, AML-Amoxyl, E-Erythromycin, CH-Chloramphenicol, APX-Ampiclox, LEV-Levofoxacin, AM-Ampicillin, FOX-Cefoxitin, AMX-Amoxicillin and CAZ-Ceftazidime.

2.3 Selected Bacteria for Plasmid Analysis

Bacteria isolates that were resistant to at least three antibiotics were selected for plasmid analysis. They included S. aureus, E. aerogenes, C. freundii, K. pneumoniae, P. aeruginosa, Salmonella species, Proteus species, S. Marcescens, E. coli, Enterococcus species and coagulase-negative Staphylococcus.

2.4 Plasmid DNA Isolation

Multidrug-resistant bacteria isolates were revived in blood agar and MacConkey agar and subsequently, they were respectively grown
overnight in Luria-Bertani (LB) broth at 37°C with aeration using an orbital shaker and plasmid DNA was extracted from lyzed isolates using the ZYPPY™ Plasmid Miniprep Kit, (Inqaba Biotech., South Africa) following alkaline lysis method described previously [11].

2.5 Quantification of Plasmid DNA

The Nanodrop 1000 spectrophotometer (Thermo Scientific, Inqaba Biotech™ South Africa) was used to quantify the amount of plasmid DNA extracted from each isolate.

2.6 Polymerase Chain Reaction (PCR)

Quantified plasmids DNA of resistant isolates were amplified by standard PCR (Thermocycler, 9700). The forward and reverse primers sequences of SHV were F-5’-CGCCTGTGTATTATCTCCCT-3’, R-3’CGAGTAGTCCACCACCAGATCCT-5’, CTX-M: F-5’-CGCTTTGCGATGTGCAG-3’, R-3’-ACCGCGATATCGTTGGT-5’, MecA: F-5’-TAGAAATGACTGAACGTCCG-3’ and R-3’-TTGCGATCAAATGTTTACCGTAG-5’. They were subjected to 35, 25, 30 cycles for SHV, CTX-M and MecA genes, respectively.

2.7 Electrophoresis

Following PCR, electrophoresis was carried out in a horizontal gel apparatus (Scie-Plas Limited, Southern, Warwickshire, UK). Electrophoresis was conducted in 1.5% agarose gel (Inqaba Biotech., South Africa) and stained in ethidium bromide. The appropriate molecular masses of plasmids were determined by comparing with lambda DNA Hind III digest (Promega –USA) as a standard marker.

2.8 Plasmid Curing

This was carried out following methods described previously [12]. Briefly, 10ml of each bacterial culture inoculated into peptone water and incubated for 24hrs was introduced into a set of 20 test tubes, respectively. Ethidium bromide in various concentrations of 0, 20, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600,650, 700, 750 and 800 µl/ml were then introduced accordingly into the test tubes and incubated for 24 hrs at 37°C to determine the sub-lethal concentrations of ethidium bromide. After 24hrs of incubation, 1ml aliquot from each test tube was inoculated onto nutrient agar plates and incubated, after which colonies were selected and inoculated onto freshly prepared Muller Hinton agar plates. Then, antibiotic discs of prior resistance were aseptically introduced into the plates, ensuring that the discs made appropriate contact with the surface of the agar. These were incubated for 24 hrs at 37°C after which plates were examined for cured colonies.

2.9 Data Analysis

Data generated were managed and analysed with descriptive statistics, Chi-square and Fisher exact tests using SPSS version 21 while the level of significance was set at 0.05 (95%).

3. RESULTS

3.1 Antibiotic Resistance Pattern of Selected Bacteria for Plasmid Analysis

S. aureus, E. aerogenes, C. freundii, K. pneumoniae, P. aeruginosa and Salmonella species were each resistant to seven antibiotics, respectively while Proteus species were resistant to eight antibiotics. Furthermore, S. marcescens and E. coli strains were resistant to five antibiotics each while Enterococcus species and coagulase-negative Staphylococcus were each resistant to three antibiotics as shown in Table 1.

