

Full Length Research Paper

# A study of multiple resistance and role of extended spectrum $\beta$ -lactamases in environmental *Pseudomonas* isolates

Chikwendu C. I.<sup>1\*</sup>, S. N. Ibe<sup>2</sup> and G. C. Okpokwasili<sup>2</sup>

<sup>1</sup>Department of Microbiology, School of Science, Federal University of Technology, P. M. B. 1526, Owerri, Imo State, Nigeria.

<sup>2</sup>Department of Microbiology, Faculty of Science, University of Port Harcourt, Choba, Rivers State, Nigeria.

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The activity of 13 antimicrobials was studied, to determine multiple antimicrobial resistances, against 208 *Pseudomonas* strains (125 *Pseudomonas* sp and 83 *Pseudomonas aeruginosa*) isolated from river and aquaculture samples. Isolates were confirmed with API 20 NE. Isolates from both sources reported high resistance rates for similar antibiotics. These were: Ampicillin (100, 100%), cefuroxime (96.8, 81.9%), streptomycin (93.6, 78.6%), cefotaxime (93.6, 77.1%), for river water and aquaculture samples respectively. While resistances were high for ofloxacin, norfloxacin, mezlocillin, ceftazidime, kanamycin and enrofloxacin (70 to 85.5%) among the river water isolates, they were moderate among the aquaculture isolates (50 to 69.4%). Ninety seven (97.2%) percent of isolates from river water and 78.9% from aquaculture were resistant to at least three or more antimicrobials and were termed multi-resistant. Results of plasmid profiling showed the presence of multiple plasmids ranging in size between 639 and 11845 bp. Preliminary tests for  $\beta$ -lactamase detection by double-disk approximation were positive with high resistances to imipenem, aztreonam, cefpodoxime and cefotaxime. PCR of amplified genomic DNA was positive for the detection of *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub>  $\beta$ -lactamase genes. However, up to 50% of all isolates tested were found to be resistant to the  $\beta$ -lactamase inhibitor of combination amoxicillin+clavulanic acid. The results infer the presence of multi resistant organisms as well as genes conferring  $\beta$ -lactamase resistance in environmental samples.

**Key words:** *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>,  $\beta$ -lactamase, multi-resistant, *Pseudomonas* sp., environmental sources.

## INTRODUCTION

Bacterial resistance to antibiotics constitutes an emerging clinical problem owing to the wide availability of antibiotics and often, their misuse (Davies and Amabile-Cuevas, 2003). According to French (2005), the emergence of antibiotic resistant pathogenic bacteria in clinical environments has become a serious problem world wide. However, drug resistant bacteria have also been detected from natural environments, where no direct exposure to antibiotics is known (Goni-Urizza et al., 2000; Schwartz et al., 2003). The presence of such non-clinical

resistant bacteria poses a risk to humans and the environment as they may act as resistance reservoirs, contributing to the maintenance and spread of antibiotic resistance genes (Goni-Urizza et al., 2000).

Due to their association with infectious diseases, most research on antibiotic resistance in environmental sources has been conducted on bacterial indicators of faecal contamination, particularly, faecal coliforms and enterococci (Goni-Urizza et al., 2000; Gallert et al., 2005). Only a few of the research has focused on other human pathogenic bacteria from non-clinical environments, including *Pseudomonas* (Blasco et al., 2008; Jensen et al., 2001; Akinbowale et al., 2006). Concern as to spread of resistance from indigenous environmental

\*Corresponding author. E-mail: [chinwechikwendu@yahoo.com](mailto:chinwechikwendu@yahoo.com).

bacteria to pathogenic organisms, thereby compromising antimicrobial treatment of pathogenic organisms has also been raised (Armstrong et al., 1995; Davies, 1996).

Pseudomonads are gram-negative, aerobic rods, which form fluorescent colonies on suitable agar. They are positive for oxidase, catalase, citrate, arginine decarboxylase and negative for the fermentation of glucose. According to Byrd et al. (1991), *Pseudomonas* sp. when compared with other gram-negative bacteria appears to possess the ability to remain viable and cultivable for unusually long periods of exposure to adverse conditions. In recent years, particular attention has been paid to this micro organism because of its increasing significance as a human pathogen and its high resistance to most antibiotics.

