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Plasmid Profiling and Antibiotic Resistance of Extended Spectrum Beta Lactamases Producing *Pseudomonas aeruginosa* Expressing Amp C Beta-Lactamase Enzyme

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Abstract: This study determined the plasmid profile and antibiotic resistance patterns of extended spectrum beta lactamases producing *Pseudomonas aeruginosa* expressing Amp C beta lactamase enzyme using standard procedures. Results obtained revealed that 32 (43.2%) had one plasmid, 37(50%) had two plasmids while the remaining five carried three plasmids each. The plasmid sizes ranged from 800bp to 25000-bp while eighteen different profiles were encountered. 10.8% of the strains (8) presented profile 1, 2.7% of the strains (2) presented profile 2, 4, 8 and 17. 16.2% of the strains (12) presented profile 3, 4.05% of the strains (3) presented profile 5, 9, 11, 14, 15 and 18. 6.8% of the strains (5) presented profile 6 and 10. 8.1% of the strains (6) presented profile 7 and 5.4% of the strains (4) presented profile 12, 13 and 16. The treatment of the plasmid positive isolates with acridine orange, SDS and ETBR suggest SDS as the most efficacious curing agents. The antibiotic resistance pattern transferred from three of the five donor strains to the transconjugants were partial and were associated with the transfer of R plasmids of sizes 24, 23, 4, 2, 25kbp. The frequencies of transfer of R plasmids to the transconjugants ranged from 1.4×10^{-5} to 1.7×10^{-7} per donor strains.

Key words: Plasmid · ESBL · Amp C · *Pseudomonas aeruginosa* · Curing Agents

INTRODUCTION

The role of *Pseudomonas aeruginosa* in a variety of human infections ranging from neonatal sepsis, burn sepsis and acute and chronic lung infections has been documented [1]. This organism is a common opportunistic pathogen, leading to infections in patients with defects in host defenses, such as chronic neutropenias and defects of neurtrophil function, hematologic cancers, human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome (AIDS) and diabetes mellitus. In addition chronic pulmonary disease is common in patients with cystic fibrosis.

The National Nosocomial Infections Surveillance (NNIS) System reports *P. aeruginosa* to be the second most common organism isolated in nosocomial pneumonia (17% of cases), the third most common organism isolated in both urinary tract infection (UTI) and surgical site infection (11% of cases) and the fifth most common organism isolated from all sites of nosocomial infection (9% of cases) [2]. To treat bacteriological infections, it is a common practice to employ antibiotics [3].

Bacterial resistance to different classes of antibiotics have been reported [4, 5] in *Pseudomonas aeruginosa* isolates from different sources. Most of these resistances are acquired and may be caused by production of plasmid mediated Amp C beta (β)-lactamase, extended spectrum β-lactamase and metallo β-lactamase (MBL) enzymes [6]. β-lactamases are the most widespread and effective mechanism through which bacteria can become resistant to β-lactam drugs. With the increasing use of β-lactam drugs and introduction of various inhibitor

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combinations such as amoxicillin-clavulanic acid or sulbactam, Ambler class C and Bush group I β-lactamase enzyme, known as AmpC β-lactamases have emerged and are being reported worldwide with varying prevalence rates [7-9].

Amp C beta lactamases inactivates the effects of broad-spectrum cephalosporins and penicillins while production of these enzymes in clinically significant *Enterobacteriaceae* represents an increasing problem resulting in higher patient morbidity and mortality [10]. For example, a recent study described a mortality rate of as high as 60% in patients with bloodstream infections due to ESBL-positive *Enterobacteriaceae* when adequate antibiotic therapy was not administered [11]. ESBL-positive *Enterobacteriaceae* which produce ESBLs and Amp C broad-spectrum beta-lactamases have been studied for more than two decades [12, 13]. Similarly, articles describing their prevalence in food animals and foods of animal origin have been published in recent years [14-19].

In many bacteria, Amp C enzymes are inducible and can be expressed at high levels by mutation. Overexpression confers resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime and ceftriaxone and is a problem [20] especially in infections due to *Pseudomonas aeruginosa*, where an isolate initially susceptible to these agents may become resistant upon therapy. This resistance may be located on transmissible plasmids. Transmissible plasmids have acquired genes for Amp C enzymes, which consequently can now appear in bacteria lacking or poorly expressing a chromosomal Amp C gene, such as *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. Resistance due to plasmid mediated Amp C enzymes is less common than ESBL production in most parts of the world but may be both harder to detect and broader in spectrum [14].

In Nigeria, indiscriminate use of antibiotics, poor hygienic practices in hospitals and lack of monitoring of microbial drug resistance have created suitable conditions for the emergency and uncontrollable spread of the ESBLs and Amp C enzymes [21] and thus make their detection complicated due to the variable affinity of these enzymes for different substrates and inoculum effect. Although, for more than a decade, since plasmid-mediated Amp C beta-lactamases were discovered, most clinical laboratories remain ignorant of their clinical consequences [22]. Consequently microorganisms producing these enzymes are concealed and are primarily liable for various nosocomial infections in hospitals.

