Original Research Article

Determination of Plasmid Profile Multiple Antibiotics Resistant *Pseudomonas aeruginosa* Isolated from Clinical Specimens in Healthcare Facilities across Anambra and Imo States Nigeria.

ABSTRACT

The emergence and dissemination of antibiotic resistant plasmids of *P. aeruginosa* is posing a major public threat and huge concern in hospital facilities. This study was done across Anambra and Imo States with a total number of 100 *P. aeruginosa* isolates, 50 from each State to determine the plasmid profile of multidrug resistant isolates. Methods of re-identifying *P. aeruginosa* were based upon cultural methods coupled with biochemical tests. To study the susceptibility of these isolates using disk diffusion method, different antibiotics were used. Plasmid extraction was done using alkaline lysis method after growing isolates resistant to more than four antibiotics on a nutrient broth. Agarose gel electrophoresis of plasmid DNA was carried out on 2% agarose gel slab in 1X TAE buffer. The results show that, the nutrient agar at 37˚C aerobically, *P. aeruginosa* isolates were recovered, which produced greenish-yellow pigment colonies, oxidase was positive and negative for gram stain. In Anambra, the result showed 100% resistance to Cefuroxime, Ceftazidime and Ciprofloxacin, 90% and 86% for Ceftriaxone and Piperacillin-tazobactem, 48% and 40% to Gentamicin and Amikacin respectively. Whereas in Imo state, the result showed 100% resistance to Ceftazidime and Ciprofloxacin, 80% to Ceftriaxone, Cefuroxime and Piperacillin-tazobactem while the least resistance was seen in Amikacin, and Gentamicin. Plasmid size ranging from 100bp to >1000bp was detected from most of the multidrug resistant isolates. Not all the Isolates with multidrug resistance were found to possess plasmids. It can be seen from this study that multidrug resistance in *P. aeruginosa* is not strictly plasmid-dependent (mediated).

Keywords: Plasmid; Antibiotics Resistance; *Pseudomonas aeruginosa*; Clinical Isolates; Imo; Anambra; Nigeria.

INTRODUCTION

Healthcare-associated infections (HAIs) are a leading cause of morbidity and mortality worldwide. *Pseudomonas aeruginosa* is an aerobic, motile, gram-negative rod that belongs to the family, Pseudomonadeceae. *Pseudomonas aeruginosa* is the most common gram negative

bacterium and also one of the most important opportunistic pathogens that has been associated with hospital-acquired infections such as respiratory tract infections, urinary tract infections (UTI), endocarditis, and surgical wounds infection which are often complicated and life-threatening [1, 2]. It is reported to be ubiquitous in humans, animals, and the natural environment. This is because it has minimal requirements for survival and its ability to adapt to a variety of environmental conditions. The widespread habitat of *P. aeruginosa* makes it very difficult to control the organism more especially in the hospital setting. The frequency of infections caused by *P. aeruginosa* is increasing and multidrug-resistant (MDR) isolates are emerging in patients who are hospitalized for more than one week [3]. According to Akinyoola *et al.,* [4], wound infections due to *P. aeruginosa* is the primary cause of limb amputation in children and outbreaks among burn patients is associated with death rates as high as 60%.

It is an opportunistic pathogen meaning that it exploits some break in the host defenses to initiate an infection [5], its ability to activate useful phenotypes under environmental stress and to persist in adverse conditions such as antibiotic or antiseptic substances. It is one of the important bacterial pathogens isolated from hospital samples. Other risk factors for acquiring infections include being seriously ill, being hospitalized, having undergone invasive procedures (eg, the use of catheters), having a compromised immunity and therapy with broad spectrum antibiotics [6]. Hospital environments, particularly in ICUs, are common habitats for *Pseudomonas aeruginosa* and also out breaks due to its multi resistance have been reported [7].

It has been identified as the 2nd most frequent organism causing ventilator-associated pneumonia, the 4th most common causing catheter-associated urinary tract infections, the 5th cause of surgical site infections and the 7th cause of central-line-associated bloodstream infections [8]. In Nigeria, studies have been done from different geoplitical zones. In south east,

Pseudomonas aeruginosa had been isolated from wound swabs 39.3%, and 41.9% in ear swap. From south south, *Pseudomonas aeruginosa* was isolated from 41% of cases with discharging ear [9]. A study from the South west by Odusanya in 2002 have reported *Pseudomonas aeruginosa* been isolated from urine (4.6%), reproductive tract (2.1%) and wound infections (16.3%). The mode of transmission may include patient to patient via the hands of health workers, contaminated reservoir (formites) to person and colonization with subsequent auto-infection [10]. It can survive harsh environmental conditions and displays intrinsic resistant to a wide variety of antimicrobial agents that facilitate the organisms ability to survive in hospital setting. In addition to its intrinsic resistance to various antibiotics, it also readily acquires resistance to the potentially active agents [11]. Since some of the resistance markers are carried by plasmids, the threat to human health is compounded by the possibility of transmission of markers to other gram negative pathogens [12].

Resistance to antipseudomonal antibiotics is increasing worldwide. This situation has been compounded by the lack of new classes of antipseudomonal drugs. The pathogenic success of *P. aeruginosa* is as a result of its array of virulence factors and its tendency to colonize surfaces in an intractable biofilm form, making the cells impervious to therapeutic concentrations of antibiotics. It is innately tolerant to many antimicrobials and disinfectants because of its outer membrane permeability barrier. In addition, it maintains resistance plasmids and is able to exchange same with other bacteria with which it lives as normal flora, through the mechanism of Horizontal Gene Transfer (HGT), particularly conjugation and transduction [13].

P. aeruginosa is difficult to eradicate due to a number of factors, the most important of which is the relatively poor efficacy of antibiotics against *P. aeruginosa* due to multiple resistance mechanisms expressed by the bacterium [14]. One of the clinical significances of *P. aeruginosa*

is its ability to secrete several virulence factors. These virulence factors include mucoid exopolysaccharide, lipopolysaccharide, biofilm, pili, exotoxin A, pigments, lipase, protease, hemolysin, histamine, exoenzyme S, leukocidin, and rhamnolipids [15]. These factors help the bacteria to adhere to and invade their host by damaging the host's immune responses and forming a barrier to antibiotics. Cell-associated and secreted virulence factors are encoded on plasmids or chromosomal genes.