Extended-spectrum  $\beta$ -lactamases (ESBLs) are enzymes that moderate resistance to extended-spectrum cephalosporins (ESCs), such as cefotaxime (CTX), ceftazidime and ceftazidime (CAZ), and the monobactam aztreonam (ATM) (Livermore and Brown, 2001). Such enzymes are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* and have been recently detected in *P. aeruginosa* at low frequency (Ben-marez et al., 1999; Lee et al., 2005; Poirel et al., 2004; Pagani et al., 2004; Weldhagen, 2004).

This study was therefore undertaken to study multiple resistance, as well as the presence and role of extended spectrum  $\beta$ -lactamases in environmental *Pseudomonas* isolates. The role of plasmids in the observed multiple resistance was also identified.

## MATERIALS AND METHODS

### Sample types, processing and bacterial isolates

Water samples for analysis were collected from river water and aquaculture sources in sterile 1L sample containers. Initial bacterial isolation was done by spreading 0.1 ml of samples on the dry surface of Pseudomonas-Aeromonas agar (GSP agar, Merck), for isolation of *Pseudomonas* sp., while fluorescent *Pseudomonas* isolates were determined on *Pseudomonas* centrimide agar (Oxoid). Isolates were confirmed with API 20 NE (Bio-Mérieux).

### Antimicrobial resistant testing and screening for ESBLs

Routine antibiograms were determined by the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar (Oxoid, Basingstoke). The antibiotics assayed and their concentrations were Ampicillin (10  $\mu$ g), cefuroxime (30  $\mu$ g), streptomycin (10  $\mu$ g), cefotaxime (30  $\mu$ g), mezlocillin (75  $\mu$ g) kanamycin (30  $\mu$ g), ceftazidime (30  $\mu$ g), norfloxacin (10  $\mu$ g), ofloxacin (5  $\mu$ g), enrofloxacin (5  $\mu$ g) ciprofloxacin (5  $\mu$ g), gentamycin (120  $\mu$ g), and co-trimoxazole (25  $\mu$ g) (Oxoid, Basingstoke). The results obtained were interpreted according to the CLSI standard (CLSI, 2007). Phenotypic expression of ESBL production was conducted by the double disk diffusion test with the following antimicrobials: imipenem (10  $\mu$ g), cefpodoxime (10  $\mu$ g), cefepime (30  $\mu$ g), ceftipime (30  $\mu$ g), amoxicillin +clavulanic acid (20/10  $\mu$ g) (MAST Diagnostics, England).

### Plasmid isolation and profiling

Plasmid extraction was carried out by the method described by Ehrenfeld and Clewell (1987). Plasmid profiling was carried out on 0.8% agarose gel in a 0.5% concentration of Tris-Borate-EDTA (TBE) buffer. A HIND III digest of DNA was used as molecular weight marker and the gel was electrophoresed in a horizontal tank at a constant voltage of 60 V for about 1 h 30 min. Plasmid DNA bands were identified by fluorescence of bound ethidium bromide using a short wave ultraviolet light transilluminator. Photographs were taken using a digital camera.

### PCR amplification

Chromosomal DNA was extracted by the alkaline lyses method as described by Birboim and Doly (1979). PCR reactions were conducted using bacterial genomic DNA as substrate and specific primers for the genes coding for  $\beta$ -lactamases TEM, SHV and CTX. 25  $\mu$ l reaction mixture containing 10-200 ng of purified DNA, 200  $\mu$ M of each deoxynucleoside triphosphates (dNTP) (Promega), 1.5 mM MgCl<sub>2</sub>, 1 x PCR buffer, 1 U of *Taq* polymerase (Promega) and sterile distilled water. Thermal cycling was conducted in an Eppendorf Master Cycler Gradient with the following temperature profile: initial denaturation (94°C for 5 min); 30 amplification cycles (94°C for 1 min); annealing for 1 minute (at 50°C for *bla*<sub>TEM</sub>, 54°C for *bla*<sub>SHV</sub> and 62°C for *bla*<sub>CTX</sub>); extension (72°C for 1 min) and final extension step (72°C for 10 min). The amplification products were analysed by gel electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. 100 bp DNA ladder was used as DNA molecular weight marker.

