

Plasmid profile analysis and curing of multidrug-resistant bacteria isolated from two hospital environments in Calabar Metropolis, Nigeria.

ABSTRACT

The aim of this study was to determine the plasmid profile and curing of environmental isolates showing multi-drug resistance. Isolates were obtained from air and surfaces of two hospitals using standard microbiological techniques. The isolates were then subjected to sensitivity using ofloxacin, reflacine, ciprofloxacin, augmentin, gentamycin, streptomycin, ceporex, nalidixic acid, septrin, ampicin, norfloxacin, amoxyl, erythromycin, chloramphenicol, ampiclox, levofloxacin, ampicillin, cefoxitin, amoxicillin and ceftazidime. Multi-drug resistant isolates were then selected for plasmid DNA analysis, quantification, electrophoresis and curing. 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 – 80.5ng/μl in quantity and showed the presence of *SHV*, *CTX-M* and *MecA* genes in the isolates with sizes of 154, 300 and 600-1000bp, 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 0-20μl, all isolates exhibited very heavy growth (+++), at 50-100μl, only *P. aeruginosa* showed very heavy growth (+++) while other organisms showed heavy to moderate growth. However, at 400 to 1,000μl, no growth was observed. 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.

Key words: Plasmid analysis, nosocomial infection, *mecA*, *SHV* and *CTX-M* genes.

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]. Studies have shown that β-lactam agents 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 with its attendant effect more devastating most especially on immune compromised patients [4]. Therefore, this study is aimed at evaluating the plasmid profile and curing of selected multidrug resistant bacteria isolates recovered from hospital settings.

MATERIALS AND METHODS

Source of isolates

A total of 240 swabs obtained from the hospital environment of two hospitals (General and Infectious Disease Hospital) in Calabar, Cross River State, Nigeria and were aseptically manipulated using standard microbiological techniques as previously reported [5] [6] [7] [8].

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 [9]. The antibiotics used were OFX-Ofloxacin, PEF-Reflacine, CPX-Ciprofloxacin, AU-Augmentin, CN-Gentamycin, S-Streptomycin, CEP-Ceporex, NA-Nalidixic acid, SXT-Septrin, PN-Amplicin, NB-Norfloxacin, AML-Amoxyl, E-Erythromycin, CH-Chloramphenicol, APX-Ampiclox, LEV-Levofloxacin, AM-Ampicillin, FOX-Cefoxitin, AMX-Amoxicillin and CAZ-Ceftazidime.

Selected Bacteria for Plasmid Analysis

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

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 lysed isolates

using the ZYPHY™ Plasmid Miniprep Kit, (Inqaba Biotech., South Africa) following alkaline lysis method described previously [10].

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.

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, CTX-M and *MecA* genes *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', R-3'-TTGCGATCAAATGTTTACCGTAG-5'. They were subjected to 35, 25, 30 cycles for *SHV*, *CTX-M* and *MecA* genes, respectively.

Electrophoresis

Following the different complete cycles of 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 mass of plasmids was determined by comparing with lambda DNA Hind III digest (Promega –USA) as a standard marker.

Plasmid Curing

This was carried out following methods described previously [11]. 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 800µl/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 24hrs at 37°C after which plates were examined for cured colonies.

Data Analysis

Data generated were managed and analysed using SPSS version 21 while then level of significance was set at 0.05 (95%).

RESULTS

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 respectively while *Enterococcus species* and coagulase-negative *Staphylococcus* were each resistant to three antibiotics as shown in Table 1.