From another point of view, *P. aeruginosa* is notorious for its resistance to antibiotics and therefore is a dangerous and dreaded pathogen. It is one of the leading causes of severe infections, such as pneumonia or bacteremia, which are associated with high mortality rates and are often difficult to treat. Reports of useful antipseudomonal agents are limited (some β-lactams, fluoroquinolones, aminoglycosides and polymyxins as last-resort drugs) and *P. aeruginosa* exhibits high intrinsic resistance to penem antibiotics such as faropenem, ritipenem, sulopenem, tetracycline, and penicillins [16]. The persistent exposure of bacterial strains to a multitude of β-lactams has induced a dynamic and continuous production and mutation of β-lactamases in the bacteria, expanding their activity even against the third and fourth generation cephalosporins, such as ceftazidime, cefotaxime, and cefepime, and also against aztreonam.

Despite advances in medical and surgical care and introduction of wide variety of antimicrobial agents having antipseudomonal activities, life-threatening infection caused by *P. aeruginosa* continues to cause complications in hospital-acquired infections [15]. *P.aeruginosa* readily colonizes hospitalized and immunocompromised individuals, although it rarely causes infection in immunocompetent persons, the resultant infections comprise about 10% of HAIs in the United States of America [17].

Despite improvements in antibiotic therapy, *Pseudomonas aeruginosa* is intrinsically resistant to a number of antimicrobial agents including multiple classes of antimicrobial agents.

Subsequently, outbreaks due to multidrug resistant *Pseudomonas aeruginosa* have been reported in various nosocomial settings such as Intensive Care Units. Tracking the antibiotic susceptibility of *P. aeruginosa* isolated in Anambra and Imo States will help to produce antibiogram charts, which may guide clinicians to good initial treatment regimens in these States. Plasmid profile analysis examines the total bacterial plasmid content, which will also help to conceive ideas on how to advise the patients and health care workers on how to promote good hygienic practices in our hospitals.

2.0. MATERIALS AND METHODS

Collection of isolates

A total number of 100 *Pseudomonas aeruginosa* isolates were collected from health care facilities across Imo and Anambra States. These isolates were collected with mueller hinton agar slant and were subjected to further identification procedures and antibiotic sensitivity testing.

Identification of *Pseudomonas aeruginosa* isolates

The presumptive *Pseudomonas aeruginosa* were subcultured on a solidified 20ml nutrient agar and were incubated at 37°C for 24hrs. This is to identify the growth charaters like colony morphology, grape-like odour. Then, the isolates were maintained in nutrient agar slant at 4°C. Biochemical test was done to confirm the identified *Pseudomonas aeruginosa*. The biochemical test was oxidase test, using oxidase stripe.

Antimicrobial susceptibility testing

Pure cultures of bacterial isolates were subjected to antimicrobial susceptibility test using agar diffusion method. An aliquot of 0.1ml of the broth culture suspended in sterile normal saline was spread over the nutrient agar plate with the help of a swab stick. Antimicrobial susceptibility testing was performed using commercially available antimicrobial discs (Oxoid UK). The following discs [Piperacillin–tazobactam (100/10 µg), Ceftazidime (30 µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Cefuroxime (30 µg), Ceftriazone (30 µg) and Gentamicin (10 µg)] were used to determine the sensitivity and resistant pattern of the isolates and was carried out as described by the Kirby- Bauer disc diffusion method. After incubation, the diameter of the zone of inhibition was measured with a transparent meter rule and compared with zone diameter interpretative chart as recommended by the Clinical Laboratory Standard Guidelines [18], to determine the sensitivity and resistant pattern of the isolates to antibiotics.

Extraction of plasmid DNA of the isolates using alkaline lysis method

Fifty (50) multidrug resistance *P. aeruginosa* isolates were selected for plasmid extraction, 25 from each state using alkaline lysis method. Pure *Pseudomonas aeruginosa* isolates were grown on nutrient medium overnight at 37°C for 24 hours. 1.5mL of the bacteria culture was added into the 2mL Eppendorf tubes and centrifuged at 10,000rpm for 1 minute to produce cell pellet, the supernatant was removed and this was repeated twice. Phosphate-Buffered-Saline (PBS) solution was filled up to the brim of the tube, votexed and centrifuged. After the pellet has properly air dried, 150uL of the resuspension buffer was added and vortexed. Two hundred microliter (200uL) of lysis solution was added to the freshly bacterial suspension and mixed. Three hundred microliter (300uL) of neutralizing solution was added and mixed and centrifuged at

14,000 rpm for 5minutes and the supernatant containing the DNA was transferred to a new 1.5mL Eppendorf tube. Double volume of isopropanol was added into the tube, mixed and incubated at -80^{o}C for 30 minutes. The mixture was later centrifuged and the supernatant discarded. 600uL of 70% ethanol was added, centrifuged and the supernatant discarded. The white colour pellet formed was left to air dry for 10 minutes. The air dried pellet was dissolved in 30uL of Tris-EDTA buffer. The presence of plasmids was confirmed by running the DNA isolates on 2% Agarose Gel Electrophoresis

Agarose gel electrophoresis

Gel electrophoresis of the plasmid DNA were carried out on 2% agarose gel slab in 1X TAE buffer. 10ul of ethidum bromide stain was added to the gel prior to electrophoresis. The sample and loading dye were taken in 8.2 ratio, mixed well and loaded into the wells using a 20uL pipette, 5uL of the control DNA ladder (Molecular Weight Marker) was loaded. The gel ran for 40 minutes at a constant voltage of 100V and 400 mA. The stained gel was visualized with a short wave ultraviolet transilluminator and the photograph of the plasmid band was taken [19].

3.0. RESULTS

Table 1 shows the antibiotic resistance pattern of the 50 *Pseudomonas aeruginosa* isolates from Anambra State in percentage distribution. The 50 *Pseudomonas aeruginosa* showed resistance to ciprofloxacin (100%), ceftazidime (100%), and cefuroxime (100%). This was followed by ceftriazone (90%), piperacillin-tazobactam (86%). The least resistance of these isolates were only seen with amikacin (40%) and gentamicin (48%) was also recorded.

Table 1: Antibiotic resistance pattern of the 50 *Pseudomonas aeruginosa* isolates from Anambra State.