## RESULTS

A total of 208 *Pseudomonas* sp were isolated from the aquatic sources. Table 1 shows a list of the different primers used in screening for genotypic expression of  $\beta$ -lactamase production by the isolates. It also shows their sequences and amplicon sizes in bp.

The river water isolates were tested against 12 antimicrobials, and the results showed high resistance rates (70 to 100%) to 83% of the antimicrobials and moderate resistance (56.9 to 58.7%) to 16.7% of the antimicrobials (Table 2). They were most resistant to enrofloxacin, ceftazidime, mezlocillin, norfloxacin, ofloxacin, cefuroxime, streptomycin, cefuroxime and ampicillin (70 to 100%), and most susceptible to ciprofloxacin and gentamycin.

The aquaculture water isolates were tested against 13 antimicrobials. The results also showed high resistance rates (77 to 100%) to 30.8% of the test antimicrobials, moderate resistance (50-69.4%) to 61.5% and low rates (36.1%) to 7.69% of the test antimicrobials (Table 3). The greatest resistance among the aquaculture isolates was exhibited against norfloxacin, cefotaxime, streptomycin and ampicillin (69.4 to 100%). The results showed that isolates from both environmental sources reported high resistant rates for similar antimicrobials.

*P. aeruginosa* isolates however expressed higher resistance rates than the *Pseudomonas* sp in both environmental samples. A comparative analysis of

**Table 1.** The primers and primer sequences used to screen for ESBL genes.

Primer	Gene	Sequence/size (bp)	Reference
757(FP)	blaTEM	5'-GCGGAACCCCTATTTG-3' / 964	Olesen et al. (2004)
821(RP)	blaTEM	5'-TCTAAAGTATATATGAGTAAACTTGGTCTGAC-3' / 964	Olesen et al. (2004)
1436(FP)	blaSHV	5'-TTCGCCTGTGTATTATCTCCCTG-3' / 854	Hasman et al. (2005)
1437(RP)	blaSHV	5'-TTAGCGTTGCCAGTYTCG-3' / 854	Hasman et al. (2005)
1354(FP)	blaCTX	5'-ATGTGCAGYACCAGTAARGTKATGGC-3' / 593	Miro et al. (2002)
1355(RP)	blaCTX	5'-TGGGTRAARTARGTSACCAGAAYCAGCGG-3' / 593	Miro et al. (2002)

FP - Forward primer and RP - Reverse primer.