Bacterial plasmids serve as the scaffold on which are assembled arrays of antibiotic resistance genes, by transposition (transposable elements and ISCR mediated transposition) and site-specific recombination mechanisms (integron gene cassettes). The evidence suggests that antibiotic resistance genes in human bacterial pathogens originate from a multitude of bacterial sources, indicating that the genomes of all bacteria can be considered as a single global gene pool into which most, if not all, bacteria can dip for genes necessary for survival. In terms of antibiotic resistance, plasmids serve a central role, as the vehicles for resistance gene capture and their subsequent dissemination [23].

Plasmids, which are extra chromosomal double-stranded DNA materials, have been found to be useful for pathogens' genetic diversity and prowess as infectious agents. Profiling pathogens for their harbored plasmids has been found to be very useful in epidemiological studies, diagnosis and elucidation of mechanisms of drug resistance [23]. Plasmids have also been found useful in knowing whether two or more strains of a pathogen evolve from the same microorganism, thereby providing a reliable insight into the genetic relatedness of pathogens in an environment [23, 24]. This study was aimed at determining the plasmid profile and antibiotic resistance patterns of extended spectrum beta lactamases producing *Pseudomonas aeruginosa* expressing Amp C beta lactamase enzyme.

MATERIALS AND METHODS

Bacterial Strains: The bacterial strains used in this study were supplied by Mrs O.D.Popoola of the Department of Microbiology (Medical Microbiology unit), Nnamdi Azikwe University, Awka, Anambra, Nigeria.

Antimicrobial Susceptibility Testing: Antimicrobial susceptibility was performed by the Kirby-Bauer disk diffusion method for various antibiotics, namely: Ampicillin (30µg), Amikacin (30µg), Co-trimoxazole (25µg), Ciprofloxacin (5µg), Ceftizoxime (30µg), Cefuroxime (30µg), Kanamycin (30µg), Piperacillin (100µg), Gentamicin (10µg), Piperacillin/ tazobactam (100µg/10µg) and Carbenicillin (100µg) (Abtek, U.K.). The results were interpreted based on the CLSI interpretative standard chart [25]

Amp C Disc Test: Screening for Amp C β-lactamase production was performed by Cefoxitin disk test. Isolates that yielded a zone diameter less than 18 mm (screen positive) were further subjected to confirmatory testing.

The disk antagonism test was used for detection of inducible Amp C β-lactamase in all the isolates of *P. aeruginosa*. A test isolate (with a turbidity equivalent to that of 0.5 McFarland standards) was spread over a Mueller Hinton agar (Oxoid, U.K) plate. Cefotaxime (30µg) and cefoxitin (30µg) (Abtek, U.K) disks were placed 20 mm apart from centre to centre. Isolates showing blunting of the cefotaxime zone of inhibition adjacent to the cefoxitin disk were screened as positive for Amp C β-lactamase. Further confirmation of Amp C production was tested by a modified three-dimensional test as described [26]. This method was particularly helpful in detecting non inducible Amp C B-lactamases. The extended spectrum beta-lactamase (ESBL) status of these strains was established by combined disk diffusion method per CLSI recommendations [25] using Cefotaxime (30µg) and Ceftazidime (30µg) disks alone and in combination with clavulanic acid. Metallo β-lactamase production was detected by Imipenem-EDTA disk test. Two 10 µg imipenem disks were placed on the plate and appropriate amounts of 10 µl of 0.5M EDTA solution were added to one of them to obtain the described concentration (750 µg). The inhibition zones of imipenem and imipenem-EDTA disks were compared after 18 hours of incubation in air at 35°C. If the increase in inhibition zone with imipenem and EDTA disk was ≥ 7 mm, then the imipenem disk alone was considered to be the MBL producer [26].