3.2 Quantification of Plasmid DNA

Various quantities of plasmid DNA ranging from 1.2ng/µl- 80.5ng/µl were extracted from selected resistant isolates recovered from both hospitals. Of the thirty plasmid DNA samples obtained, eighteen were from Gram negative bacteria while twelve were Gram positive bacteria. Isolates with serial numbers 1-12, 13-26 and 28 were obtained from General hospital while isolates with serial numbers 14-18, 27 and 29-30 were obtained from Infectious Disease Hospital (Table 2). Out of the 18 Gram negative bacteria, 4 were E. coli strains, 3 were P. aeruginosa and 3 were K. Pneumoniae strains, 2 were Salmonella species, 1 was S. marcescens, 3 were Proteus species, 1 C. freundii and 1 E. aerogenes. Furthermore, of the 12 Gram positive bacteria employed, 10 were S. aureus strains while one was Streptococcus species and the other Coagulase-negative Staphylococcus. In addition, higher quantities of plasmid DNA were recovered from Gram positive bacteria (S. aureus strains) while moderate to low quantities were observed predominantly in Gram negative bacteria (Table 2).
Table 1. Antibiotic resistance pattern of selected bacteria for plasmid analysis

| Bacteria isolates          | Antibiotics resistance |
|----------------------------|------------------------|
| *E. coli* (4)              | AM, AMX, CAZ, PN, CEP |
| *P. aeruginosa* (3)        | AM, AMX, CAZ, PEF, AU, SXT, PN |
| *Salmonella species* (2)   | AM, AMX, CEP, PN, CAZ, PEF, SXT |
| *Proteus species* (3)      | AM, AMX, CAZ, OFX, AU, PEF, CEP, PN |
| *Serratia marcescens*      | AM, AMX, CAZ, PN, CEP |
| *Citrobacter freundii*     | AM, AMX, CAZ, CEP, PN, CN, S |
| *Staphylococcus aureus*    | AML, CH, APX, FOX, AM, E |
| *Streptococcus species*    | CPX, CH, LEV |
| *Enterobacter aerogenes*   | PEF, AU, CEP, PN, AM, AMX, CAZ |
| *Coagulase-negative*       | AM, FOX, APX |
| *Staphylococcus* (1)       |                        |

KEY: OFX-Ofloxacin, PEF-Reflacine, CPX-Ciprofloxacinc, AU-Augmentin, CN-Gentamycin, S-Streptomycin, CEP-Ceporex, NA-Nalidixic acid, SXT-Sepritin, PN-Ampiclin, NB-Norfloxacin, AML-Amyxyl, E-Erythromycin, CH-Chloramphenicol, APX-Ampiclox, LEV-Levofloxacinc, AM-Ampicillin, FOX-Cefoxin, AMX-Amoxicillin, CAZ-Ceftazidime

3.3 Distribution of SHV, CTX-M and MecA Genes among Test Isolates

The extracted plasmid DNA samples when tested in combination with appropriate specific primers for the genes of interest respectively on PCR and subjected to electrophoresis exhibited varying bands on the trans-illuminator indicating the presence of SHV, CTX-M and MecA genes, respectively. The entire test isolates employed in this analysis possessed SHV genes while only 2(50%) of *E. coli* strains possessed CTX-M genes. All the 3(100%) isolates of *P. aeruginosa* studied possessed CTX-M genes while 2(66%) isolates of *K. pneumoniae* species and a species of *E. aerogenes* studied possessed CTX-M genes. Furthermore, of the two *Salmonella species* studied, only 1(50%) possessed CTX-M genes. *S. marcescens* and *C. freundii* species showed no genes for CTX-M. However, out of the 10 isolates of *S. aureus* screened, 1(10.0%) and 10(100%) possessed CTX-M, SHV and mecA genes, respectively. Furthermore, a strain of Coagulase-negative *Staphylococcus* possessed the mecA gene (Table 3).