**Table 2.** Frequency of antimicrobial resistance of *Pseudomonas* sp isolated from river water.

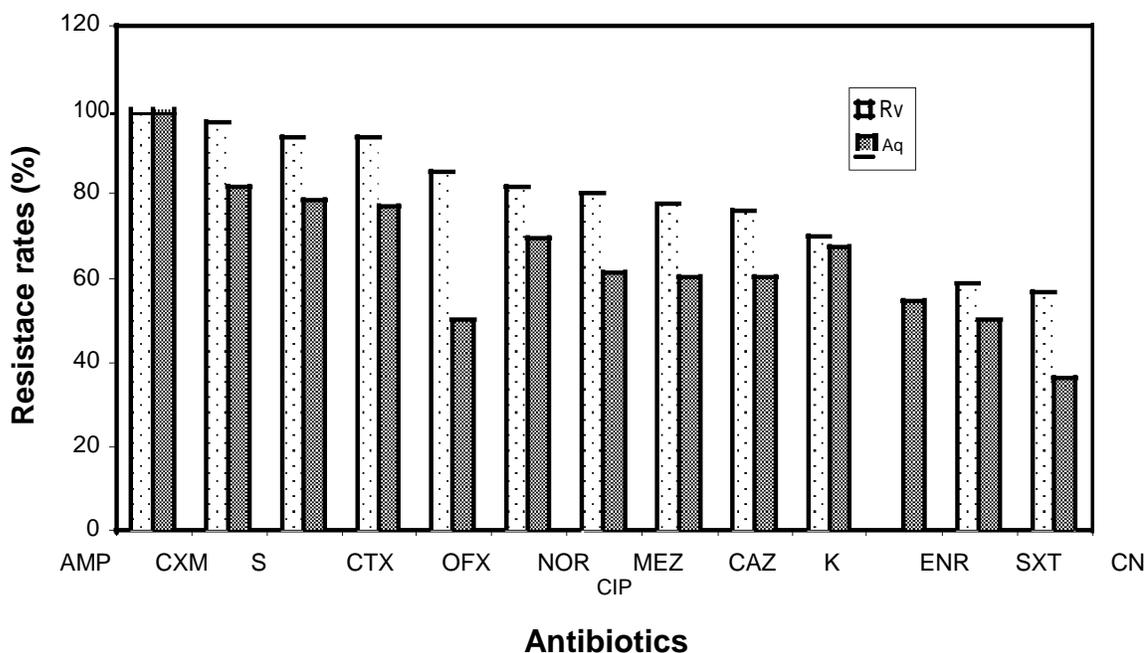
Antibiotics	No. of isolates	<i>Pseudomonas</i> Sp	No. of isolates	<i>Pseudomonas aeruginosa</i>	Total no. of isolates	Total resistance
AMP	90	90(100)	35	35(100)	125	125(100)
CXM	90	87(96.7)	35	34(97.1)	125	121(96.8)
S	90	82(91.1)	35	35(100)	125	117(93.6)
CTX	90	83(92.2)	35	34(97.1)	125	117(93.6)
OFX	75	62(82.1)	35	32(91.4)	110	94(85.5)
NOR	75	59(78.7)	35	31(88.6)	110	90(81.8)
MEZ	15	12(80.0)	ND	ND	15	12(80.0)
CAZ	90	67(74.4)	35	30(85.7)	125	97(77.6)
K	90	70(77.8)	35	25(71.4)	125	95(76.0)
ENR	70	49(70.0)	20	14(70.0)	90	63(70.0)
CN	53	28(52.8)	10	9(90.0)	63	37(58.7)
CIP	75	45(60.0)	27	13(48.1)	102	58(56.9)

AMP, Ampicillin; CXM, cefuroxime; S, streptomycin; CTX, cefotaxime; OFX, ofloxacin; NOR, norfloxacin; MEZ, mezlocillin; CAZ, ceftazidime; K, kanamycin; ENR, enrofloxacin; CN, gentamycin; CIP, ciprofloxacin. No (%) of resistant isolates.

**Table 3.** Frequency of antimicrobial resistance of *Pseudomonas* sp isolated from aquaculture water.

Antibiotics	No. of isolates	<i>Pseudomonas</i> Sp	No. of isolates	<i>Pseudomonas aeruginosa</i>	Total no. of isolates	Total resistance
AMP	64	64(100)	19	19(100)	83	83(100)
CXM	64	49(76.6)	19	19(100)	83	68(81.9)
S	64	51(79.7)	6	4(66.7)	70	55(78.6)
CTX	64	45(70.3)	19	19(100)	83	64(77.1)
NOR	53	35(66.0)	19	15(78.9)	72	50(69.4)
ENR	64	39(60.9)	19	17(89.5)	83	56(67.5)
MEZ	15	11(73.3)	3	0(0.0)	18	11(61.1)
K	39	21(53.8)	19	14(73.7)	58	35(60.3)
CAZ	64	34(53.1)	19	16(84.2)	83	50(60.2)
SXT	31	17(54.8)	ND	ND	31	17(54.8)
OFX	53	22(41.5)	19	14(73.7)	72	36(50)
CN	33	15(45.5)	13	8(61.5)	46	23(50)
CIP	53	16(30.2)	19	10(52.6)	72	26(36.1)

AMP, Ampicillin; CXM, cefuroxime; S, streptomycin; CTX, cefotaxime; OFX, ofloxacin; NOR, norfloxacin; MEZ, mezlocillin; CAZ, ceftazidime; SXT, cotrimoxazole; K, kanamycin; ENR, enrofloxacin; CN, gentamycin; CIP, ciprofloxacin. No (%) of resistant isolates.



