Beta-lactamase Genes in Multi-resistant *Aeromonas* spp. isolated from River and Aquaculture Water Sources in Nigeria

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Abstract: This study examined multi-resistant Aeromonas spp. isolated from river and aquaculture water sources for determinants of resistant genes. These species can provide a reservoir for resistant genes capable of transfer to other water-borne and human pathogens. The isolates were confirmed with API 20NE. Resistance profiles of 206 Aeromonas isolates were determined for 11 antimicrobials by the Kirby-Bauer technique. PCR was used to determine the genetic determinants responsible for the ESBL phenotypes using primers for bla_{T}pjvi, bla_{S}nv and blacrx beta-lactamase genes. Phenotypic expression of ESBL production was done by the double disk diffusion method and plasmid curing was effected with acridine orange. The Aeromonas spp. comprised of the following: Aeromonas hydrophilia/caviae, A. sobria and A. salmonicida. Isolates expressed high resistant rates (75-100%) to 63.6% of the antimicrobials and moderate resistant rates (53.3-60.0%) to 27.3% of the antimicrobials tested. There were also high occurrences of multiple resistances with 100% of the isolates being resistant to 2 or more anti'niorobials. Aeromonas spp. from river water expressed higher resistant rates than those from aquaculture water samples. Phenotypic screening for carriage or presence of ESBL gene showed that all the isolates tested positive for the presence of ESBL gene and were resistant to amoxicillin/clavulanate. Amongst the 13 isolates analysed for the 3 (3-lactamase genes, the bla_{TEM} was most prevalent with 30.8% of isolates possessing it, while 23.1% and 7.7% possessed bla_{S}Hv, and blacrx respectively. Antimicrobial resistance profile, post curing, showed 38% and 100% of isolates remained ESBL producers and inhibitor resistant respectively. The study infers the presence and diversity of ESBL genes in Aeromonas spp. isolated from river and aquaculture water settings in Nigeria.

Keywords: antimicrobial resistance, beta-lactamase, plasmid

Introduction

The genus *Aeromonas* consists of bacteria that are Gram-negative rods and are considered ubiquitous in aquatic environments (Janda and Abbott, 2010), since they have been found to inhabit surface water (river, lakes), sewage, drinking water (tap, bottled and mineral) (Abulhamd, 2010; Pablos *et al.*, 2011; Odeyemi *et al.*, 2012), thermal waters and sea waters (Biscardi *et al.*, 2002; Maalej *et al.*, 2003; Pablos *et al.*, 2009). Some species, mainly the psychrophilic *Aeromonas salmonicida* and the mesophilic *Aeromonas hydrophila* and *Aeromonas veronii* are known causative agents of fish disease (Janda and Abbott, 2010) and are also important human opportunistic pathogens with ability to cause various types of diseases, including intestinal, blood, skin and soft tissue and trauma-related infections (Vivekanandhan *et al.*, 2002; Aminov, 2009; Lamy *et al.*, 2009; Janda and Abbott, 2010).

Aeromonas spp. have also been found to be environmental reservoirs of resistance determinants to different classes of antibiotics (Cattoir *et al.*, 2008; Goni-Urizza *et al.*, 2000a; L'Abée-Lund and Sørum 2000). According to Hernould *et al.* (2008), intrinsic resistance to β-lactams among the genus may arise from the expression of chromosomal β-lactamases and/or efflux pumps. In addition to that, environmental contamination with antibiotics and other pollutants also contribute to the maintenance and spread of antibiotic resistance genes (Goni-Urizza *et al.*, 2000a). According to Kruse and Sørum (1994), one mechanism that allows the perpetuation of such genes is the spread of resistance plasmids between unrelated bacteria in natural environments. Bacteria with intrinsic or acquired resistance to antibiotics are commonly found in aquatic environments, where *Pseudomonas, Serratia* and *Aeromonas* are commonly identified.