A representation of the bands corresponding to the various genes is shown in Fig. 1. Chi square and Fischer exact tests were employed to compare the observed with the expected genes and it showed that the SHV gene was significant at 95% (p =0.001 and <0.0001, for Chi and fisher exact test, respectively). CTX-M gene was not significant (p > 0.05).

3.4 Effect of Treatment with Ethidium Bromide on Growth of Bacteria Isolates

Resistant bacteria isolates found to possess resistant plasmids when subjected to treatment with ethidium bromide exhibited various degrees of growth as presented on Table 4. At 0-20 µl concentration, all the test organisms employed in this study exhibited very heavy growth but at 50-100 µl, only *P. aeruginosa* had very heavy growth while *S. marcescens*, *C. freundii*, *S. aureus*, *Enterococcus species*, *E. aerogenes*, *E. coli*, *K. pneumoniae*, *Salmonella species*, *Proteus species*, Coagulase-negative *Staphylococcus* at 50 µl exhibited heavy growth. Meanwhile, *S. marcescens*, *C. freundii*, *S. aureus*, *Enterococcus species* and *E. aerogenes* exhibited moderate growth at 100µl while Coagulase-negative *Staphylococcus* and *Salmonella species* exhibited slight growth at the same concentration. At 200 µl, only *P. aeruginosa* had heavy growth while *E. coli* and *Proteus species* exhibited moderate growth. At 250 µl, only *P. aeruginosa* exhibited moderate growth. *E. coli* and *Proteus species* exhibited slight growth while the rest showed no growth. Furthermore, at 300 µl, only *P. aeruginosa* exhibited slight growth while the rest showed no growth. Consistently at 400-1000 µl, none of the isolates exhibited growth as presented in Table 4.

3.5 Susceptibility Pattern of Cured Isolates to Antibiotics

The susceptibility pattern of cured isolates when subjected to antibiotics was determined. All the cured *E. coli*, *Streptococcus*, *Salmonella*, *Proteus*, *Serratia*, *C. freundii* and *E. aerogenes* species showed susceptibility to all the antibiotics they were previously resistant to. Interestingly, one of the cured *P. aeruginosa* strains showed resistance to Ampicillin whereas the rest were susceptible to amoxicillin, cefazidime, reflacine, gentaminc, seprtin, ampicolin and ampicillin. In
addition, all *S. aureus* strains showed resistance to all the antibiotics previously except one isolate which showed moderate resistance to Penicillin and Cefoxitin.

**Table 2. Nanodrop quantified plasmid DNA**

| S/no | Organisms            | Quantity (ng/μl) of plasmid DNA | S/no | Organisms            | Quantity (ng/μl) of plasmid DNA |
|------|----------------------|----------------------------------|------|----------------------|----------------------------------|
| 1    | *E. coli*            | 27.7                             | 16   | *E. coli*            | 8.0                              |
| 2    | *P. aeruginosa*      | 20.5                             | 17   | *Proteus species*    | 12.8                             |
| 3    | *K. pneumoniae*      | 22.6                             | 18   | *Salmonella species* | 1.2                              |
| 4    | *P. aeruginosa*      | 8.4                              | 19   | *Staphylococcus aureus* | 74.0                             |
| 5    | *Salmonella species* | 10.5                             | 20   | ‾         | 5.6                              |
| 6    | *K. pneumoniae*      | 13.2                             | 21   | ‾         | 4.3                              |
| 7    | *Serratia marcescens*| 11.0                             | 22   | ‾         | 6.0                              |
| 8    | *Proteus species*    | 7.1                              | 23   | ‾         | 10.3                             |
| 9    | *Enterobacter aerogenes* | 5.5                             | 24   | ‾         | 80.5                             |
| 10   | *E. coli*            | 7.0                              | 25   | ‾         | 10.7                             |
| 11   | *K. pneumoniae*      | 67.0                             | 26   | ‾         | 6.6                              |
| 12   | *E. coli*            | 9.9                              | 27   | ‾         | 22.3                             |
| 13   | *Citrobacter freundii* | 5.3                             | 28   | *Streptococcus species* | 6.5                              |
| 14   | *Proteus species*    | 5.0                              | 29   | *Staphylococcus aureus* | 5.8                              |
| 15   | *P. aeruginosa*      | 18.8                             | 30   | *Coagulase-negative* | 53.9                             |
|      |                      |                                  |      |                      |                                  |