**Figure 1.** Comparative rates of resistance of *Pseudomonas* isolates from river and aquaculture water samples. Rv: River water, Aq: Aquaculture, AMP: Ampicillin, CXM: cefuroxime, S: streptomycin, CTX: cefotaxime, OFX: ofloxacin, NOR: norfloxacin, MEZ: mezlocillin, CAZ: ceftazidime, SXT: cotrimoxazole, K: kanamycin, ENR: enrofloxacin, CN: gentamycin, CIP: ciprofloxacin.

**Table 4.** Results of phenotypic screening of isolates for ES -L gene production.

Source	Isolate	No. tested	IMI resistant {No. (%)}	Producing ES -Ls {No. (%)}	Inhibitor resistant {No. (%)}
River water	<i>Pseudomonas</i> sp	60	28 (46.7)	60 (100)	28 (46.7)
	<i>P. aeruginosa</i>	21	6 (28.6)	21 (100)	10 (47.6)
Aquaculture	<i>Pseudomonas</i> sp	28	12 (42.9)	12 (42.9)	6 (21.4)
	<i>P. aeruginosa</i>	16	11 (68.8)	16 (100)	16 (100)

IMI, imipenem; ES Ls, extended spectrum beta lactamases.

resistance rates in both samples showed higher resistance in river water isolates than the aquaculture isolates (Figure 1). The aquaculture isolates showed only moderate resistance to ofloxacin, ceftazidime, mezlocillin and enrofloxacin (50 to 67.5%), while resistance rates to these same antimicrobials were high among the river water isolates (70 to 85.5%). The aquaculture isolates were also more susceptible to ciprofloxacin and Gentamicin than the river water isolates. For both environmental sources, multiple resistances were recorded in up to 98% of the isolates screened, with the all the isolates being resistant to 2 or more antimicrobials each.

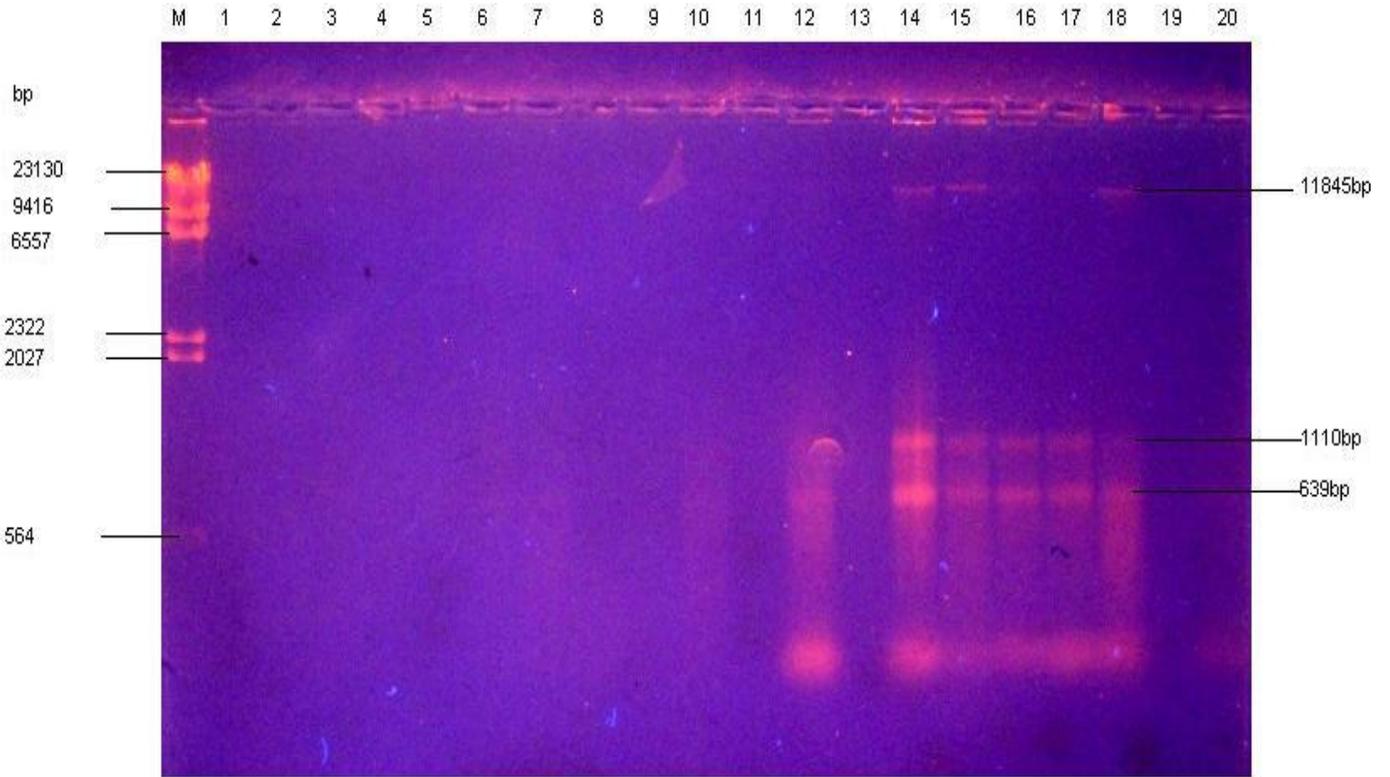
Phenotypic screening of isolates for ES -L gene production showed a high number to be ES -L gene producers as well as high rates of resistance to both imipenem and the -lactamase inhibitor (Table 4).

