Journal of Sustainability Science and Management *eISSN: 2672-7226*
Volume 14 Number 2, April 2019 : 21-35

PATHOGENIC STATUS, ANTIBIOGRAM, ADHESIVE CHARACTERISTICS, HEAVY METAL TOLERANCE AND INCIDENCE OF INTEGRONS OF INFECTED FISH-ISOLATED *Aeromonas* sp.

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Abstract: Motile aeromonad infection is a common disease in freshwater fish that is difficult to control. This study assessed the pathogenic properties of *Aeromonas* spp. isolated from major carps at Indian farms. Five selected virulent isolates were screened for antibiogram, biofilm-producing potential, heavy metal tolerance and the presence of integrons. *A. sobria*, along with two isolates each of *A. hydrophila* and *A. veronii* that were found to be pathogenic in an in vivo challenge study, were observed to be resistant to 12 types of antibiotics. However, they did show susceptivity to fluoroquinolones and aminoglycosides. Norfloxacin, chloramphenicol, ciprofloxacin, levofloxacin, amikacin and gentamicin effectively inhibited the growth of all five *Aeromonas* isolates. The isolates were tolerant to Cu^{2+} but not Hg^{2+} in the heavy metal assay. *A. sobria* and both *A. hydrophila*isolates were found to be tolerant to Cr^{6+}. The *A. sobria* and one *A. hydrophila* isolate (ATCC 0637P) were strong producers of biofilm. The presence of amplicons for integrons I and II were detected in both *A. sobria* and *A. hydrophila* isolates. This investigation produced a baseline information on the prevalence of potential pathogenic *Aeromonas* in aquaculture systems that could pose a serious risk if they were passed to animals and humans through close contact.

KEYWORDS: Antibiogram, biofilm, norfloxacin, chloramphenicol, integrons,

Introduction

Aeromonas are gram-negative, non-spore forming rod-shaped facultative anaerobes that inhabit a wide range of aquatic environments. They are known to be opportunistic pathogens in fish (Praveen *et al.,* 2016) and humans (Gosling, 1996). Virulent strains of *Aeromonas* spp. have been isolated from chlorinated portable water (Janda & Duffy, 1988; Alvandi & Anathan, 2003; Sen & Rodgers, 2004), and this bacteria is in the process of being placed in the contaminant candidates' list of the United States Environmental Protection Agency (USEPA, 2006).

Motile *Aeromonas* spp. can cause septicaemia in fish and show a high degree of antibiotic resistance (Lee *et al.,* 2009; Igbinosa *et al.*, 2013; Laith & Najiah, 2013). However, in most situations, infections are still treated with antibiotics despite the low efficacy of the drugs. The freshwater environment is also mostly contaminated with agricultural, industrial and domestic waste, which may further inhibit the effectiveness of antimicrobial agents.

Aeromonas bacterias are an important model in surveying antibiotic resistance in freshwater aquaculture (Huddleston *et al.*, 2006; Igbinosa *et al.,* 2013). In Indian major carps (*Labeo rohita*, *Cirrhinus mrigala* and *Catla catla*), they frequently cause diseases that result in huge losses to farmers. Acute outbreaks of *A. hydrophila*, *A. sobria* and *A. veronii* are major causes of carp mortality (Mohanty *et al.,* 2008; Praveen *et al.,* 2016). Antibiotic resistance can be attributed to the bacterias' dynamism in adapting to its environment. These microrganisms may have acquired their resistance viacontamination of the farms' water source or ineffictive use of anti-microbial drugs (Igbinosa, 2014).

Antibiotic-resistant aquatic pathogens, especially aeromonads, are very dangerous because of their capability to infect humans and animals (Kampfer *et al.,* 1999; Vila *et al.,* 2002). The presence of integrons aggravates

the problem because they allow the transfer of antibiotic-resistant genes between microbes, making it more difficult to curb this characteristic (Igbinosa *et al.*, 2013; Zanella *et al.*, 2012). Hence, there is a need for periodic surveillance of antibiotic resistance in microrganisms at fish farms, particularly those related to clinical infections. This may guide farmers on the correct use of antibiotics and prevent the re-emergence of deadly diseases.

The formation of biofilm also facilitates resistance to antimicrobials and cause persistent infections (Donlan, 2001). To ability of bacteria to adhere to a surface is one of the initial steps in the formation of biofilm and this is commonly observed in *Aeromonas* spp. (Lynch *et al.*, 2002; September *et al.*, 2007). Besides antibiotic resistance, conducting aquaculture in a polluted environment may also lead to the emergence of strains that are resistant to heavy metals.