Aeromonas spp., non-cholera vibrios and *Plesiomonas shigelloides* belong to the expanding group of water and food borne pathogens. They are widely distributed in aquatic environments and are increasingly regarded as important pathogens of aquatic animals, causing significant economic losses in the aquaculture industry worldwide. In addition, these bacteria have been implicated as opportunistic pathogens mainly causing gastroenteritis in humans (Austin and Austin, 1993).

This study was therefore aimed at identifying the antimicrobial resistance profile of multiple resistant *Aeromonas* isolates from river and aquaculture water sources as well as the presence of β-lactamase enzyme determinants among the multiple resistant isolates.

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Materials and Methods

Sample types and processing

River and aquaculture water samples for analyses were collected with sterile 1L sample containers. Initial bacterial isolation was carried out by placing an aliquot (0.1ml) each of river and aquaculture water samples on the dry surface of the *Pseudomonas – Aeromonas* (GSP agar, Merck) selective agar plates and spread round the plates using sterile hockey sticks. Plates were then turned upside down, labelled and incubated at 28 ± 2°C for 24 – 48 hours. *Aeromonas* isolates were bright yellow in colour on the *Pseudomonas – Aeromonas* selective agar plates. Two or three well-spaced colonies per plate were then picked, purified and confirmed with API 20 NE (Bio-Mérieux).

Detection of antimicrobial resistant phenotypes

Antimicrobial phenotypes were determined by the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar (Oxoid, Basingstoke). The antibiotics assayed were: ampicillin (10 µg), mezlocillin (75 µg), streptomycin (10 µg), cefuroxime (30 µg), ofloxacine (5 µg), cefotaxime (30 µg), cotrimoxazole (25 µg), gentamicin (120 µg), ceftazidime (30 µg), enrofloxacine (5 µg) and ciprofloxacine (5 µg) (Oxoid, Basingstoke). Resistance results were interpreted according to CLSI standards (CLSI 2007). The double disk diffusion technique was used to determine phenotypic expression of ESBL production by the isolates.

Plasmid isolation, profiling and curing

Plasmid extraction was performed using the method of Ehrenfeld and Clewell (1987) and profiling was carried out on 0.8% Agarose gel in a 0.5% concentration of Tris-Borate-EDTA (TBE) buffer. A HIND 111 digest of λ DNA was used as molecular weight marker and the gel was electrophoresed in a horizontal tank at a voltage of 60V for 1h 30min. Plasmid DNA bands were identified by fluorescence of bound ethidium bromide using a short wave ultra violet light transilluminator. Photographs were taken with a digital camera. Thirteen (13) representative isolates were chosen and analysed for the presence or otherwise of plasmids.

Plasmid curing was carried out by the method of Salisbury *et al.* (1972), using acridine orange dye.

PCR detection of β -lactamase genes

Aeromonas genomic DNA was extracted by the alkaline lyses method of Birboim and Doly (1979). Bacterial chromosomal DNA was used as substrate for the PCR and specific primers for the β-lactamase genes TEM, SHV and CTX were used for the detection of resistance genes (Table 1). PCR was performed in 25 µl of a reaction mixture containing DNA (10-200ng), 200 µM of each deoxynucleoside triphosphates (dNTP) (Promega), 1.5 mM $MgCl_2$, 1X PCR Buffer, 20 pMol (each) of the primers, 1 unit of *Taq DNA* polymerase (Promega) and sterile distilled water. Thermal cycling was conducted in an Eppendorf Master Cycler Gradient at an initial denaturation temperature of 94°C for 5 minutes, followed by 30 amplification cycles of 1 minute at 94°C; 1 minute at (50°C for blaTEM, 54°C for blaSHV and 62°C for blaCTX), and 1 minute at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on 1.5% agarose gel and visualized by staining with ethidium bromide. One hundred (100) bp DNA ladder was used as DNA molecular weight standard.