Plasmid analysis revealed the presence of plasmids

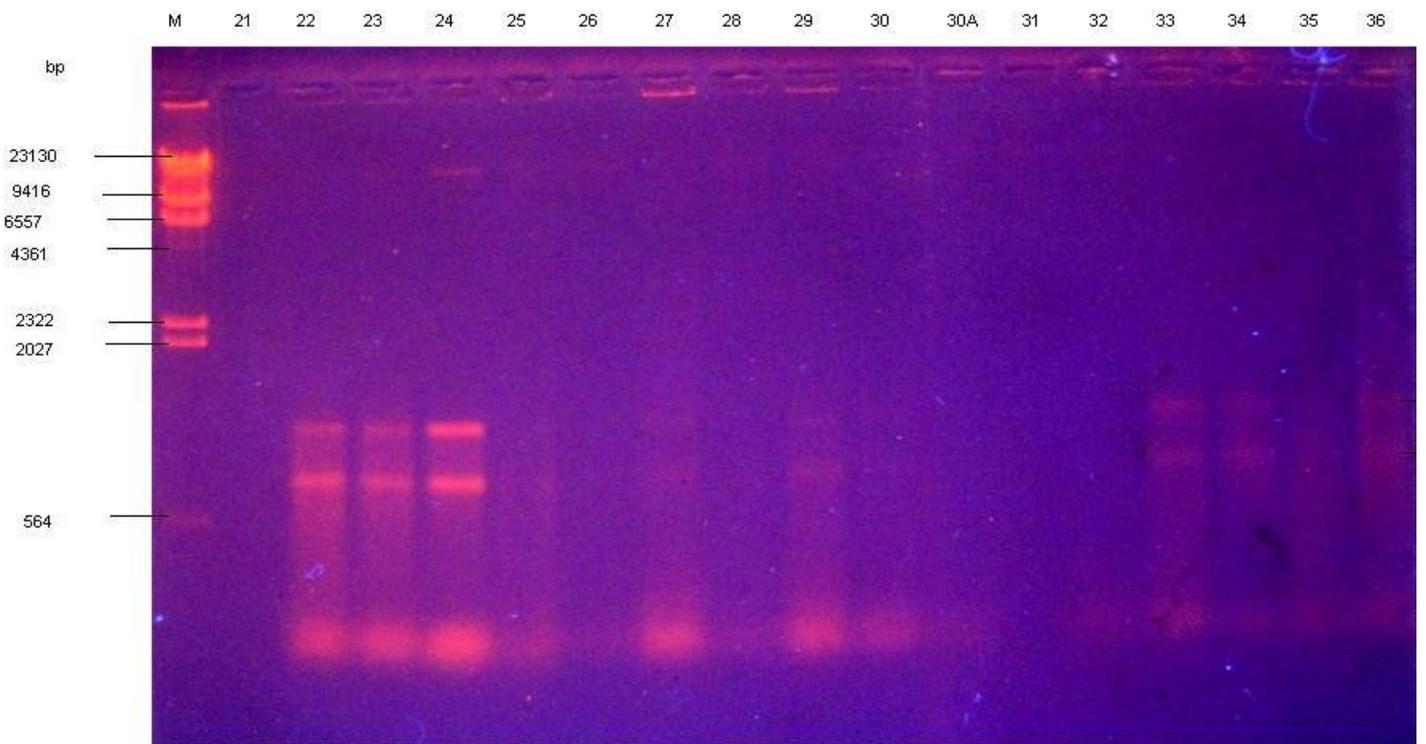
ranging in size from 794-11845bp in more than 50% of all the tested isolates. 85.7 and 58.3% of the aquaculture and river water isolates respectively possessed 2-3 plasmids each (Figures 2 and 3). Results of the gel electrophoresis of the PCR amplified genomic products showed amplification bands for two of the ES -L genes tested: *bla<sub>SHV</sub>* and *bla<sub>TEM</sub>*. 16.7% of the isolates were positive for both ES -L *bla<sub>SHV</sub>* and *bla<sub>TEM</sub>* genes at 854 and 964 bp respectively.