In line with the United Nations' World Health Organisation and Food and Agriculture Organization guidelines to prevent waterborne diseases in developing countries, and as part of India's National Surveillance Programme for Aquatic Animal Diseases (NSPAAD), this study aims to (i) evaluate the level of antimicrobial resistance in aeromonads from infected fish; (ii) evaluate their biofilm forming potential and heavy metal tolerance; and, (iii) determine the presence of class 1 and 2 integron-associated gene cassettes.

Materials and Methods

Case History, Sampling Strategy and Bacterial Isolation

The fish samples were collected from three major aquaculture states of India (Andhra Pradesh, Odisha and West Bengal), where nine farms reported mortality or suspected cases of bacterial infections between June 2014 andMay 2016 (Figure 1).

The Indian major carps, including the rohu (*L. rohita*), catla (*C. catla*) and mirigal (*C. mrigala*), along with gold fish that showed red patches and ulcerations on their operculum and body surface, as well as fin and tail rot, were caught for sampling.

The mortality rates varied from case to case. Samples (blood, kidney and swabs from ulcers or haemorrhagic areas) were obtained at each farm from three infected fishes that were anaesthetised with 100 ppm of ethyl 3-aminobenzoate methanesulfonate (Sigma Aldrich, Missouri, USA).

The isolation of pathogens was perfomed according to Koch's postulates. Fish samples were inoculated in tryptone soy broth (TSB) (HiMedia, Mumbai, India) and incubated at 28 °C for 24 hours.

The culture was streaked onto tryptone soy agar (HiMedia, Mumbai, India) to isolate and tentatively identify *Aeromonas* colonies using biochemical characterisations as described by Abbott *et al.* (2003) and Sahoo *et al.* (2016). Finally, the isolates were tested for virulence in *L. rohita* juveniles through intraperitoneal challenge.

Intraperitoneal Challenge

A total of 50 *L. rohita* (apparently healthy juveniles) were obtained from the institute farm. The fish weighing approximately 20 g were stocked in ten numbers of 100-L capacity FRP tanks (five fish each) in a wet laboratory under continuous aeration and commercial feed was provided. They were acclimatized for a week before being intraperitoneally challenged with *A. hydrophila* (four strains), *A. veronii* (three strains), *A. sobria* (one strain) and *A. caviae* (one strain) at an arbitrary dose of 1x 107 CFU/20 g fish. The fish in one tank served as control and received only PBS injection. The fish were observed for any mortality up to day 10.