Table 1: The primers used to screen for ESβL 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'-TTAGCGTTGCCAGTGYTCG-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)

KEY: FP - Forward primer; RP - Reverse Primer

Results and Discussion

A total of 206 isolates of *Aeromonas* spp. were recovered from both 100 river and aquaculture water samples. The *Aeromonas* spp. comprised of the following: *Aeromonas hydrophila/caviae, A. sobria* and *A. salmonicida*, as confirmed by API 20NE.

Results of screening against eleven (11) antimicrobials showed that the isolates expressed high resistances to Ampicillin, mezlocillin, streptomycin, cefuroxime, ofloxacine, cefotaxime and cotrimoxazole (75.0-100.0%), resistances to gentamycin, ceftazidime and enrofloxacine were moderate, (53.3-60.0%), while resistance to ciprofloxacine was low (39.9%) as shown in Table 1. There were significant differences (P=0.05) in resistance between isolates from the river and aquaculture water sources.

The *Aeromonas* spp. isolated expressed high occurrences of multiple resistances, with 100% of the organisms being resistant to at least 2 or more antimicrobials for both sample types (Figure 1).

Aeromonas spp. isolated from river water expressed greater resistance than those isolated from aquaculture water for the same antimicrobials. However, both river water and aquaculture water isolates expressed the least resistance rates to ciprofloxacin (Figure 2).

Two plasmids of sizes 639bp and 1110bp were isolated from one *Aeromonas* isolate, out of 13 isolates analysed for the presence of plasmids. Two aquaculture and 4 river water isolates showed amplification for the 3 β-lactamase genes tested, with bla_{TEM} being the most prevalent at 30.8%, while 23.1% and 7.7% of the isolates possessed bla_{SHV} and bla_{CTX} β-lactamase genes respectively (Table 2; Figures 2 and 3).

Fifty-one resistance patterns were identified amongst the *Aeromonas* spp. isolated from the river water samples, while 49 resistance patterns were identified amongst the isolates from aquaculture water samples (Table 3).

Aeromonas spp. are known to be intrinsically susceptible to all antibiotics active against non-fastidious gram negative bacilli except for many β-lactams, due to the production of multiple inducible chromosomally encoded β -lactamases (Jones and Wilcox, 1995; Rossolini *et al.,* 1996). In the present study, all *Aeromonas* spp. isolated were resistant to Ampicillin, this is similar to the observation of Goni-Urizza *et al.* (2000a) and Odeyemi and Ahmad (2017). Ampicillin is inactivated by chromosomal beta-lactamases produced by many enterobacteriaceae and *Aeromonas* (Walsh *et al.,* 1995). This supports the assertion that *Aeromonas* is naturally resistant to ampicillin (Walsh *et al.,* 1995). They were also highly resistant to cefuroxime (85.9%) and cefotaxime (76.7%).

Resistance to ceftazidime was moderate at 57.8%. Resistance to third generation cephalosporins is known to be associated with the de-repression of the chromosomal enzyme (Jones and Wilcox, 1995; Rossolini *et al.,* 1996). According to Jones and Wilcox (1995), β-lactam agents should be avoided in the treatment of *Aeromonas* spp. infections even if MICs are still in the susceptible range, since resistant mutants over producing their chromosomal β-lactamases may be selected during therapy.

The *Aeromonas* spp. isolated were also poorly susceptible to streptomycin, similar to the results of Goni-Urizza *et al.* (2000a). About 75% of the isolates were also resistant to co-trimoxazole in contrast to the assertion that the agent is generally efficient at eliminating *Aeromonas* due to the strong synergy between the drugs (Jones and Wilcox, 1995). The trend however is similar to that of Ko *et al.* (1996), who reported 39 – 50% resistance to co-trimoxazole for his isolates.