Table 1: Antibiotic resistance pattern of selected bacteria for plasmid analysis

Bacteria isolates	Antibiotics resistance
<i>E. coli</i> (4)	AM, AMX, CAZ, PN, CEP
<i>P. aeruginosa</i> (3)	AM, AMX, CAZ, PEF, AU, SXT, PN
<i>Salmonella species</i> (2)	AM, AMX, CEP, PN, CAZ, PEF, SXT
<i>Proteus species</i> (3)	AM, AMX, CAZ, OFX, AU, PEF, CEP, PN
<i>Serratia marcescens</i>	AM, AMX, CAZ, PN, CEP
<i>Citrobacter freundii</i>	AM, AMX, CAZ, CEP, PN, CN, S
<i>Staphylococcus aureus</i> (11)	AML, CH, APX, FOX, AM, E
<i>Streptococcus species</i> (1)	CPX, CH, LEV
<i>Enterobacter aerogenes</i> (1)	PEF, AU, CEP, PN, AM, AMX, CAZ
Coagulase-negative <i>Staphylococcus</i> (1)	AM, FOX, APX

KEY: OFX-Ofloxacin, PEF-Reflacine, CPX-Ciprofloxacin, AU-Augmentin, CN-Gentamycin, S-Streptomycin, CEP-Ceporex, NA-Nalidixic acid, SXT-Septrin, PN-Amplicin, NB-Norfloxacin, AML-Amoxyl, E-Erythromycin, CH-Chloramphenicol, APX-Ampiclox, LEV-Levofloxacin, AM-Amicillin, FOX-Cefoxitin, AMX-Amoxicillin, CAZ-Ceftazidime.

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 General hospital and Infectious Disease hospital respectively. 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 as presented on 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 as shown in Table 2.

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	<i>E. coli</i>	27.7	16	<i>E. coli</i>	8.0
2	<i>P. aeruginosa</i>	20.5	17	<i>Proteus species</i>	12.8
3	<i>K. pneumoniae</i>	22.6	18	<i>Salmonella species</i>	1.2
4	<i>P. aeruginosa</i>	8.4	19	<i>Staphylococcus aureus</i>	74.0
5	<i>Salmonella species</i>	10.5	20	"	5.6
6	<i>K. pneumoniae</i>	13.2	21	"	4.3
7	<i>Serratia marcescens</i>	11.0	22	"	6.0
8	<i>Proteus species</i>	7.1	23	"	10.3
9	<i>Enterobacter aerogenes</i>	5.5	24	"	80.5
10	<i>E. coli</i>	7.0	25	"	10.7
11	<i>K. pneumoniae</i>	67.0	26	"	6.6
12	<i>E. coli</i>	9.9	27	"	22.3
13	<i>Citrobacter freundii</i>	5.3	28	<i>Streptococcus species</i>	6.5
14	<i>Proteus species</i>	5.0	29	<i>Staphylococcus aureus</i>	5.8
15	<i>P. aeruginosa</i>	18.8	30	Coagulase-negative <i>Staphylococcus</i>	53.9

Distribution of *SHV*, *CTX-M* and *MecA* genes among Test Isolates

The extracted plasmid DNA samples when tested in combination with appropriate primers specific for the genes of interest respectively on PCR system 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*. In addition, 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 as shown in Table 3.

A representation of the bands corresponding to the various genes is shown in Figure 1. The statistical analysis of Chi square and Fischer exact tests employed to compare the observed with the expected showed that the *SHV* gene was significant at 95% probability level with 0.001 and <0.0001 respectively. Meanwhile, the *CTX-M* gene at 95 % probability level was not significant.

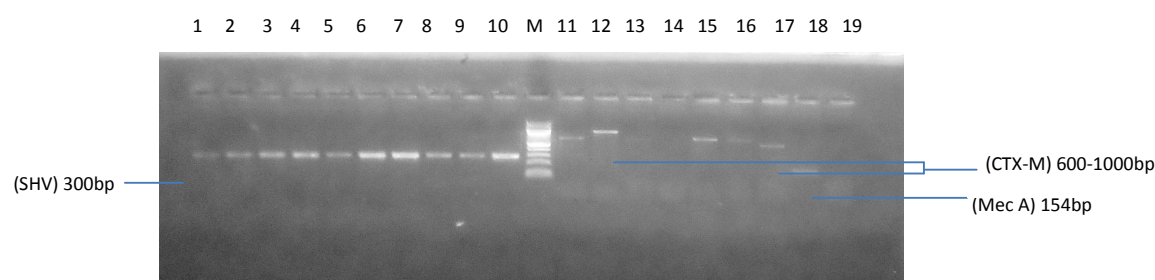


Fig 1: Agarose gel electrophoresis demonstrating the amplified *SHV*, *CTX-M* and *MecA* from both Gram negative and positive bacteria.