![Agarose gel electrophoresis demonstrating the amplified SHV, CTX-M and MecA from both Gram negative and positive bacteria](image)

**KEY:** Lanes 1-10 represent the SHV bands, Lanes 12, 13, 16-18 represent the CTX-M bands, 14 and 15 are negative for CTX-M. Lane 19 represents the Mec A gene while Lane 20 represents the negative. Control M is the 100 bp ladder

**Table 3. Distribution of SHV, CTX-M and mecA genes among test isolates**

| Organisms               | No examined (n-30) | SHV gene a(n -26, 86.7%) | CTX-M gene b(n -12, 40%) | MecA gene (n -11, 91.7%) |
|-------------------------|--------------------|--------------------------|--------------------------|--------------------------|
| *E. coli*               | 4(13.3)            | 4(100)*                  | 2(50)*                   | NA                       |
| *P. aeruginosa*         | 3(10.0)            | 3(100)                   | 3(100)                   | NA                       |
| *K. pneumoniae*         | 3(10.0)            | 3(100)                   | 2(66.7)                  | NA                       |
| *Salmonella species*    | 2(6.7)             | 2(100)                   | 1(50)                    | NA                       |
| *Proteus species*       | 3(10.0)            | 3(100)                   | 1(33.3)                  | NA                       |
| *Serratia marcescens*   | 1(3.3)             | 0(0.0)                   | 1(100)                   | NA                       |
| *Citrobacter freundii*  | 1(3.3)             | 1(100)                   | 0(0.0)                   | NA                       |
| *Staphylococcus aureus* | 10(33.3)           | 10(100)                  | 1(10.0)                  | 10 (100)                 |
| *Streptococcus species* | 1(3.3)             | 0(0.0)                   | 0 (0.0)                  | 0(0.0)                   |
| *Enterobacter aerogenes*| 1(3.3)             | 0(0.0)                   | 1(100)                   | NA                       |
| *Coagulase-negative*    | 1(3.3)             | 0(0.0)                   | 0(0.0)                   | 1 (100)                  |

*a*Represents significant Chi square and Fisher exact (p<0.0001) while b represent non-significant Chi square and Fischer (P > 0.05). NA-Not applicable
Table 4. Effect of ethidium bromide on growth of resistant bacteria isolates

| Test isolates                  | 0µL | 20µL | 50µL | 100µL | 200µL | 250µL | 300µL | 400µL | 800µL | 1000µL |
|-------------------------------|-----|------|------|-------|-------|-------|-------|-------|-------|--------|
| E. coli (4)                   | +++ | +++  | ++   | ++    | ±     | -     | -     | -     | -     | -      |
| P. aeruginosa (3)             | +++ | +++  | +++  | +++   | +     | ±     | -     | -     | -     | -      |
| K. pneumoniae (3)             | +++ | +++  | ++   | ++    | ±     | -     | -     | -     | -     | -      |
| Salmonella species (2)        | +++ | +++  | ±    | -     | -     | -     | -     | -     | -     | -      |
| Proteus species (3)           | +++ | +++  | ++   | +     | ±     | -     | -     | -     | -     | -      |
| Serratia marcescens (1)       | +++ | +++  | +    | ±     | -     | -     | -     | -     | -     | -      |
| Citrobacter freundii (1)      | +++ | +++  | +    | ±     | -     | -     | -     | -     | -     | -      |
| Staphylococcus aureus (11)    | +++ | +++  | +    | ±     | -     | -     | -     | -     | -     | -      |
| Streptococcus species (1)     | +++ | +++  | +    | ±     | -     | -     | -     | -     | -     | -      |
| Enterobacter aerogenes (1)    | +++ | +++  | +    | ±     | -     | -     | -     | -     | -     | -      |
| Coagulase negative Staphylococci | +++ | +++  | ±    | -     | -     | -     | -     | -     | -     | -      |