The high rate of resistance to cefuroxime and ofloxacin among the environmental *Aeromonas* isolates in this study is in contrast to Huddleston *et al.* (2006), whose isolates showed no resistance to cefuroxime and ofloxacin. Some other results, (Kämpfer *et al.,* 1999; Overman and Janda, 1999; Vila *et al.,* 2002; Warren *et al.,* 2004) also showed no or low incidence of resistance with cefuroxime and ofloxacin. However, Goni-Urizza *et al.* (2000b) found resistance to ofloxacin in 59% of his isolates. Generally, the environmental *Aeromonas* isolates from the river and aquaculture water samples in this study expressed very high levels of resistance to the test antimicrobials. A possible explanation for these findings could be that some of the isolates may include clinical isolates that could have been previously exposed to antibiotics (Kämpfer *et al.,* 1999; Ko *et al.,* 1996; Vila *et al.,* 2002).

In other studies carried out, aeromonads were isolated from waters that were highly polluted by industrial effluent or raw sewage (Goni-Urizza *et al.,* 2000b). The samples in this study were collected from river water sources that received both human and animal sewage. Domestic activities like bathing and washing of clothes were some of the activities carried out near the river water sources. One of the sampling points on the river also receives effluent discharge from the Federal Medical Centre, Owerri. Aquaculture fishes are also routinely fed with waste materials including chicken droppings and sewage.

The *Aeromonas* isolates expressed high incidences of multiple resistances with 100% of the isolates being resistant to at least 2 or more antimicrobials for both the river water and aquaculture samples. Previous studies have also shown the occurrence multiple resistances in *Aeromonas* (Miranda and Castillo, 1998; Odeyemi and Ahmad, 2017). The isolates exhibited a large number of resistance patterns (51 and 49 amongst the river water and aquaculture isolates respectively), which is an indication of the high variability in character associated with these environmental isolates.

Plasmids have been detected in *Aeromonas* spp. as reported previously in other studies (Akinbowale *et al.,* 2006; Rhodes *et al.,* 2000; Furushita *et al.,* 2003). Similar results include plasmids in 33.3% of *Aeromonas* sp from tilapia skin lesions (Son *et al.,* 1997), plasmid-associated ampicillin and tetracycline resistance associated with a plasmid in a single strain only (Son *et al.,* 1997), and 56.6% overall prevalence of plasmids in fresh water fish isolates (Radu *et al.,* 2003). Some isolates in Jacobs and Chenia (2007) also appeared to carry multiple plasmids simultaneously as in the present study. According to Jacobs and Chenia (2007), this multiplicity and intensity of plasmids harboured by the *Aeromonas* isolates may be related to their copy number in the host cell.

Aeromonas spp. are known to harbour self – transmissible (Sørum *et al.,* 2003) and non – conjugative but mobilizable plasmids (L'Abee-Lund and Sørum, 2002) carrying antimicrobial resistance determinants and their associated mobile genetic elements. Thus the exchange of genetic information between *Aeromonas* spp. isolates is not unlikely, and might occur with neighbouring microflora in environmental and clinical niches (Rhodes *et al.*, 2000).