Class of antibiotic	Type of antibiotic	No (%) of Resistance	No (%) of intermidiate	No (%) of susceptible
Quinolones	Ciprofloxacin(5 µg)	50(100)	0(0)	0(0)
Aminoglycosides	Gentamicin (10 µg)	24(48)	16(32)	10(20)
	Amikacin (30 µg)	20(40)	18(36)	12(24)
Cephalosporin	Ceftriazone (30 µg)	48(90)	2(10)	0(0)
	Ceftazidime (30 µg)	50(100)	0(0)	0(0)
	Cefuroxime (30 µg)	50(100)	0(0)	0(0)
Penicillin	Piperacillin–tazobactam (100/10 µg),	43(86)	0(0)	7(14)

Table 2 shows the antibiotic resistance pattern of the 50 *Pseudomonas aeruginosa* isolates from Imo State in percentage distribution. The 50 *Pseudomonas aeruginosa* showed resistance to Ciprofloxacin (100%), Ceftazidime (100%). This was followed by Ceftriazone (80%), Piperacillin-tazobactam (80%) and Cefuroxime (80%). The least resistance of these isolates was only seen with Amikacin (32%) and Gentamicin (34%) was also recorded in (Table 2).

Table 2: Antibiotic resistance pattern of the 50 *Pseudomonas aeruginosa* isolates from Imo State.

Class of antibiotic	**Type of antibiotic**	**No (%) of Resistance**	**No (%) of intermidiate**	**No (%) of susceptible**
Quinolones	Ciprofloxacin(5 µg)	50(100)	0(0)	0(0)
Aminoglycosides	Gentamicin (10 µg)	17(34)	18(36)	15(30)
	Amikacin (30 µg)	16(32)	18(36)	16(32)

Cephalosporin	Ceftriazone (30 µg)	46(80)	4(20)	0(0)
	Ceftazidime (30 µg)	50(100)	0(0)	0(0)
	Cefuroxime (30 µg)	46(80)	4(20)	0(0)
Penicillin	Piperacillin–tazobactam (100/10 µg),	46(80)	0(0)	4(20)

Table 3 shows the 25 selected antibiotic profile of multi drug resistance *Pseudomonas aeruginosa* isolates from Anambra State. Here all the isolates showed resistant to Ciprofloxacin, Ceftazidime and Cefuroxime, 20 out of 25 isolates were resistant to gentamicin, 23 out of 25 isolates were resistant to Piperacillin-tazobactam, 10 out of 25 isolates were resistant to Amikacin, 23 out of 25 were resistance to Ceftriazone. Out of the 25 isolates profiled, 7 isolated where resistant to all the antibiotics used (Ap4, Ap5, Ap18, Ap24, Ap29, Ap43, Ap47), 13 isolates were resistant to 6 antibiotics (Ap1, Ap11, Ap14, Ap19, Ap22, Ap25, Ap30, Ap36, Ap38, Ap39, Ap41, Ap46 and Ap49), 3 isolates were resistant to 5 antibiotics (Ap2, Ap32 and Ap35) and 2 isolates was resistant to 4 antibiotics (Ap12 and Ap31).

Table 3: Antibiotic profile of *Pseudomonas aeruginosa* isolates from Anambra State

Isolates	Resistant pattern	Sensitive pattern
Ap1	Cip, Cxm, Cn, Lyn,Tzp,Caz	Ak
Ap2	Cip, Cxm, Lyn, Tzp, Caz	Cn, Ak
Ap4	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	-
Ap5	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	-
Ap11	Cip, Cxm, Cn, Lyn, Tzp, Caz	Ak
Ap12	Cip, Cxm, Cn, Tzp, Caz,	Lyn, Ak
Ap14	Cip, Cxm, Cn, Lyn, Tzp, Caz	Ak
Ap18	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	-
Ap19	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ap22	Cip, Cxm, Lyn,Tzp, Ak, Caz	Cn
Ap24	Cip, Cxm, Cn, Lyn,Tzp, Ak, Caz	-
Ap25	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak

Table 3 continues: Antibiotic profile of *Pseudomonas aeruginosa* isolates from Anambra state

Isolates	Resistance pattern	sensitive pattern
Ap29	Cip, Cxm, Cn, Lyn,Tzp, Ak, Caz	-
Ap30	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ap31	Cip, Cxm, Tzp, Caz	Cn, Lyn, Ak
Ap32	Cip, Cxm, Lyn,Tzp, Caz	Cn, Ak
Ap34	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ap35	Cip, Cxm, Cn, Lyn, Caz	Tzp, Ak
Ap38	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ap39	Cip, Cxm, Lyn,Tzp, Ak, Caz	Cn
Ap41	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ap43	Cip, Cxm, Cn, Lyn,Tzp, Ak, Caz	-
Ap46	Cip, Cxm, Cn, Lyn, Ak, Caz	Tzp
Ap47	Cip, Cxm, Cn, Lyn,Tzp, Ak, Caz	-
Ap49	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak

Table 4 shows the 25 antibiotic profile of multi drug resistance *Pseudomonas aeruginosa* isolates from Imo State. In Imo State, all the isolates showed resistance to Ciprofloxacin and Ceftazidime, 23 out 25 isolates were resistant to cefuroxime, ceftriazone and piperacillin-

tazobactam, 22 out of 25 isolates were resistant to gentamicin and 8 out of 25 isolates were resistant to amikacin. Out of 25 isolates profiled, 6 isolates were resistant to all the antibiotics used (Ip2, Ip12, Ip23, Ip24, Ip40), 14 isolates were resistant to 6 antibiotics (Ip1, Ip4, Ip5, Ip7, Ip8, Ip11, Ip26, Ip30, Ip31, Ip32, Ip34, Ip42, Ip43, Ip47), 4 isolates were resistant to 5 antibiotics (Ip15, Ip18, Ip35, Ip50) and 1 isolate was resistant to 4 antibiotics (Ip39).

Plasmid profile was done on selected isolates resistant to more than four antibiotics.