KEY: +++ -- Very heavy growth (100%), ++ -- Heavy growth (70-90%), + -- Moderate growth (30-50%), ± -- Slight growth (20–25%) and (-) -- No growth (0%)

4. DISCUSSION

Plasmids have been described as extrachromosomal elements capable of independent replications. These DNA molecules are different from the chromosomal DNA and are present in bacteria [12]. The relationship between multiple antibiotic resistance patterns and plasmid profiles observed generally suggests the significant role of plasmids in the development of multidrug resistance in bacteria species [13]. As revealed by Pattnaik et al. [14] plasmid profile analysis is a method used in determining the presence, type, size and number of plasmids in bacterial isolates. Furthermore, the presence of resistant genes in the plasmids of bacteria explains to a large extent the drug resistance among these isolates.

Agarose gel electrophoresis of plasmid DNA showed that out of the four strains of E. coli subjected to analysis, all of them harboured the SHV genes while only two possessed the CTX-M genes. CTX-M gene in recent years has been implicated in extended spectrum β-lactamases (ESBL) production in E. coli due to its wide spread use which is usually via plasmid transmission between unrelated strains. The finding of CTX-M in these isolates is consistent with reports of Oteo et al. [15] and Amaya et al. [5] who revealed that 30.8% and 26% of E. coli strains observed in their studies respectively possessed blaCTX-M enzymes which hydrolyse mainly cefotaxime but weakly active against Ceftazidime. Though, Oteo et al. [15] observed that a few CTX-M enzymes especially CTX-M-15 and CTX-M-32 exhibited increased hydrolytic activities against Ceftazidime.

The finding of the SHV in E. coli strains is in accordance with report of Hosoglu et al. [16] who reported SHV mediated resistance in 28.6% of E. coli strains. In addition, Kaye et al. [17] reported ampC type of extended spectrum β-lactamase gene in E. coli strains employed in their studies. As revealed by David [18] P. aeruginosa species possess unique characteristics including its ability to acquire resistance either via mutation or extra-chromosomal elements as well as efflux pump mechanisms. Interestingly, all the P. aeruginosa strains analysed for plasmid in this study possessed both the SHV and CTX-M genes. According to Picao et al. [19] the presence of CTX-M gene in P. aeruginosa confirms the assertion that this gene is not exclusive to Enterobacteriaceae family. As revealed by Odumosu et al. [20]. The finding of SHV and CTX-M genes in P. aeruginosa isolates is consistent with reports of Du et al. [21] and these further confirm the isolation of CTX-M and SHV ESBLs from isolates in this study.
The three isolates of *K. pneumoniae* subjected to plasmid analysis all possessed *SHV* gene while only two possessed the *CTX-M* gene. Studies have shown that these strains of microorganisms constitutively express chromosomal class A β-lactamases including *SHV, CTX* and *TEM* and are wide spread globally [22]. The finding of constitutively express chromosomal class A β-lactamases in these genes in the plasmid DNA harboured by *K. pneumoniae* in this study rather suggest that the production of the *SHV* and *CTX-M* β-lactamases in these organisms is both chromosomal and plasmid encoded. Which could have resulted from environmental interactions with other plasmid harbouring members of the *Enterobacteriaceae* family probably due to the selection pressure emanating from the massive prescription and misuse of broad spectrum antibiotics [22]. These ESBL (*CTX-M*) encoding genes as reported serve as additional resistance determinants to aminoglycosides and fluoroquinolones since they are often co-transferred on the same plasmid [23,24,22].