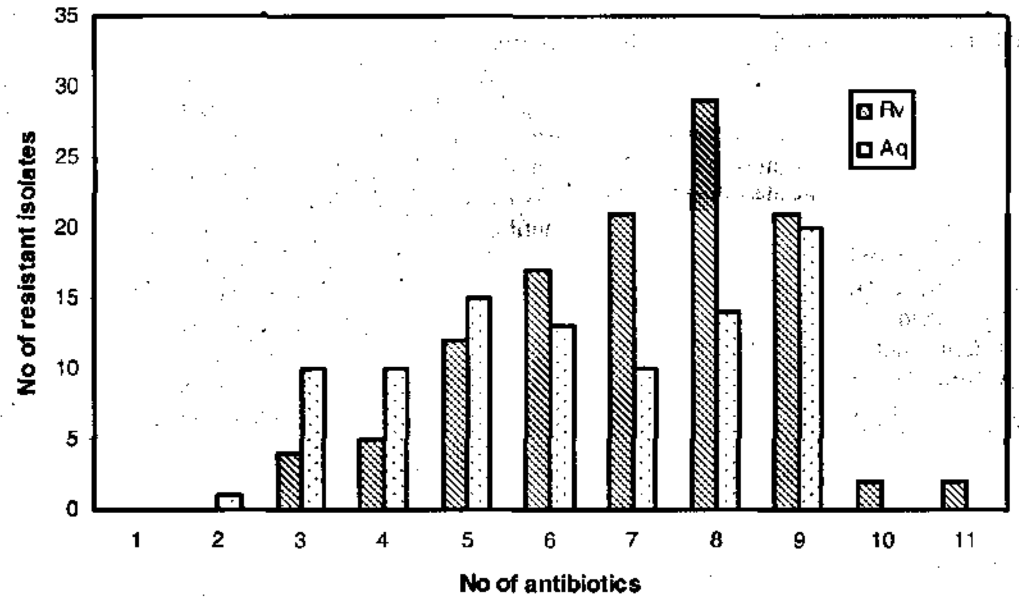
Six of the 13 isolates analysed for the bla_{TEM}, bla_{SHV} and bla_{CTX} beta-lactamase genes were found to possess them in different proportions, with bla_{TEM} being the most predominant. A post curing analysis of antimicrobial resistance of the isolates also showed that all the *Aeromonas* isolates were inhibitor resistant, being resistant to both amoxicillin-clavulanic acid, while 15.4% of the isolates remained resistance to cefotaxime-clavulanic acid. In previous studies, resistance to β-lactam and β-lactamase inhibitor combinations in *Escherichia coli* has been reported to be due to hyper-production of class A β-lactamases, like TEM-1 or SHV-1, class D plasmid-mediated enzyme or chromosomal or plasmidic class C β-lactamase and/or to modified outer membrane permeability (Reguera *et al.*, 1991; Oliver *et al.*, 1999; Chaibi *et al.*, 1999; Thomas and Moland, 2000)

According to Schmidt *et al.* (2001), the presence of a diversity of resistance genes amongst aeromonads might constitute a pool of resistance genes capable of moving among bacteria in the aquatic environment and possibly being transferred to other fish pathogens. Indeed the presence of resistance genes and associated mobile genetic elements in the absence of antibiotic pressure is a course for concern (Jacobs and Chenia, 2007). The mobilization of these genes in the aquatic environment and transmission to the human compartment is not unlikely and might provide a reservoir of resistance genes capable of transfer to other water-borne and human pathogens (Schimidt *et al.*, 2001). Furthermore, the aquatic environment has been referred to as an important reservoir of novel antibiotic resistance genes (Cattoir *et al.*, 2008).

Table 1: Frequency of antimicrobial resistance of *Aeromonas* spp. isolated from river water and aquaculture sources.

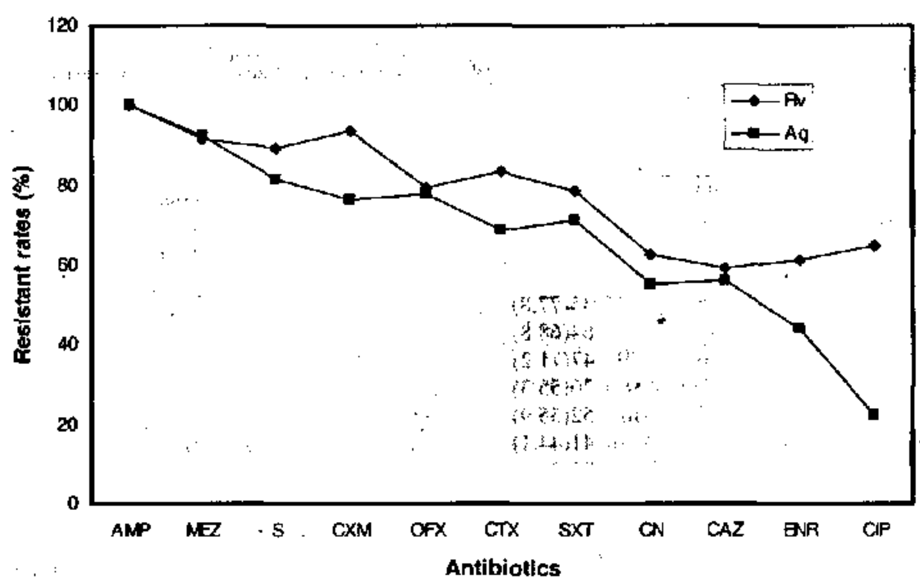
Source of environmental *Aeromonas* spp. isolates						
	River water samples		Aquaculture water samples		River and aquaculture samples	
Antibiotics	No. of isolates	No. (%) of resistant isolates	No. of isolates	No. (%) of resistant isolates	Total no. of isolates	Total (%) resistance
AMP	113	113(100)	93	93(100)	206	206(100)
MEZ	70	64(91.4)	66	61(92.4)	136	125(91.9)
S	113	101(89.4)	93	76(81.7)	206	177(85.9)
CXM	113	106(93.8)	93	71(76.3)	206	177(85.9)
OFX	58	46(79.3)	45	35(77.8)	103	81(78.6)
CTX	113	94(83.2)	93	64(68.8)	206	158(76.7)
SXT	70	55(78.6)	66	47(71.2)	136	102(75.0)
CN	83	52(62.7)	47	26(55.3)	130	78(60.0)
CAZ	113	67(59.3)	93	52(55.9)	206	119(57.8)
ENR	104	64(61.5)	93	41(44.1)	197	105(53.3)
CIP	65	42(64.6)	93	21(22.6)	158	63(39.9)