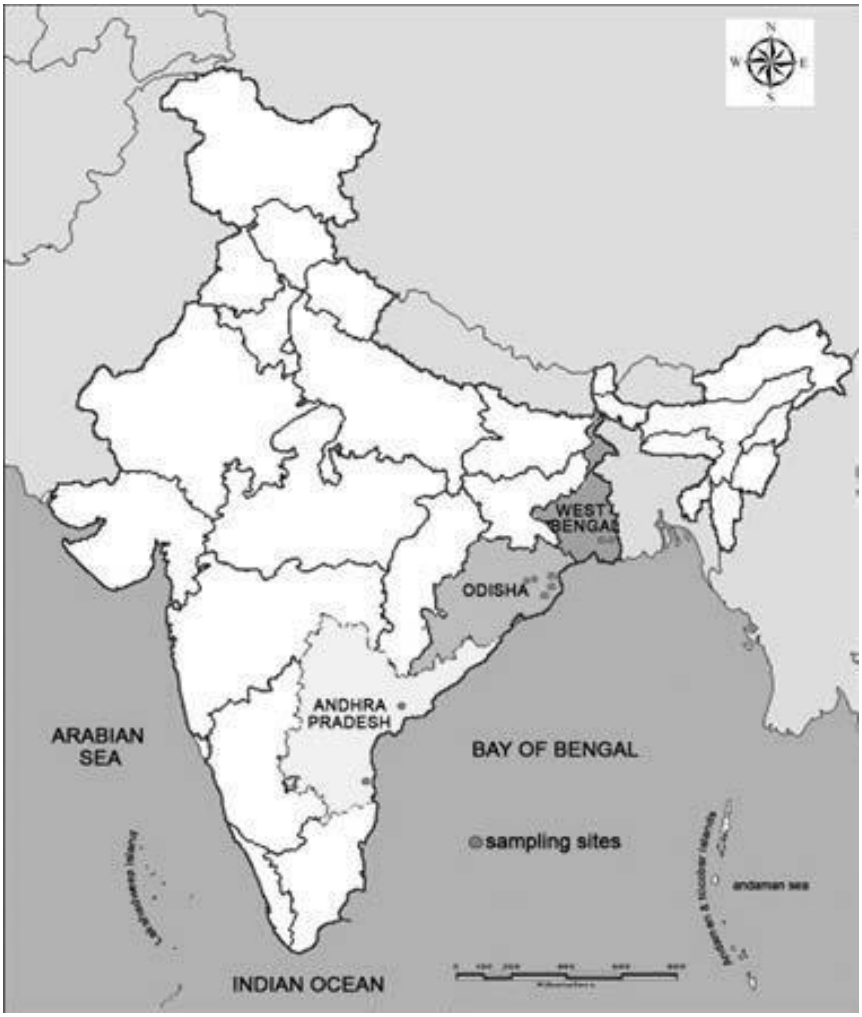

Figure 1: Map showing the sites where bacterial isolates were collected in three Indian states.

Universal PCR and Sequence Analysis for Bacterial Identification

The bacterial DNA was extracted using the phenol-chloroform method by Sambrook and Russell (2001). The DNA pellet was vacuum-dried and re-suspended in Tris–EDTA (TE) buffer, and concentration and purity were evaluated using the NanoDrop® ND-1000 spectrophotometer V3.7 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The reaction mixture (25 µl) was prepared as follows: 19.50 µl nuclease free water, 2.50 µl 10X assay buffer, 0.50 µl dNTPs, 0.50 µl 16S forward primer and 0.50 µl 16S reverse primer, 0.25 µl *Taq* DNA polymerase (Bangalore Genei, Bangalore, India) and 1.25 µl of sample DNA (25-50 ng/µl). The PCR conditions were as follows: preheating at 94°C, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 46°C for 45 seconds, and extension at 72°C for 1½ minutes before the final extension of 72°C for 10 minutes. The PCR amplified products were subjected to electrophoresis in 1% agarose with a 1 kb DNA ladder as marker (Bangalore Genei, Bangalore, India). The gel was visualised and documented using an imaging system (Alpha Innotech, San Leandro, California, USA). PCR amplicons sized between

1.4 kb and 1.5 kb were cut out and purified using a kit (Bangalore Genei, Bangalore, India) before being sent for sequencing (Xcelris Genomics, Ahmedabad, Gujarat, India).

The consensus 16S rRNA gene sequences obtained from sequencing were compared with known *Aeromonas* strains in GenBank using the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information website. The obtained sequences were also deposited into GenBank.

Antimicrobial Susceptibility Testing

Susceptibility of bacterial isolates were tested against the antibiotics listed in Table 1. Antibiogram study was performed using the disc diffusion method on Mueller-Hinton Agar (MHA) (Hi Media, Mumbai, India) (Cruikshank *et al.*, 1975) and resistance was determined using the zone inhibition method described by Igbinosa *et al.* (2013).

Briefly, the bacterial cultures were inoculated in TSB and incubated for 24 hours at 28°C. A total of 100 µl from each culture ($1.5x10^8$ cfu/ml in McFarland standard 0.5) was spread on MHA plates using hockey stick cell spreaders. The antibiotic discs were impregnated onto the plates after they became dry, followed by 24 hours of incubation at 28 °C.

The zone of inhibition was measured and compared according to the manufacturer's instructions and the strains were classified as sensitive, intermediate or resistant as in Table 1. The antibiotics used were classified into different groups as per the standard definition of acquired resistance (Magiorakos *et al.*, 2012). The bacteria strains were classified as multi-drug resistant (MDR) based on acquired non-susceptibilitytoat least one agent in three or more antimicrobial categories for *Enterobacteriaceae* as described by Magiorakos *et al.* (2012). The antibiotics were selected on the basis of their use in the aquaculture industry.

Table 1: Antibiotic susceptibility pattern of *Aeromonas* isolates.