Table 4: Antibiotic profile of *Pseudomonas aeruginosa* isolates from Imo State

Isolates	Resistance pattern	Sensitive pattern
Ip1	Cip, Cxm, Cn, Lyn, Tzp, Caz	Ak
Ip2	Cip, Cxm, Cn,Lyn, Tzp, Ak, Caz	-
Ip4	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip5	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip7	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip8	Cip, Cxm, Cn,Tzp, Ak, Caz	Lyn

Ip11	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip12	Cip, Cxm, Cn, Lyn,Tzp, Ak, Caz	-
Ip15	Cip, Cn, Lyn,Tzp, Caz	Cxm, Ak
Ip18	Cip, Cxm, Lyn,Tzp, Caz	Cn, Ak
Ip21	Cip, Cxm, Cn, Lyn,Tzp, Ak, Caz	-
Ip23	Cip, Cxm, Cn, Lyn,Tzp, Ak, Caz	-

Table 4 countinues: Antibiotic profile of multi drug resistance *Pseudomonas aeruginosa* isolates from Imo State

Isolates	**Resistance pattern**	**Sensitive pattern**
Ip24	Cip, Cxm, Cn, Lyn,Tzp, Ak, Caz	-
Ip26	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip30	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip31	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip32	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip34	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip35	Cip, Cxm, Cn, Tzp, Caz	Lyn, Ak
Ip39	Cip, Lyn,Tzp, Caz	Cxm, Cn, Ak
Ip40	Cip, Cxm, Cn, Lyn,Tzp, Ak, Caz	-

Ip42	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip43	Cip, Cxm, Cn, Lyn,Tzp, Caz	Ak
Ip47	Cip, Cxm, Cn, Lyn, Ak, Caz	Tzp
Ip50	Cip, Cxm, Cn, Lyn, Caz	Tzp, Ak

Table 5 shows the plasmid distribution among isolates in Anambra State. Out of 25 isolates profiled, six isolates harboured no plasmid at all (Ap5, Ap18, Ap25, Ap34, Ap39, Ap46), but were all resistant to Ciprofloxacin, Ceftriazone, Ceftazidime and Cefuroxime. Nine isolates harboured one plasmid each (Ap1, Ap12, Ap14, Ap24, Ap30, Ap32, Ap38, Ap43, Ap49) and were all resistant to Ciprofloxacin, Ceftazidime, Cefuroxime and Piperacillin-tazobactam. Eight isolates haboured two plasmids (Ap2, Ap11, Ap19, Ap29, Ap31, Ap35, Ap41, Ap47) and were all resistant to Ciprofloxacin, Ceftazidime and Cefuroxime. Two isolates harboured three plasmids (Ap4 and Ap22) and were all resistant to Ciprofloxacin, Amikacin, Ceftriazone, Ceftazidime, Cefuroxime and Piperacillin-tazobactam.

Table 5: Plasmid Distribution Among Isolates In Anambra State

Isolate code	Resistant pattern	No of plasmids	Plasmid profile bp
Ap1	Cip, Cxm, Cn, Lyn, Tzp, Caz	1	>1000
Ap2	Cip, Cxm, Lyn, Tzp, Caz	2	100, 600
Ap4	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	3	100, 500, >1000
Ap5	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	0	-
Ap11	Cip, Cxm, Cn, Lyn, Tzp, Caz	2	100, 500
Ap12	Cip, Cxm, Cn, Tzp, Caz	1	400
Ap14	Cip, Cxm, Cn, Lyn, Tzp, Caz	1	100
Ap18	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	0	-
Ap19	Cip, Cxm, Cn, Lyn, Tzp, Caz	2	150, 500
Ap22	Cip, Cxm, Lyn, Tzp, Ak, Caz	3	100, 600, >1000
Ap24	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	1	100
Ap25	Cip, Cxm, Cn, Lyn, Tzp, Caz	0	-
Ap29	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	2	100, 400
Ap30	Cip, Cxm, Cn, Lyn, Tzp, Caz	1	200
Ap31	Cip, Cxm, Tzp, Caz	2	100, >1000
Ap32	Cip, Cxm, Lyn, Tzp, Caz	1	500
Ap34	Cip, Cxm, Cn, Lyn, Tzp, Caz	0	-
Ap35	Cip, Cxm, Cn, Lyn, Caz	2	100. 400
Ap38	Cip, Cxm, Cn, Lyn, Tzp, Caz	1	100
Ap39	Cip, Cxm, Lyn, Tzp, Ak, Caz	0	-
Ap41	Cip, Cxm, Cn, Lyn, Tzp, Caz	2	600, >1000
Ap43	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	1	>1000
Ap46	Cip, Cxm, Cn, Lyn, Ak, Caz	0	-
Ap47	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	2	400, 600
Ap49	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	1	100

Table 6 shows the plasmid distribution among isolates in Imo state. In Imo State, two isolates haboured no plasmid (Ip7 and Ip30) but were resistant to 6 antimicrobials. Eight isolates haboured one plasmid (Ip1, Ip4, Ip5, Ip11, Ip12, Ip18, Ip21, Ip34, Ip47) and were all resistant to Ciprofloxacin, Cefuroxime, Cefriazone, Ceftazidime. Nine isolates haboured two plasmids (Ip2, Ip15, Ip24, Ip26, Ip32, Ip35, IP40, Ip43, Ip50) and were all resistant to Ciprofloxacin, Gentamicin, Ceftazidime, and Cefuroxime. Five isolates haboured three plasmids (Ip8, Ip23, Ip31, Ip39, Ip42) and were all resistant to Ciprofloxacin, Ceftazidime and Piperacillin-tazobactam. Plasmids isolated size ranges from 100 to >1000bp.

Table 6: Plasmid distribution among isolates in Imo State

Isolate code	Resistance pattern	No of plasmids	Plasmid profile bp
Ip1	Cip, Cxm, Cn, Lyn, Tzp, Caz	1	500
Ip2	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	2	100, 400
Ip4	Cip, Cxm, Cn, Lyn, Tzp, Caz	1	100
Ip5	Cip, Cxm, Cn, Lyn, Tzp, Caz	1	100
Ip7	Cip, Cxm, Cn, Lyn, Tzp, Caz	0	-
Ip8	Cip, Cxm, Cn, Tzp, Ak, Caz	3	100, 500, >1000
Ip11	Cip, Cxm, Cn, Lyn, Tzp, Caz	1	600
Ip12	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	1	100
Ip15	Cip, Cn, Lyn, Tzp, Caz	2	100, 300
Ip18	Cip, Cxm, Lyn, Tzp, Caz	1	300
Ip21	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	1	100
Ip23	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	3	100, 600, >1000
Ip24	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	2	400, >1000
Ip26	Cip, Cxm, Cn, Lyn, Tzp, Caz	2	100, 400
Ip30	Cip, Cxm, Cn, Lyn, Tzp, Caz	0	-
Ip31	Cip, Cxm, Cn, Lyn, Tzp, Caz	3	400, 600, >1000
Ip32	Cip, Cxm, Cn, Lyn, Tzp, Caz	2	100, 400
Ip34	Cip, Cxm, Cn, Lyn, Tzp, Caz	1	300
Ip35	Cip, Cxm, Cn, Tzp, Caz	2	400, >1000
Ip39	Cip, Lyn, Tzp,Caz	3	100, 400, >1000
Ip40	Cip, Cxm, Cn, Lyn, Tzp, Ak, Caz	2	300, 600
Ip42	Cip, Cxm, Cn, Lyn, Tzp, Caz	3	100, 400, 600
Ip43	Cip, Cxm, Cn, Lyn, Tzp, Caz	2	100, 600
Ip47	Cip, Cxm, Cn, Lyn, Ak, Caz	1	100
Ip50	Cip, Cxm, Cn, Lyn, Caz	2	600, >1000

