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Isolation, Identification and Antimicrobial Susceptibility Pattern of Extended-Spectrum-β-lactamase *Aeromonas* spp. from Fish Samples of Chhattisgarh State, India

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A B S T R A C T

A total of 200 fish samples were examined for the presence of extended spectrum β-lactamases (ESBL) producing *Aeromonas* spp. in raw fish samples collected from retail markets of Durg, Raipur, Rajnandgaon and Balod districts of Chhattisgarh state. A total of 70 (35%) fish samples were found contaminated with *Aeromonas* spp. Among 70 *Aeromonas* isolates, 16 (22.9%) were phenotypically identified as ESBL producer. Out of these 16 isolates, 11 and 5 were from fresh water and marine fish samples, respectively. Further antimicrobial sensitivity testing revealed that the isolates were resistance for ampicillin (88.6%), cephalexin (64.3%) followed by oxytetracycline (48.6%). Among 62 ampicillin resistant isolates, 39 isolates were recovered from fresh water fish samples and 23 from marine water fish samples. Multiple antibiotic resistant (MAR) index for *Aeromonas* isolates varied between 0.11 and 0.88. The study indicates the presence of multidrug resistant ESBL producing *Aeromonas* spp. in fish samples.

Introduction

Aeromonas are ubiquitous bacteria found in a variety of aquatic environments and food products including seafood, raw meats, packaged ready-to-eat meats, milk and milk products (Igbinosa *et al.*, 2012). *Aeromonas* are increasingly being regarded not only as important pathogens of fish and other cold-blooded organisms, but also as the opportunistic pathogens in both immuno-competent and immuno-compromised humans (Daskalov, 2006). *Aeromonas* species are responsible for intestinal and extra-intestinal infections like septicaemia, cellulites, wound infections, urinary tract infections, peritonitis, hepatobiliary tract infections, and soft tissue infections in humans (Khajanchi *et al.*, 2010). *Aeromonas* can even cause more severe forms of infections such as haemolytic uremic syndrome (HUS) and necrotizing fasciitis. Five *Aeromonas* species viz. *A. hydrophila*, *A. caviae*, *A. veronii*, *A. jandaei*, and *A. schubertii* are most commonly implicated in human intestinal infections. *A. hydrophila* and *A. salmonicida* are important fish pathogens

and result in huge economical losses in the fishing industry (Tomas, 2012).

The abuse of antibiotics in the modern era, lead the microorganisms to develop antibiotic resistance as a part of natural selection which allows them to survive in different environments. Bacteria like *Aeromonas* are able to adapt to changes in the environment such as an increase in antibiotic concentration, which often results in the development of mutations allowing them to survive in unfavourable conditions.

Also, bacteria are able to transfer resistant genes to one another through vertical and horizontal transfer which aids in their ability to adapt to their environment (Kummerer, 2009). This genetic transfer can be mediated by plasmids, bacteriophages, transposons, genomic islands and transformations (Lupo *et al.,* 2012).

Antibiotic resistance in *Aeromonas* spp. is usually chromosomally mediated, but β-lactamases produced by aeromonas may occasionally be encoded by plasmids or integrons (Aravena-Roman *et al.,* 2012). *Aeromonas* are known to produce one or more unrelated inducible β-lactamases with activity against a wide variety of β-lactam antibiotics, including penicillins, cephalosporins, and extended-spectrum cephalosporins.

The emergence of ESBL producing *Aeromonas* spp. in the aquaculture and in foods of animal origin is a growing problem worldwide.

Keeping this in view, the present study was carried out with the objective to determine the prevalence and multidrug resistant pattern among isolates of *Aeromonas* spp. recovered from fish samples in different districts of Chhattisgarh, India.

Materials and Methods

Sample collection

A total of 200 fish samples comprising of fresh (n=120) and marine (n=80) water origin were collected randomly from Durg, Raipur, Rajnandgaon and Balod districts of Chhattisgarh, India between August 2017 and July 2018. All the fish samples were aseptically collected from retail fish shops following the protocol given by International Commission on Microbiological Specifications for Foods (ICMSF 1978).

Isolation and biochemical characterization

Isolation of *Aeromonas* spp. was carried out as per the method outlined by Balakrishna *et al.,* (2010) with slight modifications. Briefly, 25 g of fish sample was taken, blended and discharged in 225 ml of Alkaline Peptone Water (APW) (Himedia, India) for enrichment and incubated at 37^0C for 20-24 hours. The *Aeromonas* colonies appeared as yellow coloured, small and smooth similar to honey drop. For confirmation of *Aeromonas* spp. esculin hydrolysis, catalase test and oxidase test were performed.

Antibiotic sensitivity test and Multiple Antibiotic Resistance (MAR) index

All biochemically confirmed *Aeromonas* isolates were further tested for antimicrobial drug susceptibility pattern on Mueller-Hinton agar (MHA) (HiMedia, India) by using disc diffusion method (CLSI 2012). The antibiotics used were oxytetracycline (30µg), cephalexin (30 µg), ciprofloxacin (5 µg), gentamicin (30 µg), cefotaxime (10 µg), ampicillin (10 µg), ceftazidime (30 µg), aztreonam (30 µg), cefixime (5 µg) (HiMedia, India). Diameter of complete inhibition of zones was measured and compared with the interpretation chart, and graded as resistant, intermediate and sensitive.