KEY: AMP, Ampicillin; MEZ, mezlocillin; S, streptomycin; CXM, cefuroxime; OFX, ofloxacine; CTX, cefotaxime; SXT, cotrimoxazole; CN, gentamycin; CAZ, ceftazidime; ENR, enrofloxacine; CIP, ciprofloxacine.



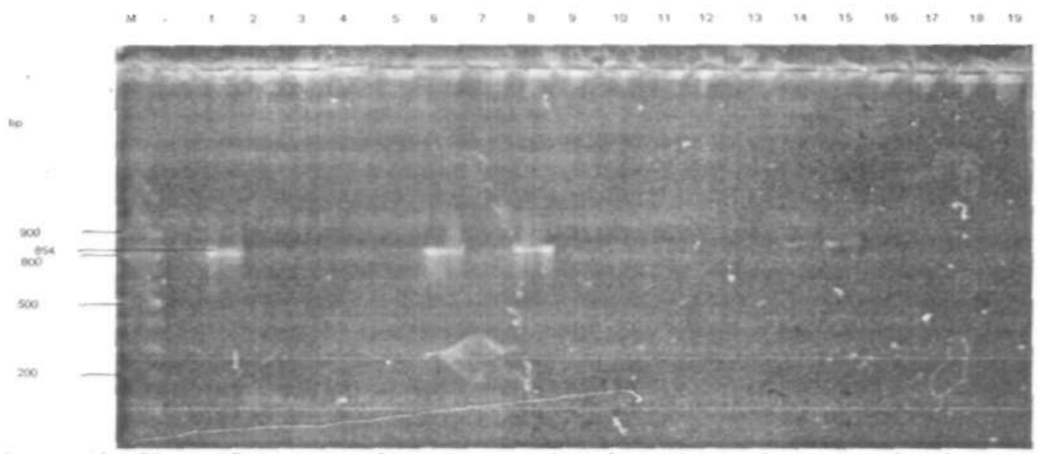
KEY: Rv, River water; Aq, Aquaculture water.