Plasmid Extraction, Profiling and Curing: Conjugation experiment was carried out according to Willets [27] using rifampicin-resistant *E. coli* DH5α as the recipient cell and five selected representatives of *Pseudomonas aeruginosa* as donor strains. The initial donor-to-recipient ratio of 1:20 was used for mating. Transconjugants were selected on MH agar plate containing 300 µg/mL of rifampicin and 50 µg/mL of ampicillin. Antibiotic susceptibility assay was carried out on the transconjugants. Plasmid extraction of donor strains and transconjugants was done using the alkaline lysis method of Takahashi and Nagano [28]. Plasmid DNA bands were detected by electrophoresis on 0.8% horizontal agarose gel pre-stained with ethidium bromide (0.5 µg/mL) and visualized under UV light. The sizes of the plasmid DNA bands were determined by extrapolation based on the mobility of Hind III digested λ DNA co-electrophoresed with the plasmid DNA samples [29-31]. Plasmid extraction of 74 isolates was carried out Three treatments were tested to evaluate curing efficiency of acridine orange, ethidium bromide and sodium dodecyl sulfate (SDS). The strains were grown in LB broth +2.5% NaCl at 30°C for 24 h. One set of 10.0-ml test tubes was prepared with 2.0 ml of LB broth with 10, 5, 2.5 and 1.25% SDS, another set with acridine orange at 25, 50, 100 and 200 µg/ml and a third set with 50, 100 and 200 µg/ml of ethidium bromide. All the tubes were inoculated with 200µl of the cultured bacterial broth and incubated at 30±°C for 24 h under constant agitation. Serial dilutions in sterile saline solution 2.5% were done and 100µl were plated in LB agar +2.5% NaCl, the plates were incubated at 30±°C for 24 h. Five colonies were selected from treatments with the highest concentration of curing agent and antibiograms were performed for the antibiotics to which they were originally resistant. Colonies that showed change in the size of the inhibition halo were submitted to plasmid extraction as explained previously. From the previous experiment, the most efficient curing agent was selected and the rest of the strains were tested following the methodology already described, the bacterial cells were centrifuged at 2200×g for 1 min, the supernatant was discarded and the pellet was resuspended in 1.0 ml saline solution (2.5%), this washing was repeated two more times. The resuspended bacteria were serially diluted and plated in LB agar+2.5% NaCl; after incubation, 13 colonies per tested strain were selected and inoculated in a Mueller Hinton agar plate with the corresponding antibiotics, to identify bacterial colonies that presented a change in the susceptibility using Kirby-Bauer method. Each well was inoculated with 50 µl of 0.5McFarland turbidity standard of a bacterial suspension, one colony suspended. The zone of inhibition was estimated or each strain before curing, to evaluate change in the resistance pattern due to the loss of the plasmid. Those strains that presented lower resistance patterns were selected. From these strains, the plasmid DNA was extracted and DNA bands were recorded electrophoretically (as described previously).

RESULTS AND DISCUSSION

Bacterial Strain Characterization and Antibiotic Susceptibility: All the strains grew on Cetrimide agar, were Gram-negative rod shape bacteria, motile by means of single polar flagella, oxidase-positive, do not ferment glucose and were positive to pyocyanin production [32]. Of the 74 strains tested, 72 were resistant to ampicillin (97.3%), 58 to amikacin (78.4%), 66 to carbenicillin (89.2%), 71 to cefoxitin (95.9%), 67 to ceftazidime (90.5%), 68 to ceftzoxime (91.9%), 60 to cefuroxime (81.1%), 42 to cotrimoxazole and ciprofloxacin (56.8%), 28 to gentamicin (37.8%), 53 to imipenem (71.6%), 17 to combination of pipreacillin and tazobactam (22.9%) and 66 to kanamycin

Table 1: Prevalence of antibiotic resistance among the studied *Pseudomonas aeruginosa* strains (N=74)

	Bacterial Resistance patterns	
Antibiotics	%	
Ampicillin	72	97.3%
Amikacin	58	78.4%
Carbenicillin	66	89.2%
Cefoxitin	71	95.9%
Ceftazidime	67	90.5%
Ceftizoxime	68	91.9
Cefuroxime	60	81.1
Cotrimoxazole	42	56.8
Ciprofloxacin	42	56.8
Gentamicin	28	37.8
Imipenem	53	71.6
Piperacillin+tazobactam	17	22.9
Kanamycin	66	89.2

Table 2: Prevalence of Amp C beta lactamase producing *Pseudomonas aeruginosa*

N	EP	IA	INA
74	30	7	23

N = Total number of isolates, EP = Amp C producers, IA = inducible Amp C, INA = inducible and non inducible Amp C.

Table 3: β-lactamase mediated resistance mechanism in Amp C producing *Pseudomonas aeruginosa* (n = 30).

AMP C	AMPC + ESBL	AmpC +MBL
30	5	21

(89.2%) (Table 1). 30 of the 74 isolates were Amp C Beta lactamase producers, 7 and 23 produced inducible and non inducible Amp C beta lactamases respectively (Table 2). Table 3 represents the beta lactamase mediated resistance mechanism in Amp C producing *Pseudomonas aeruginosa.* Five of the 30 Amp C beta lactamases produced extended spectrum beta lactamases while 21 of the same isolates produced metallo betalactamse enzymes in addition to the AMP C enzymes. In this study, we found a relatively lower prevalence of Amp C β-lactamase producing *P. aeruginosa* (40.5%). Production of multiple β-lactamases by *P. aeruginosa* has tremendous therapeutic consequences and poses a significant clinical challenge if it remains undetected. Since these organisms also carry other drug-resistant genes and the only viable treatment option remains the potentially toxic polymyxin B and colistin [32], early identification of the infections due to these organisms is necessary as the appropriate treatment might reduce the spread of these resistant strains as well as reduce the mortality in hospitalized patients. This emphasizes the need for the detection of isolates that produce these enzymes to avoid therapeutic failures and nosocomial outbreaks. Since there is no standard guideline for detection of most of these β-lactamase enzymes in *P. aeruginosa*, the comparison between studies becomes difficult as the patient population in particular centers and the methods of study differ.