Formula a/b was applied to calculate MAR Index for *Aeromonas* isolates. In this index, "a" is considered as the number of antibiotics to which an isolate was resistant and "b" is the number of antibiotics to which the isolates were exposed (Krumperman, 1983).

Phenotypic characterization of ESBL producers

As per CLSI (2012) protocol, *Aeromonas* isolates with a zone of inhibition of ≤ 17 mm for aztreonam and ceftazidime, and ≤ 22 mm for cefotaxime in disc diffusion susceptibility testing were selected for detection of ESBLs production. For this purpose, cefotaxime (10µg) and cefotaxime + clavulanic acid (30 + 10 µg) (HiMedia, India) were used. Discs were positioned on the inoculated MHA plates at a distance of 25 mm apart and incubated overnight. The *Aeromonas* isolates resistant to either of the cephalosporin discs and sensitive to their respective cephalosporin + clavulanic acid discs with diameter of more than 5 mm were considered as presumptive ESBL producers (Krumperman, 1983).

Statistical analysis

In the present study, the recovery of various isolates from the diverse origin was expressed in percent. Wherever necessary, the attempts were made to undertake statistical analyses for significant results.

Results and Discussion

In the present study, a total of 70 (35%) isolates were confirmed as *Aeromonas* after morphological and biochemical characterization (Table 1). Similar prevalence of 33.6% of *Aeromonas* spp. in fish samples was also reported by Vivekanandhan *et al.,* (2003). However significantly higher prevalence of 67.9% of *Aeromonas hydrophila* was reported in fish samples by Ullmann *et al.,* (2005) in Berlin, Germany. Domestic sewage is one of the major source to contribute many of the pathogens to the aquatic environment. Sewage contamination of the seawater plays an important role in the expansion of a putatively virulent *A. hydrophila* strain (Thayumanavan *et al.,* 2003). Considering the psychrotrophic nature and role of *A. hydrophila* as a pathogen of emerging importance, the considerably high levels of this organism in a popular food item such as fish raises serious concern.

Results of antimicrobial susceptibility test revealed that isolates were found to be highly sensitive for cefixime (67%) followed by gentamicin (65.7%) and ceftazidime (50%). Whereas 48.6%, 42.9% and 38.6% isolates were found sensitive for ciprofloxacin, oxytetracycline and aztreonam.

On contrary 88.6%, 64.3%, and 48.6% isolates were found highly resistant against ampicillin, cephalexin and oxytetracycline, respectively. Among 62 ampicillin resistant isolates, 39 isolates were recovered from fresh water fish samples, and 23 from marine water fish samples.

Results from antimicrobial pattern of *Aeromonas* spp. suggests that geographical locations and local selective pressure influence the antibiotic resistance levels. Increased levels of resistance among isolates of *A. hydrophila* to commonly used antibacterial agents have been observed. In the present study most of the isolates were resistant to ampicillin and oxytetracycline. Our study suggest that presence of antibiotics resistant *Aeromonas* spp. in aquatic environment especially seawater poses danger to seafood which directly affect consumers. Like other enteric gram-negative bacilli, the emergence of resistance among *Aeromonas* spp. may be due to the indiscriminate use of antibiotics (Hatha *et al.,* 2005). Results of MAR index calculated for 70 *Aeromonas* isolates in this study was recorded as 0.88 (for

2 isolates), 0.77 (for 3 isolates) followed by 0.66 (for 9 isolates), 0.55 (for 9 isolates), 0.44 (for 13 isolates), 0.33 (for 12 isolates) and 0.22 (for 15 isolates). However 0.11 MAR index was recorded in other remaining 7 isolates. The MAR index above 0.2 for the isolates indicated injudicious use of antibiotics. Many strains are known to harbour mobile elements that encode antibiotic resistance and can be transferred among themselves or to other bacterial species to establish multiple antibiotic resistances. The prolonged use of antibiotics has been identified as a major factor responsible for the increased incidence of antibiotics resistance.

In this study, among 70 *Aeromonas* isolates, 16 (22.9%) were phenotypically identified as ESBL producers. Out of these 16 isolates, 11 and 5 were from fresh water and marine fish samples respectively. The occurrence of ESBL producing *Aeromonas* spp. in the fish and fishery products may be due to post harvest contamination such as infected handlers, uncleaned vessels and repeated use of contaminated water in the fishery outlets (Le *et al.*, 2015). *Aeromonas* spp. plays an important role as vectors in dissemination of β-lactamases into the natural environment. Detection of ESBL producers *Aeromonas* isolates in the present study supports this hypothesis.

Table.1 Occurrence of ESBL producers among fresh water and marine water isolates of *Aeromonas* spp.

Type of sample	No. of samples collected	No. of samples positive	ESBL production (%) by phenotypic method
Fresh water	120	39 (32.5%)	11 (28%)
Marine water	80	31 (38.8%)	5 (16%)
Total	200	70 (35%)	16 (22.9%)

In conclusion, the overall prevalence of *Aeromonas* spp. in fish samples was found to be 35% and MAR index calculated for isolates indicates injudicious use of antibiotics. *Aeromonas* spp. isolated from fish samples were found to produce ESBL.

These multidrug-resistant and ESBL producing *Aeromonas* spp. isolates can be transmitted to the human population after consumption of fish and their products. The study revealed that fish samples are frequently contaminated with antimicrobial resistant bacteria due to the intensive use of antimicrobial agents in aquaculture.

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