Antimicrobial agents	Disc Concentration	Resistant (R)/Intermediate Sensitive (IS)/Sensitive (S)				
		A. hydrophila (KT026460)	*A. hydrophila* (KX219575)	*A. veronii* (KT026458)	*A. veronii* (KT026457)	*A. sobria* (KU499851)
Aminoglycosides						
Amikacin (Ak)	30 mcg	S	S	S	S	S
Gentamicin (Gen)	10 mcg	S	S	S	S	S
Tobramycin (Tob)	10 mcg	S	R	S	S	S
β- lactams						
Piperacillin (Pi)	100 mcg	S	S	R	S	S
Monobactams						
Azteronam (At)	30 mcg	R	R	R	R	R
Penicillins						
Ampicillin (Amp)	10 mcg	R	R	R	S	IS
Carbapenems						
Imipenem (Ipm)	10 mcg	R	IS	S	IS	IS
Cephalosporins						
Ceftazidime (Caz)	30 mcg	R	R	IS	IS	IS
Fluoroquinolones						
Ciprofloxacin (Cip)	5 mcg	S	S	S	S	S
Levofloxacin (Le)	5 mcg	S	S	S	S	S
Extended Spectrum Cephalosporin						
Cefuroxime (Cxm)	30 mcg	R	R	R	S	IS
Cephalothin (Cep)	30 mcg	R	R	R	S	R
Cephotaxime (Ctx)	30 mcg	R	R	R	S	IS
Phenicols						
Chloramphenicol (C)	25 mcg	S	S	S	S	S
Amoxyclav (Amc)	30 mcg	R	R	R	IS	R
Cloxacillin (Cox)	1 mcg	R	R	R	R	R
Co-Trimoxazole (Cot)	25 mcg	IS	R	R	S	S
Polymixins						
Colistin (Cl)	10 mcg	S	S	R	S	R
Tetracyclines						
Tetracycline (Te)	30 mcg	S	S	S	IS	R
Doxycycline Hydrochloride (Do)	30 mcg	S	S	S	S	R
Macrolides						
Erythromycin (E)	15 mcg	IS	IS	S	R	R
Cefoperazone (Cpz)	75 mcg	IS	S	S	S	IS
Lincomycin (L)	2 mcg	R	R	R	R	R
Methicillin (Mec)	5 mcg	R	R	R	S	S
Norfloxacin (Nx)	10 mcg	S	S	S	S	S
Oxacillin (Ox)	1 mcg	R	R	R	R	R
Oxytetracycline (O)	30 mcg	S	S	S	R	R
Pencillin G (P)	10 unit	R	R	R	R	R
Streptomycin						
Streptomycin (S)	10 mcg	S	S	S	S	IS
Sulphatriad (S3)	300 mcg	R	S	R	R	IS
Glycylcyclines						
Vancomycin (Va)	30 mcg	R	R	S	R	R

Heavy Metal Tolerance Analysis

Heavy metal resistance test was performed according to Miranda and Castillo (1998). The heavy metals used in this study were mercury (Hg^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}), and the agar dilution method was followed for this. TSA medium was incorporated with different concentrations of $HgCl_2$ (SRL, Mumbai, India), $K_2Cr_2O_7$ (Qualigens Fine Chemicals, Mumbai, India) and $CuSO_4$ (Hi Media, Mumbai, India). By serial two-fold dilutions, the concentration of Cr^{6+} ranged from 25 to 400 µg/mL, while Hg^{2+} and Cu^{2+} ranged from 2.5 to 40 µg/mL, and 200 to 3200 µg/ml, respectively. The overnight bacterial suspension as prepared in above concentration was spread onto the heavy metal incorporated TSA medium petri plates and incubated at 37^0C overnight. The metal resistance was defined based on the positive bacterial growth obtained at concentrations of ≥ 10 µg/ml (Hg^{2+}), 100 µg/ml (Cr^{6+}) and 600 µg/ml (Cu^{2+}) (Allen *et al.*, 1977).

Biofilm Formation Assay

Biofilm formation of *Aeromonas* isolates was quantitatively assessed using the microtitre plate method as described by Stepanovic *et al.* (2000) and Odeyemi *et al.* (2012), with slight modifications. *Aeromonas* isolates were grown in TSB for 18 hours at 37^0C and centrifuged for five minutes at 6,000 rpm. Cell pellets were washed two times in phosphate-buffered saline (pH 7.2) and the concentration was adjusted to 0.5 McFarland standards (Basson *et al.*, 2007). The 96-well 'U' bottomed polystyrene microtiter plates were filled with 200 µl of TSB and inoculated with 20 µl suspension of different *Aeromonas* isolates. *Aeromonas hydrophila* (ATCC0637P) was used as positive control while wells containing only uninoculated TSB were used as negative control.