The presence of Amp C producing isolates may be indicative of the ominous trend of more and more isolates acquiring resistance mechanisms rendering the antimicrobial armamarium ineffective. The Amp C enzymes were classified as CMY-2 [33]. The comparison of our results with the above-mentioned studies clearly shows that *Pseudomonas aeruginosa* strains producing ESBL and AmpC enzymes are less prevalent. This observation is not surprising as AmpC producers are susceptible to fourth generation cephalosporins like cefepime while ESBL producers are variably resistant to fourth-generation cephalosporins [34]. Both ESBL producers and non-producers showed high level resistance to cefepime. A high inoculum effect has been reported with cefepime for ESBL-producing and Amp C-producing isolates of Enterobacteriaceae [34]. The fact that 21 of the total 30 Amp C producers also produced metallo beta lactamases is very frightening. This is because MBLs also represent a clinical threat due to their unrivalled spectrum of activity and their resistance to therapeutic serine beta-lactamase inhibitors [31-33]

Plasmid Extraction, Profiling and Curing: Of the 74 strains analyzed, none was found without plasmid, 32 (43.2%) had one plasmid (Table 4), 37(50%) had two plasmids while the remaining five carried three plasmids each. The size of the extracted plasmids ranges from 800bp to 25000-bp. Eighteen different profiles were encountered, 10.8% of the strains (8) presented profile 1, 2.7% of the strains (2) presented profile 2,4,8 and 17. 16.2% of the strains (12) presented profile 3, 4.05% of the strains (3) presented profile 5, 9, 11, 14, 15 and 18. 6.8% of the strains (5) presented profile 6 and 10. 8.1% of the strains (6) presented profile 7 and 5.4% of the strains (4) presented profile 12, 13 and 16. The plasmid profiles in *Pseudomonas aeruginosa* have been studied [35] where a high diversity of profiles was observed [36]. Plasmid profiling has been proven useful to differentiate between Pseudomonas aeruginosa strains [35] but their discriminatory power has also been questioned [37].

The treatment with 200μgml^{-1} of acridine orange did not cure resistance to ampicillin, amikacin, carbenicillin, cefoxitin, ceftazidime and ceftizoxime. However, this concentration was enough to cure 38.3, 28.5, 19.0, 28.6, 24.5, 23.5 and 27.3% of resistance to cefuroxime,

Table 4: Plasmid profiles of the studied *Pseudomonas aeruginosa* strains

Plasmid profiles	Molecular weight of plasmids (kbp)	Frequency of plasmids occurrence
1	24	8(10.8)
2	25, 1	2(2.7)
3	25	12(16.2)
4	25, 6.5,1	2(2.7)
5	25, 1.5	3(4.05)
6	25, 2	5(6.8)
7	25, 3	6(8.1)
8	25, 5	2(2.7)
9	0.8	3(4.05)
10	23.5	5(6.8)
11	23, 4, 2	3(4.05)
12	23 4(5.4)	
13	23, 2	4(5.4)
14	20, 23.5	3(4.05)
15	23.5, 2	3(4.05)
16	24, 2	4(5.4)
17	23.5, 4, 3	2(2.7)
18	24, 23.5	3(4.05)
Total	33	74(100)

Table 5: Effect of different curing agents on the resistance patterns of the studied *Pseudomonas aeruginosa* strains Percentage of strains that lost resistance to antibiotics after treatment with

	SDS				ETBR				Acridine orange			
Antibiotics	10%	5%	2.5%	1.25%	200µg/ml	100µg/ml	50µg/ml	25µg/ml	200µg/ml	100µg/ml	50µg/ml	25µg/ml
Ampicillin	72(100)	58(80.6)	45(62.5)	26(36.1)	49(68.1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Amikacin	58(100)	47(81)	45(77)	23(40)	29(50)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Carbenicillin	66(100)	33(50%)	0(0)	0(0)	27(40.9)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Cefoxitin	71(100)	32(45)	16(23)	0(0)	39(54.9)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Ceftazidime	67(100)	50(74.6)	0(0)	0(0)	46(68.7)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Ceftizoxime	68(100)	34(50)	0(0)	0(0)	41(60.3)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Cefuroxime	60(100)	30(50)	0(0)	0(0)	30(50)	0(0)	0(0)	23(38.3)	0(0)	0(0)	0(0)	0(0)
Cotrimoxazole	42(100)	20(47.6)	0(0)	0(0)	21(50)	0(0)	0(0)	0(0)	12(28.5)	0(0)	(0)	0(0)
Ciprofloxacin	42(100)	19(45.2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	8(19.0)	0(0)	0(0)	0(0)
Gentamicin	28(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	8(28.6)	2(7.2)	0(0)	0(0)
Imipenem	53(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	13(24.5)	0(0)	0(0)	0(0)
Pip+taz	17(100)	8(47.1)	0(0)	0(0)	17(100)	0(0)	0(0)	0(0)	4(23.5)	0(0)	0(0)	0(0)
kanamycin	66(100)	24(36.4)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	18(27.3)	0(0)	0(0)	0(0)