## DISCUSSION

The environmental *Pseudomonas* isolates in this study exhibited high rates of resistance as well as multiple antibiotic resistance (MAR) to the different drugs tested. They exhibited 100% resistance to ampicillin as well as very high rates of resistance (96.8, 81.9 and 93.6,77.1%),



**Figure 2.** Electrophoretic pattern for the plasmid profile of isolates 14-19. Lanes 14-19, *Pseudomonas aeruginosa* from aquaculture.



**Figure 3.** Electrophoretic pattern for the plasmid profiles of isolates 22-33. Lane 22 – 25, samples of plasmid DNA for *Pseudomonas* sp from river water; lane 26, *P. aeruginosa* from river water; lanes 27 – 28, *Pseudomonas* sp from river water; lanes 29-32, *P. aeruginosa* from river water and lane 33, *Pseudomonas* sp from aquaculture.

to the second and third generation cephalosporins cefuroxime and cefotaxime, for river water and aquaculture samples respectively. This is similar to the results of Akinbowale et al. (2006). The resistance found in ampicillin also corresponds to what has been found in many studies in a number of countries (Chelossi et al., 2003; Saha and Pal, 2002; Hartha et al., 2005). Resistance to ceftazidime was however moderate at 77.6 and 60.2% for river and aquaculture samples respectively. The resistance patterns of *P. aeruginosa* to the cephalosporins was consistent with the one reported by Gad et al. (2007) and Yetkin et al. (2006) who showed that the percentage of resistance to cephalosporins for their isolates was in the range of 27% to 88%.

Resistance to the quinolone antimicrobials, in contrast to the assertion of low frequency of resistance by environmental isolates (McKeon et al., 1995; Guardabassi et al., 1998), was high for ofloxacin and norfloxacin and moderate for ciprofloxacin (56.9% and 36.1%) for river water and aquaculture samples respectively. Gad et al. (2007) also reported moderate activity of quinolones towards *P. aeruginosa*. While other authors reported similar high rates of resistance to the quinolones (Bratu et al., 2005; Sahm et al., 2001), Blasco et al. (2008) and Corona-Nakamura et al. (2001), showed that *P. aeruginosa* was absolutely susceptible to ciprofloxacin. Chelossi et al. (2003) however reported resistance to nalidixic acid in 70% of their isolates. These discrepancies according to Gad et al. (2007) can be attributed to the continuous development of multi-drug resistant (MDR) strains of *P. aeruginosa* in different parts of the world.

Resistance to Gentamicin and kanamycin were moderate to high in isolates from both environmental samples. This also agrees with the results of Gad et al. (2007) and Muller-Premru and Gubina, (2000). Sokari et al. (1988) while working on diverse Nigerian waters found a lower incidence of resistance in *E. coli* than among *Pseudomonas* sp. A high incidence of antibiotic resistance in *Pseudomonas* sp has also been reported by Ibiebele and Sokari (1989) for drinking-well waters and by Jones (1986) and Jones et al. (1986a) for lake waters. Boon and Cattanch (1999) found out that native bacteria had a greater incidence of antibiotic resistance than faecal bacteria isolated from the same waters. This could be explained according to Jones et al. (1986b) and Leff et al. (1993), if antibiotic-resistant *Pseudomonas* sp made up a significant proportion of the culturable native bacteria.