The plates were incubated at 37^0C for 24 hours for the bacteria to adhere to abiotic material. The wells were washed three times with sterile phosphate-buffered saline (PBS) after aspirating the contents gently. The wells were air dried and stained with 200 µl of 1% crystal violet for 30 min. The wells were then washed with distilled water and air dried. Then 200 µl of absolute ethanol was added to each well to resolubilize the dye bound to adherent cells. The absorbance of the plates was read at 570 nm using an automated microtiter plate reader (Bio-Rad, iMARK™, California, USA). Each isolate was run in triplicates and the readings were averaged. Isolates were categorized as a non-biofilm producer (ODi < ODc), weak producer (ODc < ODi < 0.484), moderate producer (ODi = 0.484 < 0.968) and strong producer (ODi > 0.968) according to Miranda and Castillo (1998) and Igbinosa *et al.* (2013).

PCR for Detecting Integrons

DNA was extracted from the five bacterial samples as detailed in Materials and Methods. Primers to amplify class I and class II integrons are shown in Table 2 (Igbinosa *et al.*, 2013). PCR was carried out with a final volume of 25 µl containing 1 µl of 100 ng template DNA, 0.5 µl of 2 mM dNTPs, 0.5 µl of 10 pico moles of each primer, 2.5 µl of 10× *Taq* buffer A (Tris-HCl, 15 mM $MgCl_2$), and 0.25 µl of 5 U/µl *Taq* DNA polymerase (Bangalore Genei, Bangalore, India).

Amplification was performed in a Veriti thermal cycler (Applied Biosystems, Forster City, California, USA) programmed for a preliminary two-minute denaturation step at 94°C, followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 56°C for 60 seconds, extension at 72 °C for 90 seconds, and finally, extension at 72 °C for 10 minutes.

Table 2: Primer sets used for the detection of class 1 and class 2 integrons.

Target Gene	**Primer sequence 5' 3'→**	**Size**
Class I integron F	GGC ATC CAA GCA GCA AG	Variable
Class I integron R	GGC ATC CAA GCA GCA AG	Variable
Class II integron F	CGG GAT CCC CGG CAT GCA CGA TTT GTA	Variable
Class II integron R	GAT GCCATCGCAAGT ACGAG	Variable

Results

Biochemical Characterization

The biochemical characteristics of *Aeromonas* sp. isolated from diseased fish were detailed in Table 3.

Table 3: Physical and biochemical characteristics of the isolated bacteria.

Characteristics	**Reaction**				
	A. hydrophila **(KT026460)**	*A. hydrophila* **(KX219575)**	*A. veronii* **(KT026458)**	*A. veronii* **(KT026457)**	*A. sobria* **(KU499851)**
Gram stain	-	-	-	-	-
Motility	+	+	+	+	-
Growth in Triple sugar iron agar	A/A	A/A	A/A	A/A	A/A
Gelatin liquefaction	+	+	+	+	-
Methyl red	-	-	-	-	-
Catalase	+	+	+	+	+
Growth on TSA	+	+	+	+	+
Growth on RSA	+ (YC)	+ (YC)	+ ((YC)	+ (YC)	+ (YC)
Citrate	+	+	+	+	+
Indole	+	+	+	+	+
Voges Proskauer reaction	+	+	+	+	+
Congo red binding	+	+	+	+	-
Acid production from Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
Galactose	+	+	+	+	+
Raffinose	-	-	-	-	-
Mannose	+	+	+	+	+
Trehalose	-	-	-	-	-
Cellobiose	+	+	-	-	-
Dulcitol	-	-	-	-	-
Xylose	-	-	-	-	-
Inulin	-	-	-	-	-
Salcin	-	-	-	-	-
Inositol	-	-	-	-	-
Arabinose	-	-	-	-	-

YC, yellow colony; A/A, glucose and lactose/sucrose fermented

Bacterial Incidence and Pathogenicity

Of the nine farms that reported an outbreak involving motile aeromonads, *A. hydrophila* seemed to be the dominant species (Table 4), followed by three cases of *A. veronii*, and one case each of *A. sobria* and *A. caviae*.