cotrimoxazole, ciprofloxacin, gentamicin, imipenem and combination of piperacillin and tazobactam as well as kanamycin. The strains of the *Pseudomonas aeruginosa* tested were resistant to concentration of acridine orange below 200 μgml^{-1} except two strains that were sensitive to gentamicin upon treatment of their plasmid. This observation may be corroborating previous reports that acridine orange is capable of treating cells with lower molecular plasmids [38].With SDS at 10%, all three strains were cured, therefore, it was selected as the curing agent since the disposal of acridine orange and ethidium bromide were more difficult. SDS was also effective at 5% concentration except that it was not able to cure resistance to gentamicin and imipenem. At 2.5% concentration, SDS cured resistance to ampicillin, amikacin as well as cefoxitin while concentration of 1.25% only cured resistance to ampicillin and amikacin. According to Bennett [23], isolates of *P. aeruginosa* maintain their resistances to lincomycin and erythromycin after 1% SDS treatment for three incubation periods while Ahmad [39] also observed that all isolates of *P. aeruginosa* isolated from different human infections appeared 100% sensitive to streptomycin for all isolates after 1% SDS treatment and during all incubation times. Our findings therefore buttressed that *P. aeruginosa* isolates responded at different rates to 10% SDS and this

may be related to the permeability through outer membrane and to the location of antibiotic resistant genes carried on different plasmids. Sonstein and Baldwin [40] elucidated that the effectiveness of SDS may be related to plasmid copy number, or the amount of enzyme which inactivate antibiotics. Adachi *et al*. [41] found that SDS was only effective in elimination of sex (F) and R-plasmids in *E. coli* in a high frequency at a concentration that is higher than 1%. Moreover, it was reported that the longer the incubation times (24 to 72 h), the higher the frequency of sensitivity. In addition, comparing the genus with other genus of bacteria, Saffawi [42] found that the SDS affected the antibiotic resistant genes in *S. aureus* isolated from different environments. However, these resistances which were cured were conferred by Am, Cm and Sm genes with 100 and 65% on penicillin gene when the SDS was used at a concentration of 0.002% (W/V). Ethidium bromide treated plasmids to varying degree of percentages. As shown in the Table 5, concentration of 10% was the only efficacious concentration for the treatment of plasmids curing 68.1, 50, 40.9, 54.9, 68.7, 60.3, 50 and 100% resistance to ampicillin, amikacin, carbenicillin, cefoxitin, ceftazidime, ceftizoxime, cotrimoxazole and ciprofloxacin as well as combination of tazobactam and piperacillin respectively. This result agrees with that of Keyser *et al*. [43] who reported that low copy number plasmid was efficiently cured by EB. The agents causing complete inhibition of plasmid replication like Acridine orange and ethidium bromide intercalate between base pairs in DNA. Furthermore, they suggested that differences in DNA polymerase and RNA polymerase are responsible for differences in EB sensitivity to bacterial strains due to differences in the rate of the agent's penetration into different strains of Enterobacteriaceae. Rubins and Rosenblum [44] speculated that further exposure to EB caused the rate of elimination to decrease and the resistance to EB to increase and the resistance levels tended to increase slightly after 24 h of growth in EB. This finding agrees with the results obtained in this study. The previous results showed that the plasmids carrying antibiotic resistance genes were not eliminated with EB. This could be due to high copy number of these plasmids in these isolates. However, these results are in sharp disagreement with that documented by Pallida *et al*. [45] who demonstrated that the percent of cured plasmid DNA is not more than 20% in optimal conditions in *P. aeruginosa.* This is because where EB is efficacious; the percentage of cured plasmid is more than 20%.

Transformation of Competent Cells: Table 6 depicts the outcomes of the conjugation experiment. Conjugal transfer of resistance to ampicillin, amikacin, ciprofloxacin, gentamicin, carbenicilllin, cotrimoxazole, kanamycin and piperacillin and tazobactam was detected in the transconjugants after the mating experiment. The antibiotic resistances were transferred from four of the five selected donor strains. The antibiotic resistance pattern transferred by these donor strains was partial and associated with the transfer of R plasmids of sizes 24, 23, 4, 2 and 25 kbp from three of the five transferable strains (Table 6). The frequencies of transfer of antibiotype or R plasmids to the transconjugants ranged from 1.4×10^{-5} to 1.7×10^{-7} transconjugants per donor strain. This study documented the involvement of plasmids as factors responsible for antibiotic resistance in some of the recovered pathogens since these resistances were partly transferred to *E. coli* DH5α by conjugation. Therefore, our findings suggest the emergence and active transfer of antibiotic resistance and R plasmids among the studied strains of *Pseudomonas aeruginosa*. In this study, antibiotics such as ampicillin, amikacin, ciprofloxacin, carbenicillin, cotrimoxazole, gentamicin, kanamycin and combination of piperacillin and tazobactam were easily transferred from a multi drug resistant *Pseudomonas aeruginosa* to *E. coli*. The co-transfer of plasmids of sizes 23 and 4kb suggests that these extrachromosomal DNAs are R plasmids. The minimum size of a plasmid with an efficient conjugation system has been reported to be >15 kbp [30]. Therefore, the presence of the 5.0-kbp plasmids in the transconjugants implies that the larger molecular size plasmids (i.e., 25, 24 and 23kbp) might serve as vehicles for the transfer of lower molecular weight plasmids such as 4kbp to *E. coli* DH5α. This also implies that the mechanism of plasmid mobilization among the studied pathogens may entail the use of larger sized plasmids as vehicles in addition to the classical transfer systems such as conjugation that require considerable genetic information, as previously reported by Smith and Linggood [46], Achmith and Helmuth [47], Christiansen *et al.* [48] and Jamieson and Bremner [30]. The result from the conjugation experiment also revealed that the varied drug-resistant exconjugants arose at a frequency range of 10^{-5} - 10^{-7} per donor cell. These evolution rates are similar to those of previously reported epidemiologically and clinically important pathogens in Nigeria.