Figure 1 shows bar chart representing the concentration of each antibiotics among the 50 *Pseudomonas aeruginosa* isolates in Anambra State in number distribution. Ceftazidime, Cefuruxime, Ciprofloxacin showed resistance to all the 50 *Pseudomonas aeruginosa* isolates, Ceftriazone showed resistance to 48 *Pseudomonas aeruginosa* isolates, Pipracillin/tazobacter showed resistance to 43 *Pseudomonas aeruginosa* isolates while Gentamicin and Amikacin showed resistance to 24 and 20 *Pseudomonas aeruginosa* isolates respectively. Sensitivity was seen in Pipracillin/tazobacter (7 isolates), Gentamicin (10 isolates) and Amikacin (12 isolates).

Figure 1: Bar chart representing the concentration of each antibiotic among the 50 *Pseudomonas aeruginosa* isolates in Anambra State

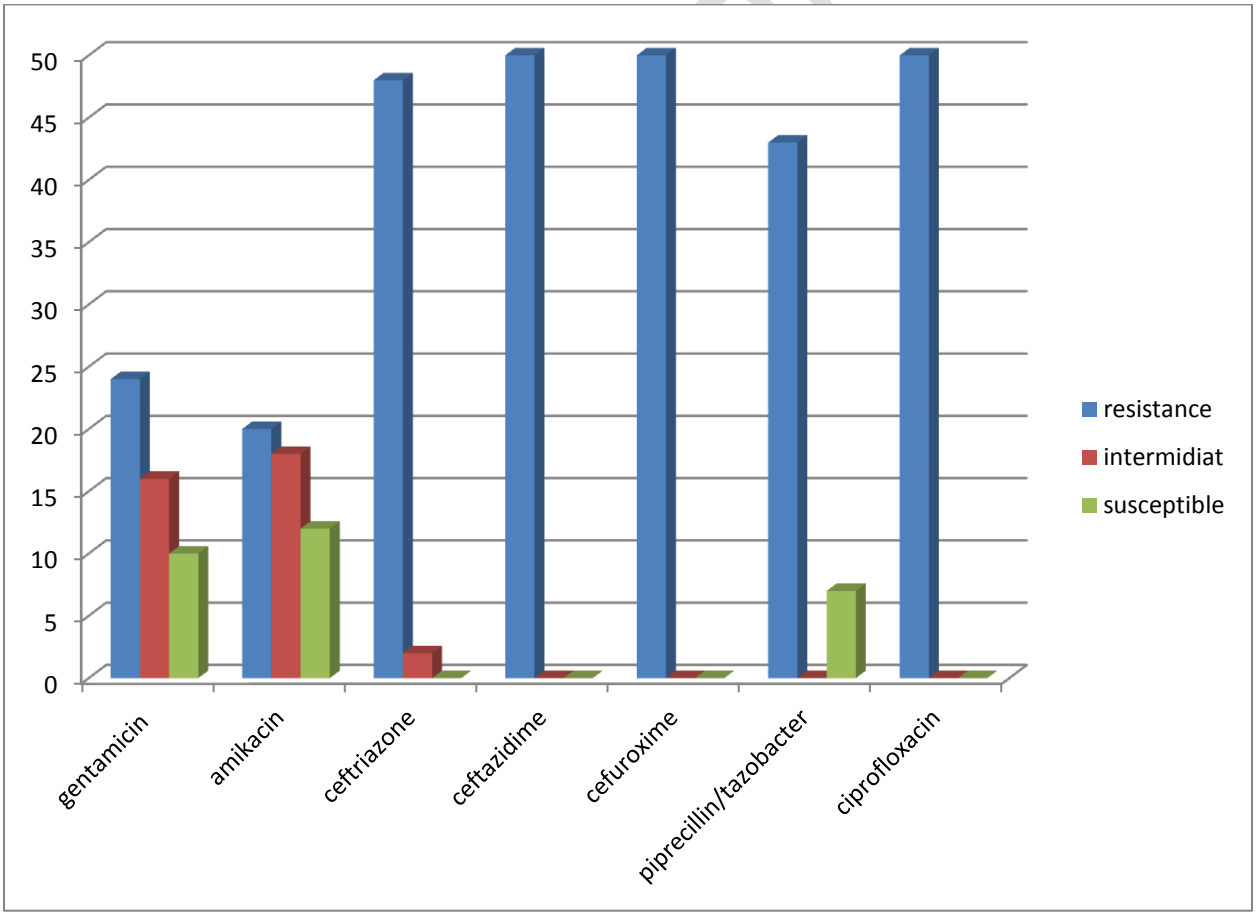


Figure 2 shows bar chart representing the concentration of each antibiotic among the 50 *Pseudomonas aeruginosa* isolates in Imo State in number distribution. Ciprofloxacin and Ceftazidime showed resistance to all the 50 *Pseudomonas aeruginosa* isolates, Ceftriazone, Cefuroxime and Piperacillin/tazobactam showed resistance to 46 *Pseudomonas aeruginosa* isolates each while Gentamicin and Amikacin showed resistance to 17 and 16 *Pseudomonas aeruginosa* isolates respectively. Sensitivity was seen in Piperacillin/tazobactam (4 isolates), Gentamicin (15 isolates) and Amikacin (16 isolates).