All experimental fishes showed pathological signs of swollen and pale gills, and haemorraghia on ventral parts of the body after 6-12 hours of injection. The fish mortalities observed in each tank between day one and day ten post-challenge were as follows:

A. hydrophila (KT026460), four; *A. hydrophila* (KX219575), three; *A. veronii* (KT026458), three; *A. veronii* (KT026457), two; *A. sobria* (KU499851), two;
A. veronii (KM277749), one; *A. hydrophila* (KT026459), four; *A. hydrophila* (KM277748), one; and, *A. caviae* (KM277747), none.

Based on pathogenicity and geographical distribution, only the first five isolates, along with one ATCC strain (0637P) of *A. hydrophila*, were considered for further study (for integron and biofilm assays).

Table 4: Physical and biochemical characteristics of the isolated bacteria.

Bacterial species identified	Accession Number	Location	Sample origin
Aeromonas caviae	KM277747	Khurda, Odisha Lat: 20º18'18.1''N; Long: 85º85'439''E	Body fluid of goldfish showing signs of haemorrhages on body surface with mortality rate of 21%
Aeromonas veronii	KM277749	Chettunnapadu, Bhimadolu, West Godavari, A.P. Lat: 16º40'830''N; Long: 81º16'123'E	Body fluid of Indian major carps showing signs of haemorrhages around eye, fin erosion, protruded eye and mortality of 4.2%
Aeromonas veronii	KT026457	Puri, Odisha	Blood of *Labeo rohita* with haemorrhages on body and 5% mortality
Aeromonas veronii	KT026458	Nellore, A.P. Lat: 14º21'653''N; Long: 80º03'065'E	Blood of *Labeo rohita* with haemorrhages on body and 28% mortality
Aeromonas hydrophila	KT026459	Kolkata, Lat: 22º46'27.1''N; Long: 88º17'47''E	Spleen of goldfish showing high mortality of 70% and haemorrhages on body and gills
Aeromonas hydrophila	KT026460	Kolkata, Lat: 22º46'49.5''N; Long: 88º18'18''E	Kidney of goldfish showing high mortality of 90% and haemorrhages on body and gills
Aeromonas sobria	KU499851	East Godavari, A.P Lat: 17 º04'227''N; Long: 81 º47'418''E	Blood of *Labeo rohita* with haemorrhages on body and 10% mortality
Aeromonas hydrophila	KX219575	Puri, Odisha, Lat: 19º92'619''N; Long: 85 º94'99''E	Body fluid of *Labeo rohita* with haemorrhages on body and 8% mortality
Aeromonas hydrophila	KM277748	Khurda, Odisha Lat: 20º18'18.1''N; Long: 85º85'439''E	Body fluid of goldfish showing signs of ulcers on body surface with sparse mortality
Aeromonas hydrophila	ATCC strain (0637P)	Procured from vendor in Mumbai (HiMedia)	Lyophilized bacteria, ATCC licence derivative.

Antibiogram Study

A. hydrophila (KT026460), *A. hydrophila* (KX219575), *A. veronii* (KT026458), *A. veronii* (KT026457) and *A. sobria* (KU499851) were found to be MDR strains as these pathogens showed non-susceptibility to more than two antibiotics in one group. The isolates were found to be more or less resistant to 12 classes of antibiotics. Surprisingly, all five *Aeromonas* isolates showed absolute sensitivity to norfloxacin, chloramphenicol, ciprofloxacin, levofloxacin, amikacin and gentamicin (Table 1).

Heavy Metal Tolerance

All the *Aeromonas* spp. isolates were tolerant to Cu^{2+} but not Hg^{2+}. Additionally, *A. veronii* isolates were also not tolerant to Cr^{6+}. However, both isolates of *A. hydrophila* and *A. sobria* were found to be tolerant to Cr^{6+} (Table 5).

Table 5: Heavy metal tolerance of *Aeromonas* species.

heavy metal	Concentration (µg/ml)	*A. hydrophila* (KT026460)	*A. hydrophila* (KX219575)	*A. veronii* (KT026458)	*A. veronii* (KT026457)	*A. sobria* (KU499851)
$HgCl_2$	2.5-40	≥ 5 µg/ml	≥ 5 µg/ml	≥ 5 µg/ml	≥ 5 µg/ml	≥ 10 µg/ml
$CuSO_4$	150-2400	600 µg/ml	600 µg/ml	600 µg/ml	600 µg/ml	600 µg/ml
$K_2Cr_2O_7$	25-400	≥ 200 µg/ml	≥ 200 µg/ml	≥ 100 µg/ml	≥ 100 µg/ml	≥ 200 µg/ml

{The concentration with respect to each bacteria (column-wise) indicates no growth of bacterial strain at that concentration or above in case of $K_2Cr_2O_7$ and $HgCl_2$. In case of $CuSO_4$ the bacterial isolates showed tolerance at 600 µg/ml}

Biofilm Formation

Although all five isolates were found to be biofilm producers on polystyrene microtitre plates, only *A. sobria* and *A. hydrophila* (ATCC 0637P) were strong producers (Table 6).