Table 6: Conjugal transfer of antibiotic resistance and plasmids to *Escherichia coli* DH5α by some of the selected bacterial isolates

Donor strains	ATB	PF(kbp)	ATB to EC	RP to EC	CF
PsE 1	Amp, Ami, Cip, Gent	24	Amp, Ami, Cip,	24	2.3×10^{6}
PsE 11	Car, Cef, Cot, Imi	23, 4, 2	Car, Cot	23,4	1.7×10^{7}
PsE 2	Gen, Imi, Kan, Cot	25, 1	Gen, Kan,	25	1.4×10^{5}
PsE 12	PT, Cip, Cef, Cefti	23	PT, Cip	-	1.3×10^{7}
PsE 14	Cef, Amp, Car, Ami	20, 23.5	-	-	-

ATB = Antibiotic resistance pattern, PF = Plasmid profile, EC= *Escherichia coli*, RP= Molecular weight of resistance plasmid transferred, CF = Conjugation frequency, PsE= *Pseudomonas aeruginosa* strain

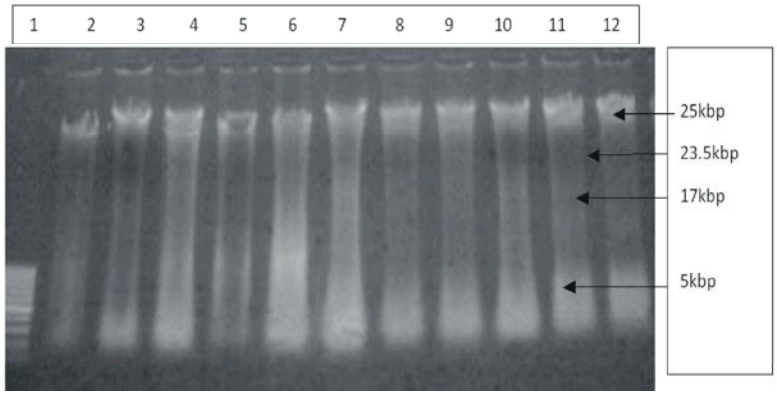

Plate 1: Plasmid DNA profiles of multi drug resistant *Pseudomonas aeruginosa* isolates
Lane 1 = 10kbp Hind III λ DNA marker, lane 2-12 = *Pseudomonas aeruginosa* isolates

CONCLUSIONS

It can be concluded based on our findings that antibiotic resistance of extended spectrum beta lactamases producing *Pseudomonas aeruginosa* expressing Amp C beta-lactamase enzyme are associated with transferable R plasmids for ampicillin, amikacin, ciprofloxacin, carbenicillin, cotrimoxazole, gentamicin and kanamycin.

REFERENCES

1. Fujitani, S., S. Kathryn, D. Moffett, L.Victor and L.Yu, 2014. *Pseudomonas aeruginosa.* Infectious Disease and Antimicrobial Agents, 45: 1-10.
2. Richards, M.J., J.R. Edwards, D.H. Culver and R.P. Gaynes, 1999. Nosocomial infections in medical intensive care units in the United States; National Nosocomial Infections Surveillance System. Crit. Care Med., 27(5): 887-892. Make references like this style.
3. Roque, A., A. Molina-Aja, C. Bolan-Mejia and B. Gomez-Gil, 2001. In vitro susceptibility to 15 antibiotics of vibrios isolated from penaeid shrimps in Northwestern Mexico. Int. J. Antimicrob. Agents, 17: 383-387.
4. Abraham, T.J., R. Manley, R. Palaniappan and K. Dhevendaran, 1997. Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* Isolated from Diseased Penaeid Shrimp., 12: 1-8.
5. Bhattacharya, M., P. Choudhury and R. Kumar, 2000. Antibiotic and metal-resistant strains of *Vibrio parahaemolyticus* isolated from shrimps. Microb. Drug Resist., 6: 171-172.
6. Manchanda, V. and N.P. Singh, 2008. Occurrence and detection of Amp C b-lactamases among Gram negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, India. J Antimicrob Chemother., 51: 415-418.
7. Ambler, R.P., 1980. The structure of β-lactamases. Philos Trans R Soc. Lond B Biol. Sci., 289: 321-331.
8. Bush, K., G.A. Jacoby and A.A. Medeiros, 1995. A Functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother., 39: 1211-1233.
9. Lee, K., M. Lee, J.H. Shin, M.H. Lee, S.H. Kang and A.J. Park, 2006. Prevalence of plasmid mediated Amp C β-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in Korea. Microb Drug Resist., 12: 44-49.
10. Paterson, D.L. and R.A. Bonomo, 2005. Extended-spectrum beta-lactamases: a clinical update. Clinical Microbiology Reviews, 18: 657-686.