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 100bp ladder.

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

Organisms	No examined (n-30)	<i>SHV</i> gene a(n -26, 86.7%)	<i>CTX-M</i> gene b(n -12, 40%)	<i>MecA</i> gene (n -11, 91.7%)
<i>E. coli</i>	4(13.3)	4(100) ^a	2(50) ^b	NA
<i>P. aeruginosa</i>	3(10.0)	3(100)	3(100)	NA
<i>K. pneumoniae</i>	3(10.0)	3(100)	2(66.7)	NA
<i>Salmonella species</i>	2(6.7)	2(100)	1(50)	NA
<i>Proteus species</i>	3(10.0)	3(100)	1(33.3)	NA

<i>Serratia marcescens</i>	1(3.3)	0(0.0)	1(100)	NA
<i>Citrobacter freundii</i>	1(3.3)	1(100)	0(0.0)	NA
<i>Staphylococcus aureus</i>	10(33.3)	10(100)	1(10.0)	10 (100)
<i>Streptococcus species</i>	1(3.3)	0(0.0)	0 (0.0)	0(0.0)
<i>Enterobacter aerogenes</i>	1(3.3)	0(0.0)	1(100)	NA
Coagulase-negative <i>Staphylococcus</i>	1(3.3)	0 (0.0)	0(0.0)	1 (100)

KEY: ^aRepresents significant Chi square and Fisher exact (p<0.0001) while b represent non-significant Chi square and Fischer (P > 0.05). NA-Not applicable.

Effect of Treatment with Ethidium Bromide on Growth of Bacteria Isolates

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

TABLE 4: Effect of ethidium bromide on growth of resistant bacteria isolates

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

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

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 previously resistant to. Meanwhile, one of the cured *P. aeruginosa* strains showed resistance to Ampicillin whereas the rest were susceptible to amoxicillin, ceftazidime, reflacine, agmentin, septrin, amplicin and ampicillin. In addition, all *S. aureus* strains showed susceptibility to all the antibiotics previously resistant to except one isolate which showed moderate resistance to Penicillin and Cefoxitin.

DISCUSSION

Plasmids have been described as extra chromosomal elements capable of independent replications. These DNA molecules are different from the chromosomal DNA and are present in bacteria [11]. 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 [12]. As revealed by Pattnaik *et al* [13] plasmid profile analysis is a method used in determining the presence, type, size and number of plasmids in bacterial isolates. According to them, the presence of resistant genes in the plasmids of bacteria explains to a large extent the drug resistance among these isolates. Therefore, figure 1 in this study is a representation of some of the resistant plasmid genes harboured by these pathogens.

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 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* [14] and Amaya *et al* [4] who revealed that 30.8% and 26% of *E. coli* strains studied in their studies respectively possessed *bla*_{CTX-M} enzymes which hydrolyse mainly cefotaxime but weakly active against Ceftazidime. Though, Oteo *et al* [14] 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* [15] who reported *SHV* mediated resistance in 28.6% of *E. coli* strains. In addition, Kaye *et al* [16] reported *ampC* type of extended spectrum β -lactamase gene in *E. coli* strains employed in their studies. They further revealed that these β -lactamase genes either act singly or in combination conferring active resistance to commonly used antibiotics. These studies confirm that these extra-chromosomal elements were acquired through interaction with mobile genetic elements including plasmids and transposons thus justifying the fact that extra-chromosomal elements are significant to the development of resistance in bacteria.