Figure 2: Bar chart representing the concentration of each antibiotic among the 50 *Pseudomonas aeruginosa* isolates in Imo State

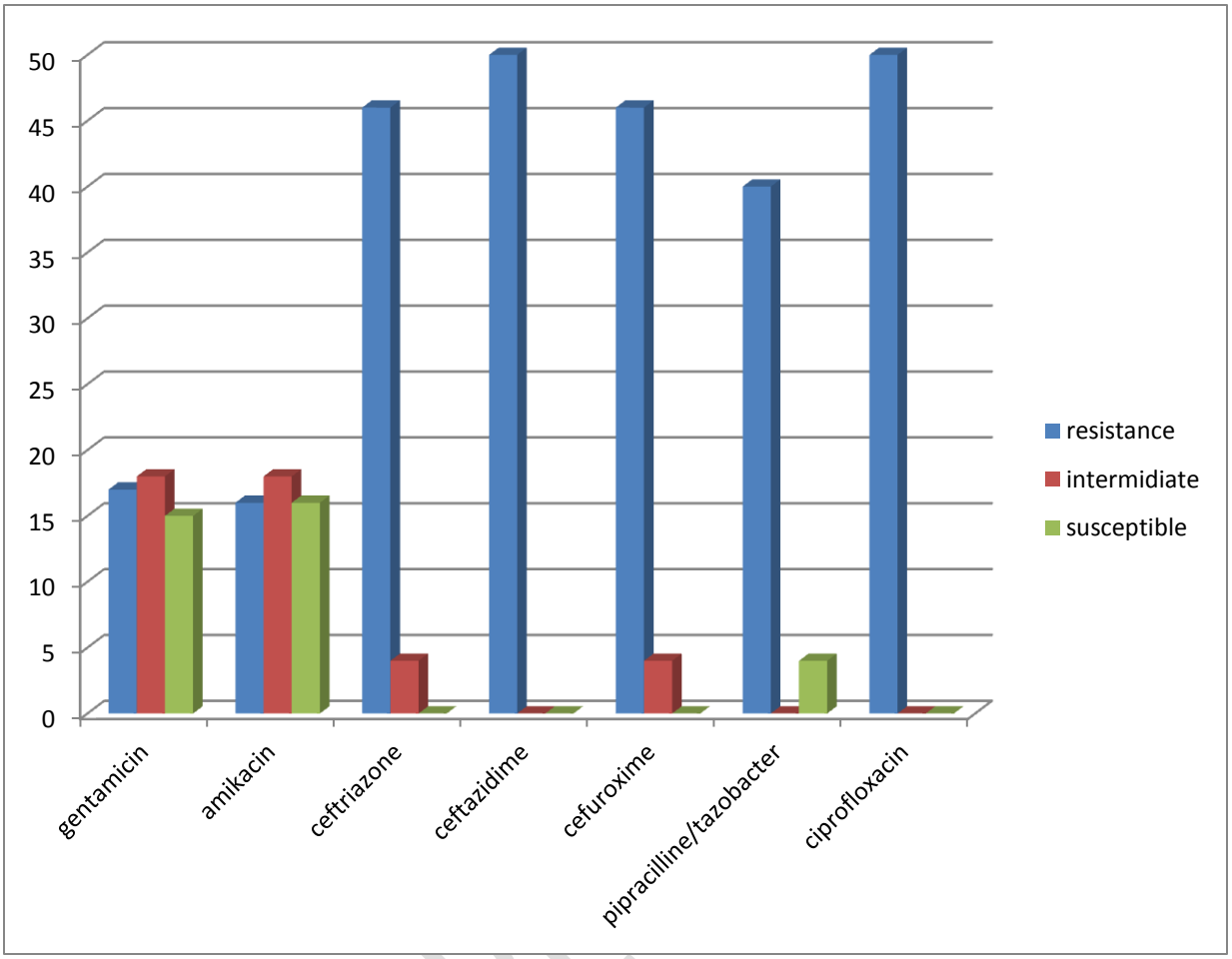
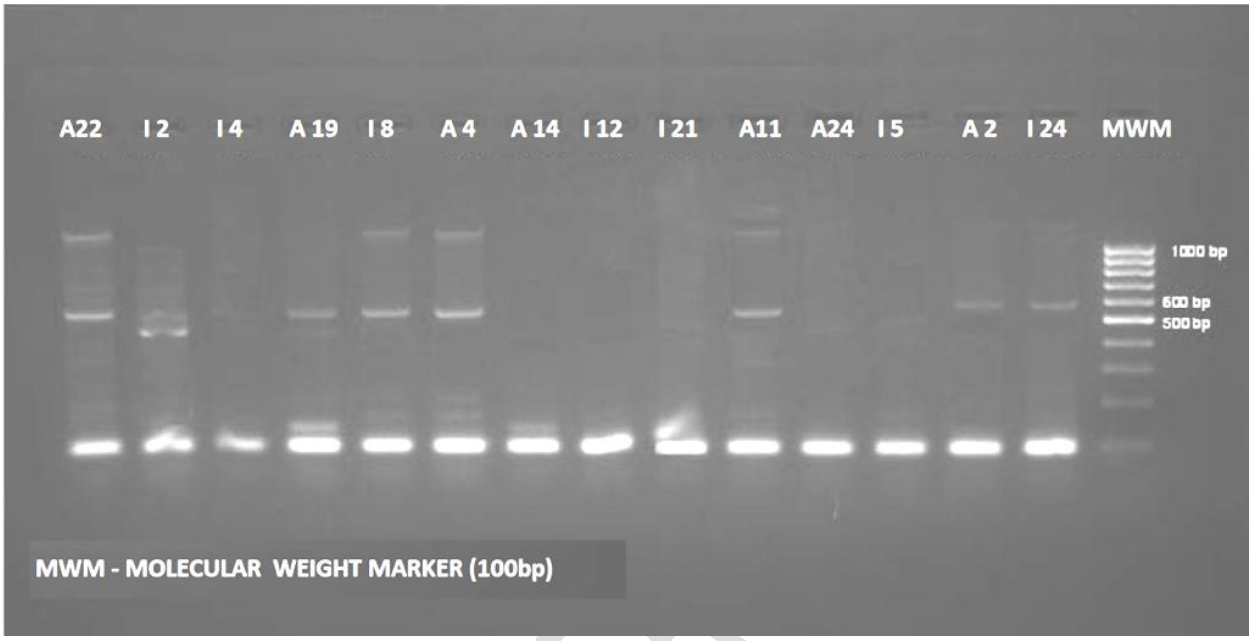


Figure 3 shows the agarose gel electrophoresis of plasmids recoverd from *Pseudomonas aeruginosa* isolates. Here, A22, I8, A4 harboured 3 plasmid bands with the molecular weight of 100, 500 and <1000 base pairs (bp). I2, A19, A11, A2 and I24 possessed 2 plasmid bands of different molecular weights whereas I4, A14, I12, A24, I5 and I21 haboured 1 plasmid band each with the molecular weight of 100 base pair

Figure 3: Agarose gel electrophoresis of plasmids recovered from *Pseudomonas aeruginosa* isolates



Lane MWM= 100bp DNA ladder marker, lane A22- I24 = *Pseudomonas aeruginosa* isolates screened.