Furthermore, of the two *Salmonella* species subjected to plasmid profiling, *SHV* and *CTX-M* genes were recovered from two and one isolate respectively. This is consistent with previous report which revealed that the four classes of β-lactamases including A, B, C and D of which *SHV* and *CTX-M* are parts; known to confer resistance to antibiotics including β-lactam drugs are plasmid mediated [25]. This is because as further revealed by them, *Salmonella* species lack chromosomally encoded elements responsible for β-lactamase production compared to other gram negative bacilli. In addition, Elumalai et al. [26] reported *blaTEM* genes encoding *TEM-1* β-lactamase as the gene that conferred resistance in *Salmonella* species to penicillins and early cephalosporins observed in their study. Rayamajhi [25] further reported that *SHV* enzymes of Class A β-lactamases often derived by one or more amino acid substitution around the active sites of the enzyme and responsible for ESBLs phenotypes are generally found in Gram negative bacteria.

Meanwhile, of the three *Proteus* species profiled for plasmids in this study, all possessed the *SHV* gene while one isolate possessed the *CTX-M* gene. This observation is consistent with that of Aragon et al. [27] who confirmed that *Proteus* species lack intrinsic chromosomally encoded β-lactamase genes and as such, depend entirely on acquisition of various β-lactamase genes to express a β-lactamase mediated resistance phenotype. It is reported that the prevalence of *blaCTX-M* and *blaSHV* among *Proteus* species with *CTX-M* present either alone or in combination with other β-lactamase genes [28]. Furthermore, *CTX-M β-lactamase* has been shown to be responsible for the resistance of *Proteus* species to antibiotics including Aminoglycosides [27].

*CTX-M* gene was discovered in *S. marcescens* in this study is consistent with the report of Yang et al. [29] who noted that these strains harbour *ESBL* genes including *CTX-M* and *SHV* with *CTX-M-3* being the most common. Wu et al. [30] further reported that strains of *S. marcescens* who concurrently harboured *CTX-M-3* and *SHV-5* conferred high level resistance to cephalosporins including cefotaxime and ceftazidime. Thus, the high resistance exhibited by this organism may have been due to the *CTX-M* genes observed in this study. However, Yatsuyanagi et al. [31] noted that *S. marcescens* possessed chromosomally encoded *ampC* and *blaTEM* genes for β-lactamases respectively. Thus, ability of *S. marcescens* to encode extended spectrum β-lactamases both chromosomally as well as via plasmid acquisition coupled with its array of virulence and resistance mechanisms makes *S. marcescens* an organism of public health importance.

*C. freundii* profiled in this study harboured only the *SHV* gene. This confirms reports of Pepperell et al. [32] who revealed that this strain of organisms harbour extended spectrum β-lactamase gene that encode *SHV* enzymes; specifically SHV-5 and SHV-12. Since there is a general believe that β-lactamases do not only confer resistance to β-lactam drugs but also to other groups of antibiotics, the resistance of this species to other antibiotics may have been as a result of this. Studies have shown that multi drug resistance in *Enterobacteriacaeae* has been strongly linked with the carriage of integrons, genetic elements capable of assimilating antibiotic resistant genes as cassettes via site specific recombination catalysed by a specific integrase enzyme [33].

The *E. aerogenes* strain employed in this study revealed the presence of *CTX-M* gene. This observation is consistent with the report of Davin-Regli and Pages [34] who noted that mobile genetic elements (plasmid) encoding *CTX-M* and *TEM* enzymes have greatly influenced the resistance of these species of organisms to antibiotics globally [33]. However, in addition to plasmid encoded ESBL production capability of this organism, it possesses efflux pumping
mechanisms and chromosomally encoded ampC gene which express low level resistance coupled with its ability to readily undergo mutation. These combined features enhance the resistance of this organism to almost all antibiotics commonly used [34].