Table 6: Biofilm producing potential of *Aeromonas* isolates.

Isolate	**$OD_{570\ nm}$**	**Biofilm producing status**
Control	0.242	_____
A. hydrophila ATCC strain	3.317	Strong
A. hydrophila (KT026460)	0.324	Weak
A. hydrophila (KX219575)	0.417	Weak
A. veronii (KT026458)	0.234	Weak
A. veronii (KT026457)	0.252	Weak
A. sobria (KU499851)	3.180	Strong

Integron Assay

PCR of class I and II integrons produced amplicons in each isolate of *A. hydrophila*, *A. sobria* and *A. hydrophila* (ATCC 0637P). No amplicons were produced in the other three isolates. The class I primers produced amplicons of varying sizes, ranging from 0.6 kb to 2.0 kb in the DNA of two isolates of *A. hydrophila* and *A. sobria*, whereas *A. hydrophila* (ATCC 0637P) generated amplicons of between 0.4 kb and 2.0 kb. On the other hand, the same strains produced very little amplicons with the class II integron primers. No detectable amplicons were noticed for other samples (Figure 2).

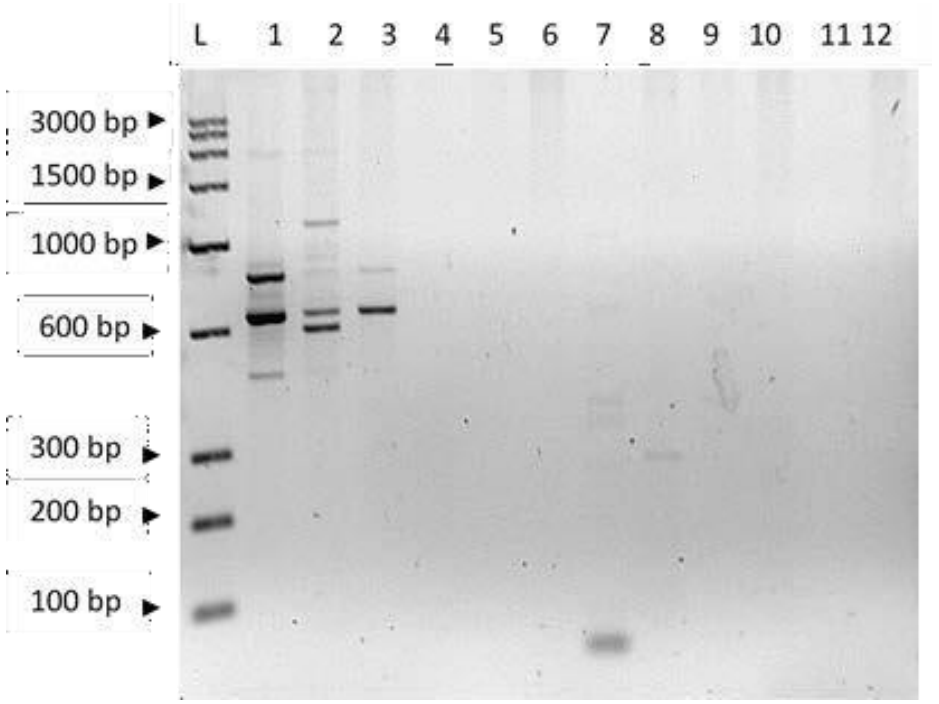

Figure 2: PCR amplification of class I integrons from the template DNA of *A. sobria*, *A. hydrophila* and *A. veronii*. Lane L represents 3.0 kb molecular weight marker. Lane 1, *A. hydrophila* (ATCC 0637P); Lane 2, *A. sobria*; Lanes 3 & 4, *A. hydrophila*; Lanes 5 & 6, *A. veronii* for class I integrons. Lanes 7-12, for class II integrons in the same sequential manner

Discussion

Aquaculture has become an important industry with the increasing demand for high quality food at low cost. Fish diseases could cause huge losses, thus increasing the need for antibiotics. But improper usage had to the emergence of antibiotic-resistant bacteria in the fish-breeding environment, which posed a huge risk to health (Aly *et al.*, 2014).