Figure 1: Multiple resistances in *Aeromonas* spp. isolated from river and aquaculture water samples



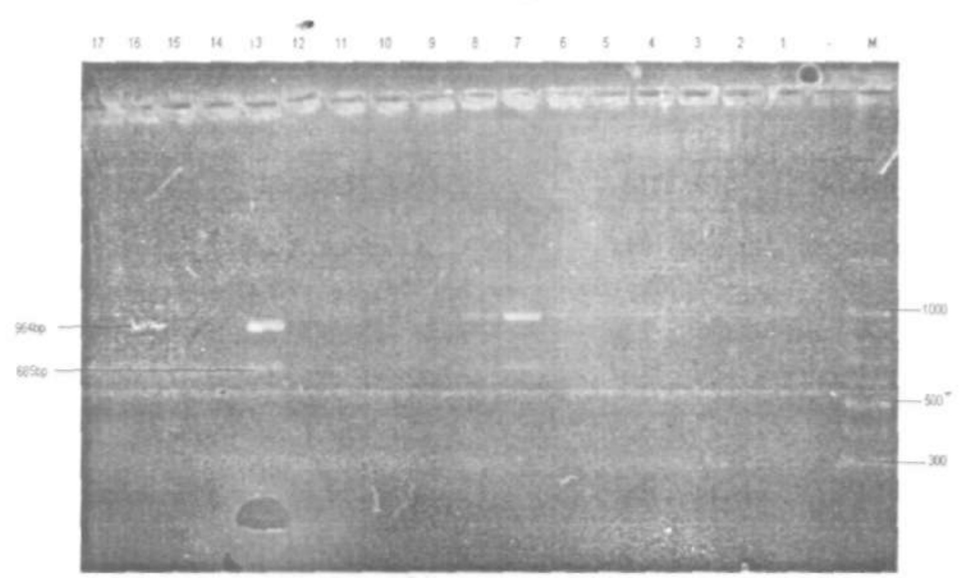
KEY: Rv, River water; Aq, Aquaculture water; AMP, Ampicillin; MEZ, mezlocillin; S, streptomycin; CXM, cefuroxime; OFX, ofloxacine; CTX, cefotaxime; SXT, cotrimoxazole; CN, gentamycin; CAZ, ceftazidime; ENR, enrofloxacine; CIP, ciprofloxacine.

Fig 2: Comparative rates of resistance in *Aeromonas* spp. isolated from river and aquaculture water samples



KEY: Lanes 1-13, PCR amplified products *of Aeromonas* spp. from river and aquaculture water; M, molecular weight marker.

Fig 3: Gel electrophoresis of the PCR amplified products for the detection of bla_{SHV} β-lactamase genes for isolates 1 – 13.



KEY: Lanes 1-13, PCR amplified products of *Aeromonas* sp from river and aquaculture water; M, molecular weight marker
Fig 4: Gel electrophoresis of the PCR amplified products for the detection of bla_{TEM} β-lactamase genes for isolates 1 – 17.

Table 2: Amplification bands of bla_{SHV}, bla_{TEM} and bla_{CTX} β-lactamase positive isolates

Isolate no	Source	Name	β-lactamase gene amplification band (bp)		
			blaSHV	blaTEM	blaCTX
1	Aquaculture	*Aeromonas* spp.	854	964	-
3	Aquaculture	*Aeromonas* spp.	-	-	593
6,10,11	River	*Aeromonas* spp.	854	-	-
7	River	*Aeromonas* spp.	-	685, 964	-
8	River	*Aeromonas* spp.	854	964	-
13	River	*Aeromonas* spp.	-	685, 964	-

Table 3: Antimicrobial resistance patterns of isolates

Isolate	Source	No. of patterns	Most predominant pattern	No. (%) of isolates with predominant pattern
Aeromonas sp	Rv	51	CAZ+CTX+CXM+ENR+CN+S+MEZ+OFX+AMP	19 (16.80)
Aeromonas sp	Aq	49	CAZ+CTX+CXM+ENR+SXT+MEZ+AMP+OFX	8 (8.60)
			CAZ+CTX+CXM+ENR+CN+S+CIP+AMP+OFX	8 (8.60)

KEY: Rv, river water; Aq, aquaculture; CAZ, ceftazidime; CTX, cefotaxime; CXM, cefuroxime; ENR, enrofloxacine; CN, gentamycin; S, streptomycin; MEZ, mezlocillin; OFX, ofloxacine; AMP, ampicillin; CIP, ciprofloxacine.

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