As revealed by David [17] *P. aeruginosa* species possess uniquely characteristics including its ability to acquire resistance either via mutation or extra-chromosomal elements as well as its inherent resistance to antibiotics via efflux pump mechanisms. Thus, all the *P. aeruginosa* strains analysed for plasmid in this study possessed both the *SHV* and *CTX-M* genes. According to Picao *et al* [18] 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* [19], *P. aeruginosa* harbours multiple copies of plasmids with resistant genes that have been implicated in multiple antibiotics resistance. The finding of *SHV* and *CTX-M* genes in *P. aeruginosa* isolates is consistent with reports of Du *et al* [20] who revealed that resistance in this strain could be due to Ambler Class A β -lactamase including *bla*_{VEB}, *bla*_{GES},

bla_{TEM}, *bla_{PER}*, *bla_{SHV}*, class B β -lactamases including *bla_{SPM}*, *bla_{IMP}* or *bla_{VIM}* as well as class D β -lactamases including *bla_{OXA}*, all of which they encountered in their work. This further confirms the isolation of *CTX-M* and *SHV* ESBLs from isolates in this study.

In addition, of the three isolates of *K. pneumoniae* subjected to plasmid analysis, all of them possessed the *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 [21]. The finding of 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. This may have resulted from interactions with other plasmid harbouring members of the *Enterobacteriaceae* family in the environment which is due to the selection pressure emanating from the massive prescription and misuse of broad spectrum antibiotics [21]. 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 [22][23][21].

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* is a part; known to confer resistance to antibiotics including β -lactam drugs are plasmid mediated [24]. 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* [25] reported *bla_{TEM}* genes encoding *TEM-1* β -lactamase as the gene that conferred resistance in *Salmonella species* to penicillins and early cephalosporons observed in their study. Rayamajhi [24] 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* [26] 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 *bla*_{CTX-M} and *bla*_{SHV} among *Proteus species* with *CTX-M* present either alone or in combination with other β -lactamase genes [27]. Furthermore, *CTX-M* β -lactamase has been shown to be responsible for the resistance of *Proteus species* to antibiotics including Aminoglycosides [26].

CTX-M gene was discovered in *S. marcescens* strain subjected to plasmid profiling in this study. This observation is consistent with the report of Yang *et al* [28] who noted that these strains harbour *ESBL* genes including *CTX-M* and *SHV* with *CTX-M-3* being the most common. Wu *et al* [29] 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* [30] noted that *S. marcescens* possessed chromosomally encoded *ampC* and *bla*_{TEM} 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* [31] 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 *Enterobacteriaceae* has been gravely 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 [32].

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 [33] 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 [32]. However, in addition to plasmid encoded *ESBL* production capability of this organism, it possess 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 [33].

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* [34] 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* [35] 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* [36] reported that *Fem* factors (*FemA*, *FemB* and *FemX*) in this species could contribute to methicillin resistance.

In addition, a strain of 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,(2010) who revealed that this strain of *Staphylococci* harboured *SCCmec* type IV; a protein that encode PBP-2A. To confirm the possession of this protein, Khadri and Alzohairy [37] further asserted that this strain of organisms is responsible for the resistance to methicillin exhibited by methicillin sensitive *S. aureus* (MSSA).

Curing agents including ethidium bromide, sodium dodecyl sulphate are often employed for the curing of plasmid related agents. For this research, ethidium bromide was employed as a curing agent. This curing agent according to Raghada *et al* [11] act by inactivating the plasmid DNA replication during cell division; resulting to the presence plasmid-less cells in the next generations. According to Spengler *et al* [38] 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 [39]. The bacteria isolates when subjected to curing agent at varying concentrations, exhibited different pattern of growth especially at low concentrations of ethidium bromide and at higher concentrations, the isolates showed no growth at all. 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* [11] this further confirms the fact that the resistant molecules were harboured 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* [38], Zaman *et al* [39] and Raghada *et al* [11] that most microorganisms possess intrinsic resistance mechanisms which enable them to deactivate drug potencies. 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.

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.

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