4.0. DISCUSSION

This study determined the plasmid profile of multi drug resistant *P. aeruginosa* isolated from clinical samples in Anambra and Imo States and found out that the level of multidrug resistance in these States were high but was still relatively low compared to other reports from Ogbolu *et al.,* [20], who showed that *P. aeruginosa* isolates are found to be multi drug resistance. *P. aeruginosa* is currently one of the most frequent nosocomial pathogen and the infection due to this organism is hard to treat due to antibiotic resistance [21]. The overall incidence of antibiotic resistance of *Pseudomonas aeruginosa* was high in this study, no single antibiotic showed 100% sensitivity to all *P. aeruginosa* isolates. In Anambra State, *P. aeruginosa* isolates showed very

high resistance level (100%) to Ceftazidime, Cefuroxime and Ciprofloxacin while in Imo state, it showed (100%) to Ceftazidime, Ciprofloxacin This is called multiple drugs resistance (MDR). MDR *P. aeruginosa* has been previously reported [22]. This resistance results from the complex interaction of several mechanisms, which tend to inactivate the antibiotics or prevent their intracellular accumulation to inhibitory levels [23]. However, in Anambra State there was also 90% resistance to Ceftriazone, and 86% resistance to Piperacillin-tazobactem, whereas in Imo State there was 80% resistance to Cefuroxime, Ceftriazone and Piperacillin-tazobactam. Its resistance to third generation Cephalosporins and Piperacillin/tazobactem is a real threat. As compared to other studies, in this study, *P. aeruginosa* showed the least resistance in Gentamicin and Amikacin in both States (for Imo state 34% and 32%, Anambra 48% and 40% respectively). This study was in agreement with the study conducted by Akingbade *et al*., [24], who stated a least resistance of 29% in Gentamicin. This study did not concur with studies conducted by Tamil and Murugan, [25], in Jamaica [26], Latin America [27], who reported Ciprofloxacin to be the most potent drug available for the treatment of *P. aeruginosa* infections. The reason for the high resistance observed in this study may be due to increase in irrational consumption of antibiotics, easy accessibility to antibiotics, sub standard diagnostic laboratories not undertaking antibiotic susceptibility testing, transmission of resistant organism between people, self medications, non compliance with medication and sales of substandard drugs. Although there was a high resistance rate from the result of this study across the two States, Anambra State was seen to have the highest resistant rate, and this maybe as a result of poor hygiene and sanitation in Anambra State, sales of antibiotics over the counter; but yet Amikacin and Gentamicin may be considered as empirical therapy of first choice for *Pseudomonas aeruginosa* in Anambra and Imo State.

In this study, plasmids analyses revealed that there were detectable plasmids in 19 (76%) out of the 25 selected multi drug resistant *Pseudomonas aeruginosa* isolates in Anambra State and 23(92%) out of the 25 selected multi drug resistance *Pseudomonas aeruginosa* isolates in Imo State. All the *Pseudomonas aeruginosa* isolates that were found to harbour plasmids were resistant to at least 4 antibiotics with sizes ranging from 100bp to >1000bp . This study was somehow related to the studies carried out by Akingbade *et al*., [24], whose plasmid size ranged from 662bp to 830bp, but was not in agreement with the study conducted by Bamidele *et al*., [25], whose estimated plasmid size ranges from <1 to >23kbp. Plasmids are mobile genetic elements and can also facilitate the dispersal of resistance genes among the bacterial population and can also serve as vehicle for other resistance mechanisms. Plasmid mediated resistance to various antimicrobial drugs have also been demonstrated by Olayinka *et al*., [21] who reported that 14 isolates out of 16 posessed plasmids 8 of which had similar plasmid band patterns of 1-3 plasmid bands having low to intermediate molecular weights and Igumbor *et al*., [28] who reported that all isolates resistant to the antibiotics used possessed plasmids of molecular weight 1.5, 1.8, 2.9, 7.4 kbp. In a study in LUTH, resistance to gentamicin, tobramycin and carbencillin were attributed to transferable plasmids [29]. In another study done in Greece, plasmids isolated from multi-resistance *P.aeruginosa* strains were found to encode high level resistance to gentamicin and tobramycin [7]. Also, outbreaks in Korea, Japan and Turkey, plasmids encoding potent beta lactamases together with aminoglycoside modifying enzymes were disseminated among *P. aeruginosa* strains rendering control more difficult [30].

Out of 25 isolates profiled in Anambra State, six isolates harboured no plasmid at all but were all resistant to Ciprofloxacin, Ceftriazone, Ceftazidime and Cefuroxime. Nine isolates harboured one plasmid each and were all resistant to Ciprofloxacin, Ceftazidime, Cefuroxime and

Piperacillin-tazobactam. Eight isolates haboured two plasmids and were all resistant to Ciprofloxacin, Ceftazidime and Cefuroxime. Two isolates harboured three plasmids and were all resistant to Ciprofloxacin, Amikacin, Ceftriazone, Ceftazidime, Cefuroxime and Piperacillin-tazobactam.

In Imo State, two isolates haboured no plasmid but were resistant to 6 antimicrobials. Eight isolates haboured one plasmid and were all resistant to Ciprofloxacin, Cefuroxime, Cefriazone and Ceftazidime. Nine isolates haboured two plasmids and were all resistant to Ciprofloxacin, Gentamicin, Ceftazidime and Cefuroxime. Five isolates haboured three plasmids and were all resistant to Ciprofloxacin, Ceftazidime and Piperacillin-tazobactam. However, there seems to be no relationship between the resistant pattern of these isolates and the number of plasmids they haboured.

CONCLUSION

This present study provides a view into the antibiotic resistant profile of *P. aeruginosa* isolated from clinical specimen. It has highlighted diverse plasmid profiles and wide spread antimicrobial resistance patterns of *P. aeruginosa* isolates in Anambra and Imo State and have been concluded that plasmids are the major vehicles that help in the spreading of resistance. The multiplicity of antibiotic resistance and plasmids among the isolates in this study is disturbing, considering the fact that it establishes their potential abilities to the spread of antimicrobial resistance. The irrational and inappropriate use of antibiotics is responsible for this development of resistance. Therefore the rational use of antimicrobial must be a priority. The incidence of *Pseudomonas aeruginosa* in hospital settings is becoming alarming in developing countries because of relaxation in proper hygiene measures and production of low quality antiseptics. Traditional

drugs like Ciprofloxacin, Ceftriazone, Ceftazidime and Piperacillin tazobactam used for the treatment of *P. aeruginosa* infections may not be reliable. Therefore new drugs should be considered for *P. aeruginosa* antibiotic therapy.