11. Tumbarello, M., M. Sanguinetti, E. Montuori, E.M. Trecarichi, B. Posteraro, B. Fiori, R. Citton, T. D'Inzeo, G. Fadda, R. Cauda and T. Spanu, 2007. Predictors of mortality in patients with bloodstream infections caused by extended-spectrum-beta-lactamase-producing *Enterobacteriaceae*: Importance of inadequate initial antimicrobial treatment. Antimicrobial Agents and Chemotherapy, 51: 1987-1994.
12. Bradford, P.A., 2001. Extended-spectrum-beta-lactamases in the 21st century: characterization, epidemiology,and detection of this important resistance threat. Clinical Microbiology Reviews, 14: 933-951.
13. Brinas, L., M.A. Moreno, M. Zarazaga, C. Porrero, Y. Saenz, M. Garcia, L. Dominguez and C. Torres, 2003. Detection of CMY-2, CTX-M-14 and SHV-12 beta-lactamases in *Escherichia coli* fecal-samples isolates from healthy chickens. Antimicrobial Agents and Chemotherapy, 47: 2056-2058.
14. Liebana, E., M. Gibbs, C. Clouting, L. Barker, F.A. Clifton- Hadley, E. Pleydell, B. Abdalhamid, N.D. Hanson, L. Martin, C. Poppe and R.H. Davies, 2004. Characterization of beta-lactamases responsible for resistance to extended-spectrum cephalosporins in *Escherichia coli* and *Salmonella enterica* strains from food-producing animals in the United Kingdom. Microbial Drug Resistance, 10: 1-9.
15. Weill, F.X., R. Lailler, K. Praud, A. Kerouanton, L. Fabre, A. Brisabois, P.A. Grimont and A. Cloeckaert, 2004. Emergence of extended-spectrum-beta-lactamase (CTX-M-9)- producing multiresistant strains of *Salmonella enteric* serotype Virchow in poultry and humans in France. Journal of Clinical Microbiology, 42: 5767-5773.
16. Hasman, H., D. Mevius, K. Veldman, I. Olesen and F.M. Aarestrup, 2005. Beta-lactamases among extended-spectrum beta-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in The Netherlands. The Journal of Antimicrobial Chemotherapy, 56: 115-121.
17. Jensen, L.B., H. Hasman, Y. Agerso, H.D. Emborg and F.M. Aarestrup, 2006. First description of an oxyimino-cephalosporin resistant, ESBL-carrying *Escherichia coli* isolated from meat sold in Denmark. The Journal of Antimicrobial Chemotherapy, 57: 793-794.
18. Wu, S., E. Chouliara, H. Hasman, A. Dalsgaard, A. Vieira and L.B. Jensen, 2008. Detection of a single isolate of CTX M-1-producing *Escherichia coli* from healthy pigs in Denmark. The Journal of Antimicrobial Chemotherapy, 61: 747-749.
19. Jacoby, G.A., 2009. Amp C β-lactamases. Clin Microbiol. Rev., 22: 161-82.
20. Arzai, A.H. and D.J.M. Adamu, 2008. Prevalence of beta-lactamase Producers among randomly selected bacterial pathogens in Kano, Nigeria. Biological and Environmental Sciences Journal for the Tropics, 5(3): 218-223.
21. Pagani, L. and G. Rossolini, 2006. CMY-16, a novel acquired Amp C-type β-lactamase of the CMY/LAT lineage in multifocal monophyletic isolates of *Proteus mirabilis* from Northern Italy. J. Antimicrob Chemother., 43: 213-216.
22. Manchanda, V. and N.P. Singh, 2008. Occurrence and detection of Amp C b-lactamases among Gram negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, India. J Antimicrob Chemother., 51: 415-418.
23. Bennett, P.M., 2008. Plasmid encoded antibiotic resistance: acquisition and Transfer of antibiotic resistance genes in bacteria. Br. J. Pharmacol., 153(4): 347-357.
24. Philippon, A., G. Arlet and G.A. Jacoby, 2002. Plasmid-determined Amp C-type β-lactamases. Antimicrob Agent Chemother., 46: 1-11.
25. CLSI, 2005. Perfomance Standards for antimicrobial disc susceptibility tests. CLSI:Wayne PA. M100-S15.
26. Yong, D., K. Lee, J.H. Yum, H.B. Shin, G.M. Rossolini and Y. Chong, 2002. Imipenem–EDTA disk method for differentiation of metallo β lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J. Clin Microbiol., 40: 3798-3801.
27. Willets, N., 1988. Conjugation Methods. Microbiol., 21: 49-75.
28. Takahashi, S. and Y. Nagano, 1984. Rapid procedure for isolation of plasmid DNA and application to epidemiological analysis. J. Clin Microbiol., 20: 608-613.
29. Meyers, J.A.D., L.P. Sanchez, P. Elwell and S. Falkow, 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol., 127: 1529-1537.
30. Jamieson, A.F. and D.F. Bremner, 1979. Characterization of plasmids from antibiotic-resistant Shigella isolates by agarose gel electrophoresis. J. Gen Microbiol., 113: 73-81.