Consistently, S. aureus strains subjected to plasmid analysis revealed that all possessed the SHV and mecA genes while only one strain possessed the CTX-M gene. The finding of SHV and CTX-M genes in S. aureus strains seem to be an amazing discovery but since these genes are mostly associated with Gram negative organisms thus, herald more research in this area. Meanwhile, the finding of mecA gene in S. aureus strains employed in this study is consistent with report of Pantosti et al. [35] who revealed that methicillin resistance in S. aureus is due to decrease affinity to β-lactam drugs mediated by low-affinity specific penicillin-binding proteins PBP-2A. Iyer et al. [36] added that all methicillin-resistant strains of S. aureus possessed mecA gene carried by “staphylococcal-cassette chromosome mec” (SCCmec) located in the bacterial cell wall; which encode PBP-2A. The possession of the SCCmec has enabled the resistance of S. aureus not only to methicillin or β-lactam drugs but to other groups of antimicrobial agents. In addition, Akcam et al. [37] reported that Fem factors (FemA, FemB and FemX) in this species could contribute to methicillin resistance. In addition, Coagulase-negative Staphylococcus strain employed in this study was found to possess mecA gene. The finding of mecA gene in Coagulase negative Staphylococcus is consistent with reports of Khadri and Alzohairy [37,38].

According to Spengler et al. [39] curing agents including ethidium bromide act on plasmid either via inhibition of plasmid efflux pumps on the plasmid membrane or inhibition of DNA gyrase responsible for plasmid DNA replication. They further added that this curing agent have a better activity compared to other physical and chemical agents including sodium dodecyl sulphate and acridine orange. The efficiency of curing has also been reported to vary depending on the type of plasmid and the bacterial host harbouring it [40]. The bacteria isolates when subjected to curing agent at varying concentrations, exhibited different pattern of growth especially at low concentrations and high concentrations. Lack of growth of the isolates at higher concentrations of ethidium bromide may be as a result of the toxic nature of this agent. The elimination of bacterial plasmid (curing) from culture is considered a significant procedure to substantiate the relationship between a genetic trait and carriage of specific plasmids by the isolates as the phenotypic characters which are associated with the plasmid are not expressed in cured derivatives. Cured cells were achieved at 50-300µl of ethidium bromide. When rejuvenated in an all purpose medium and exposed to antimicrobial agents to which they were previously resistant to, these isolates showed susceptibility to all antibiotics of prior resistance except P. aeruginosa which showed resistance to ampicillin and S. aureus to Penicillin and cefoxitin.

The susceptibility of most of the isolates to antimicrobial agents previously resistant to may be due to the curing action of ethidium bromide against these isolates and as reported by Raghada et al. [12] this further confirms the fact that the resistant genes were harbour on the plasmids. However, resistance of P. aeruginosa to ampicillin and S. aureus to penicillin and cefoxitin may be an indication that resistance of these organisms against antimicrobial agents are not only plasmid encoded. This confirms the assertion of most researchers including Spengler et al. [39], Zaman et al. [40] and Raghada et al. [12] that most microorganisms possess intrinsic resistance mechanisms which enable them to deactivate drug. Regardless of whether these organisms are normal or opportunistic pathogens and possess resistance mechanisms or not, infection control procedures should be prioritized in healthcare settings taking into consideration the fact that this environment house individuals seen to be more vulnerable to these pathogens.

5. CONCLUSION

The findings of the beta lactamase genes from environmental isolates in this study is of great public health concerns and could have considerable role in the dissemination of multidrug resistant bacteria among hospitalized patients. We therefore recommend antibiogram and molecular typing in the control and management of nosocomial infections in our hospitals. In addition, a review of hospital infection control practices will ensure that these infections are reduced drastically.

CONSENT

It is not applicable.
ETHICAL APPROVAL

Ethical approval was sought for and obtained from the Cross River State Health Research Ethics Committee (CRS-HREC). The approval number for this is RP/REC/2015/338.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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