The entire *Pseudomonas* sp isolates from both the river water and aquaculture samples were resistant to one or more antimicrobials and multiple antibiotic resistant (MAR) isolates were over 90%. These results are similar to Messi et al. (2005) whose MAR *Pseudomonas* were 55% and had over 80% being resistant to one or more antimicrobials. MDR in *P. aeruginosa* is usually defined as resistance to three or more of the following

antimicrobial agents: antipseudomonal penicillins (e.g., piperacillin), antipseudomonal cephalosporins (e.g., ceftazidime), fluoroquinolones (e.g., ciprofloxacin), carbapenems (imipenem, meropenem and doripenem), and the aminoglycosides (gentamicin, tobramycin or amikacin) (Defez et al., 2004).

Multiple resistances may represent a hazard in two fronts: antimicrobial resistant bacteria would be at a selective advantage compared to the normal flora in patients during antibiotic treatment and also resistance can be transferred to susceptible organisms. This transfer may be a particular problem if the recipients are more virulent than the donor, as pathogenic bacteria.

More than 50% of all the isolates analysed were positive for the presence of plasmids with up to half of that possessing multiple plasmids ranging from 2 – 3 plasmids. Plasmids were also detected in 46% of *Pseudomonas* isolates of Messi et al. (2005). According to them, there has been increasing evidence that *Pseudomonas* and related micro organisms might play an important role in the spread of plasmids. As reported by Boronin (1992), the plasmids are ubiquitous in *Pseudomonas*, but the frequency of their occurrence and molecular weight varies greatly in particular species and in different microbial habitats.

Phenotypic expression of  $\beta$ -lactamase production was performed by the double-disk diffusion method. Amongst the isolates tested for ES -L production, 42.9% of *Pseudomonas* isolates from aquaculture were identified as ES -L producers while 100% of the river water isolates were identified as ES -L producers (Table 4). On the average, 53.9% of the tested isolates were  $\beta$ -lactamase inhibitor resistant while 46.8% were imipenem resistant. PCR was carried out with the genomic DNA of the representative isolates to check for the expression of the three  $\beta$ -lactamase genes: *bla<sub>SHV</sub>*, *bla<sub>TEM</sub>* and *bla<sub>CTX</sub>*. Results of the study by Jiang et al. (2006) showed a high frequency of ESBLs, including *bla<sub>TEM</sub>*, *bla<sub>SHV</sub>* and *bla<sub>CTX</sub>* genes in their isolates of *P. aeruginosa* examined. The above results support the results of the present study where 42.9% of the *Pseudomonas* isolates were identified as ESBL producers. These observations suggest that ESBLs widespread in the family Enterobacteriaceae might be increasingly frequently found in *P. aeruginosa*, which could also be a reservoir for the dissemination of this kind of enzyme (Livermore, 1995).

All the isolates expressed very high resistances to the  $\beta$ -lactam -  $\beta$ -lactamase inhibitor combinations. Resistance to  $\beta$ -lactam -  $\beta$ -lactamase inhibitor combinations in *E. coli* isolates has been reported to be due to hyper production of class A  $\beta$ -lactamases (such as TEM-1 or SHV-1), class D plasmid-mediated enzyme or chromosomal or plasmidic class C  $\beta$ -lactamase and /or to modified outer membrane permeability (Chaïbi et al., 1999; Oliver et al., 1999; Reguera et al., 1991; Sirot et al., 1998). The AMP C  $\beta$ -lactamase (commonly called cephalosporinase) is not inhibited by the currently available  $\beta$ -lactamase

inhibitors clavulanic acid, sulbactam and tozobactam, therefore additionally conferring resistance to antibiotic combinations such as co-amoxicillin/clavulanic acid or ampicillin/sulbactam (Nordman and Gilbert, 1998).

Results of the above study infer not only the presence of multi resistant organisms as well as genes conferring ES -lactamase resistance in environmental samples, but also indicates the need of constant monitoring and elimination of these organisms which could serve as active reservoirs of antimicrobial resistance in the environment.

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