31. Litwin, C.M., A.L. Storm, S. Chipowsky and K.J. Ryan, 1991. Molecular epidemiology of Shigella infections: plasmid profiles, serotype correlation and restriction endonuclease analysis. J. Clin Microbiol., 29: 104-108.
32. Cheesebrough, M., 2005. Ed. Identification of bacteria. In Medical Laboratory manual for Tropical countries. Vol II London UK Butter Worth & Co. Publishers.
33. Naumiuk, L., A. Baraniak, M. Gniadkowski, B. Krawczyk, B. Rybak and E. Sadowy, 2004. Molecular epidemiology of *Serratia marcescens* in two hospitals in Gdansk, Poland, over a 5-year period. J. Clin Microbiol., 42: 3108-3116.
34. Wu, L.T., M.F. Tsou, H.J. Wu, H.E. Chen, Y.C. Chuang and W.L. Yu, 2004. Survey of CTX-M-3 extended-spectrum beta-lactamase (ESBL) among cefotaxime-resistant *Serratia marcescens* at a medical center in middle Taiwan. Diagn Microbiol Infect Dis., 49: 125-9.
35. Akingbade, O.A., S.A. Balogun, D.A. Ojo, R.O. Afolabi, B.O. Motayo, P.O. Okerentugba and I.O. Okonko, 2012. Plasmid profile analysis of multidrug resistant *Pseudomonas aeruginosa* isolated from wound Infections in South West, Nigeria. World Applied Sciences Journal., 20(6): 766-775.
36. Hassan, S., Y. Bagher, O. Parviz and R.L. Abdolaziz, 2010. Molecular epidemiology and drug susceptibility of *Pseudomonas aeruginosa* strains isolated from burn patients. Science, 41(9): 539-544.
37. Pedersen, K., 1999. The fish pathogen *Vibrio anguillarum*. Doctoral Thesis, The Royal Veterinary and Agricultural University, Denmark.
38. Zaman, M.A., M.H. Pasha and M.Z. Akhter, 2010. Plasmid curing of *Escherichia coli* cells with ethidium bromide, sodium dodecyl sulfate and acridine orange. Bangladesh J. Microbiol., 27: 28-31.
39. Ahmad, K.D., 1989. The positive control of ilv C expression in *E. coli K*-12. Ph.D. Thesis, University of Durham, England.
40. Sonstein, S.A. and J.N. Baldwin, 1972. Loss of the penicillinase plasmid after treatment of *Staphylococcus aureus* with sodium dodecyle sulfate. J. Bacterial., 109: 262-265.
41. Adachi, H., M. Nakano, M. Inuzuka and M. Tomoeda, 1972. Specific role of sex pili in the effective eliminatory action of sodium dodecyle sulfate on sex and drug resistance factors in *Escherichia coli*. J. Bacteriol., 109(3): 1114-1124.
42. Saffawi, N.T., 2001. Removal of the resistance of *S. aureus* bacteria isolated from various human infections to antibiotics by using chemical materials and physical factors. M.Sc. thesis, College of Education, University Mosul, Iraq, pp: 68-72.
43. Keyser, H.H., B.B. Bohlool, T.S. Hu and D.F. Weber, 1982. Fast growing Rhizobia isolated from root nodules of soybean. Science, 215: 1631-1632.
44. Rubins, S.J. and E.D. Rosenblum, 1971. Effect of the recipient strain and ultraviolet irradiation on transduction kinetics of the penicillinase plasmid of *Staphylococcus aureus*. J. Bacteriol., 108: 1192-1199.
45. Padilla, C., M. Salazar and O. Faundez, 1992. Range of action and genetic bacteriocine codification of *Pseudomonas aeruginosa* isolated from three different ecological niches. J. Appl. Bacteriol., 73(6): 497-500.
46. Smith, H.R. and M.A. Linggood, 1970. Transfer factors in *Escherichia coli* with particular regard to their incidence in enteropathogenic strains. J Gen Microbiol., 62: 287-299.
47. Achtman, N. and R. Helmuth, 1974. The F factor carries an operon of more than $15\times l0^6$ daltons coding for deoxyribonucleic acid transfer and surface exclusion. In: Microbiology. American Society for Microbiology. Ed., Schlessinger, D., Washington, pp: 95-103.
48. Christiansen, C., G. Christiansen, A.L. Bak and A. Stenderup, 1973. Extra-chromosomal deoxyribonucleic acid in different enterobacteria. J. Bacteriol., 114: 367-377.