REFERENCES

[1] Lambert PA. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. Journal Resource Social Medical. 2002; **95**(41): 22-26.

[2] Schaechter M, Baldauf SL, Baross JA, Baulcombe DC, Haselkom R, Hopwood DA, Ingraham JL. Encyclopedia of Microbiology USA. 2009; **3**: 314.

[3] Lister PD, Wolter DJ, Hanson ND (2009). Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clinical Microbiology Revised. 2009; **22**: 582-610.

[4] Akinyoola AL, Oginni LM, Adegbehingbe OO. Causes of limb amputation in Nigerian children. West African Journal of Medicine. 2006; **25**(4): 273-275.

[5] Pathmanathan SG, Samat NA, Mohamed R. Antimicrobial susceptibility of clinical isolates of *Pseudomonas aeruginosa* from a Malaysian Hospital. Malays Journal Medical Science. 2009; **16**(2): 27-32.

[6] Rowland B. *Pseudomonas* infections. http://www.answers.com/topic/pseudomonasinfections. 2009

[7] Tsakris A, Pournaras S, Woodsord, N. Outbreak of infection caused by *Pseudomonas aeruginosa* producing VIM-1 carbapanemase in Greece. Journal Clinical Micrbiology. 2000; **38**:1290-1292.

[8] Hidron AI. Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention. Infection Control and Hospital Epidemiology. 2008; **29**:996–1011.

[9] Wariso BA, Iba SN. Bacteriology of chronic discharging ears in Port Harcourt, Nigeria. West African Journal of Medicine. 2006; **25**:219-222.

[10] Gooze L. Bacterial infections associated with HIV. http://www.livingsite.com. 1998.

[11] Forbes BA, Sahm DF, Weissfeld AS. Bailey and Scott's Diagnostic Microbiology. 2002; **11**:389-394.

[12] Balows A, Duerden BI. Topley and Wilsions's Systemic Bacteriology. London: Arnold. 1998; **2**(9).

[13] Todar K. Todar's online textbook of bacteriology. http://www/.textbookbacteriology.net. 2008.

[14] Kanj SS, Kanafani ZA. Current concept in antimicrobial therapy against resistance gram negative organism: extended-spectrum beta-lactamase-producing Enterobacteriaceae, carbapenem-resistant Enterobacteriaceae, and multidrug-resistant *Pseudomonas aeruginosa*. Mayo Clinic Proceedings. 2011; **86**(3): 250-259.

[15] Rakesh MR, Govind LN, Kalpesh M, Rosy P, Kavu P, Vegad MM. Antibiotic resistance pattern in *Pseudomonas aeruginosa* species isolated at a tertiary care hospital, Ahmadabad. National Journal Medical Resources. 2012; **2**(2):156 -9.

[16] Santo E, Macedo C, Marin JM. Virulence factors of *Pseudomonas aeruginosa* from a university hospital in Ribeitao Preto, Sao Paulo, Brazil. Revised inst medical tropical. Sao Paulo. 2006; **48**: 185-188

[17] Qarah S, Cunha BA, Dua P, Lessnau K, Madappa T. *Pseudomonas aeruginosa* infection from http://www.emedicine.medscape. Comarticle /2262748-diagnosis. 2009.

[18] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement. Wayne: Clinical and Laboratory Standards Institute. 2015.

[19] Schaberg DR, Tompkins LS, Falkow S. Use of agarose gel electrophoresis of plasmid deoxyribonucleic acid to fingerprint gram- negative bacilli. Journal of clinical microbiology. 1981; **13**(6): 1105-1108.

[20] Ogbolu DO, Ogunledun A, Adebiyi OE, Daini OA, Alli AO. Antibiotic susceptibility patterns of *P. aeruginosa* to available antipseudomonal drugs in Ibadan, Nigeria. African Journal of Medical Science. 2008; **37**: 339-344.

[21] Olayinka AT, Olayinka BO, Onile BA. Antibiotic susecptibility and plasmid pattern of *P. aeruginosa* from the surgical unit of a university teaching hospital in north central Nigeria. International Journal of Medical Science. 2009; **1**: 79-83.

[22] Loureiro MM, De -Moraes B, Mendonca VLF, Pinheiro GS, Asensi MD. *Pseudomonas aeruginosa:* Study of antibiotic resistance and molecular typing in hospital infection cases in a neonatal intensive care unit from Riode Janeiro City, Barzel. Members Institutes of Oswaldo Criz, Riode Janeir. 2002; **97** (3): 387 - 394.

[23] Hancock RE, Speert DP. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. Drug Resistant Update. 2000; **3**:247-255.

[24] Akingbade OA, Balogun SA, Ojo DA, Afolabi RO, Motayo BO, Okerentugba PO, Okonko IO. Plasmid profile analysis of multidrug resistant *P. aeruginosa* isolated from wound infections in South West, Nigeria. World Applied Sciences Journal. 2012; **20** (6): 766-775.

[25] Tamil SS, Murugan S. Antimicrobial susceptibility patterns of *Pseudomonas aeruginosa* from diabetes patients with foot ulcers. International Journal of Microbiology. 2011; **60**: 51-95

[26] Brown PD, Izundu A. Antibiotic resistance in clinical isolates of *P. aeruginosa* in Jamaica. American Journal of public health. 2004; **16**:125-130.

[27] Jonas RN (2001). Resistance pattern among nosocomial pathogen, trends over the past few years. Chest, **119**: 397- 404.

[28] Igumbor E, Gwanzuru L, Chirara M, Obi C, Muza D. Antibiotic sensitivity and plasmid profile of *Pseudomonas aeruginosa.* Central African Journal of Medicine. 2000; **46** (11): 296-300

[29] Rotimi AO, Esho EO, Emina PA. Outbreak of multiple resistant in *Pseudomonas aeruginosa* carrying transferable resistant factor (R plasmids) in a urology clinic, Niger Qurters. Journal of Hospital Medicine. 1984; **2**:3-9.

[30] Livermore DM. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clinical Infectious Diseases. 2002; **34**:634-40.