Thus, it is essential to screen intermittently the presence of antibiotic-resistant bacterias because the pattern of resistance in a particular environment could add value to the FAO/ WHO commission recommendations to prevent waterborne diseases in developing countries.

Motile aeromonad septicaemia (MAS) is the cause of secondary infections and outbreaks in freshwater aquaculture systems (Abbott *et al.*, 2003). The failure of antibiotic treatment should be a cause for concern because the bacterias isolated in this study were observed to be MDR strains that were not affected by 12 classes of antibiotics (Magiorakos *et al.* 2012).

According to Wooley *et al.* (2004), antibiotic resistance was a major problem when dealing with *A. hydrophila* infections. MDR *A. hydrophila* had also been isolated and described in many diseased fish samples (Kaskhedikar & Chhabra, 2010; Laith & Najiah, 2013; Samal *et al.*, 2014). The presence of MDR bacteria in water was a major health concern because they could infect humans and spread in the general population and environment (Tao *et al.*, 2010).

However, six antibiotics, including gentamicin, were still effective against *Aeromonas* species (Abulhamd, 2009; Igbinosa *et al.*, 2013). *Aeromonas* had been reported to show high sensitivity to chloramphenicol (Awan *et al.*, 2009; Igbinosa *et al.*, 2013), which wasin line with our results. Tetracycline was effective against all *Aeromonas* isolates except *A. sobria*.

There were similar observations of tetracycline sensitivity in *Aeromonas* species at different regions (Mahmoud & Tanios, 2008; Awan *et al.*, 2009; Zanella *et al.*, 2012; Igbinosa, 2014).

The aminoglycosides like amikacin and gentamicin showed very strong antibacterial activity against all five *Aeromonas* isolates. This finding also corroborated the results of previous studies (Awan *et al.*, 2009; Dallal *et al.*, 2012; Igbinosa, 2014). All isolates were observed to be resistant to aztreonam from the monobactam group, which might be due to the bacterias' ability to produce betalactamase to hydrolyse the beta-lactam ring of the antibiotic, or alter their penicillin-binding proteins.

All isolates showed complete resistance to lincomycin, oxacillin and penicillin G. The mechanism of resistance could be through target site modification by methylation or mutation that prevented the binding of antibiotics to ribosomal targets (Leclercq, 2002).

An important factor in bacteria virulence is biofilm formation, which allows colonization by adhesion to epithelial cells in the intestinal villi of the fish, thus reducing sensitivity to antibiotics and masking the bacteria from the host's immune system (Davey & O'Toole, 2000; Zanella *et al.*, 2012). All five isolates showed biofilm formation, but the *A. hydrophila* ATCC strain and *A. sobria* were strong producers, thus increasing their virulence. The biofilm-forming ability in *Aeromonas* spp had been reported (Kirov *et al.*, 2002) and considered a potential pathogenic factor (Igbinosa, 2014). In addition to antibiotics resistance, all the *Aeromonas* spp. isolates studied were tolerant to Cu^{2+}, and both strains of *A. hydrophila* and *A. sobria* were found to be tolerant to Cr^{6+}. However, all the isolates could not tolerate the presence of Hg^{2+}. Similarly, *A. veronii* strains were also not tolerant to Cr^{6+}.

This might be influenced by the environment where the isolates were found, which were surrounded by agricultural fields. Fertilizer and pesticide run-off from those fields, which contain heavy metals, might enter the aquaculture system. The bacteria would develop tolerance to those metals after a certain period of time.

Integrons are described as reservoirs of mobile gene cassettes that have site-specific recombination, and several multiple antibiotic resistance integrons are important contributors to the development of resistance in *Enterobacteriaceae* and Aeromonadaceae families (White *et al.*, 2001; Jacobs & Chenia, 2007). More than 100 different antibiotic-resistant gene cassettes had been found within integrons (White *et al.*, 2001; Jacobs & Chenia, 2007; Nawaz *et al.*, 2010). The integrons found in one isolate of *A. hydrophila* and *A. sobria* might be responsible for conferring multiple antibiotic resistance markers that could be genetically transferred. Thus, these two isolates seem to be the most pathogenic, coupled with negative results from the antibiogram and heavy metal tolerance assay. Lukkana *et al.* (2012) had detected class I integron in *A. hydrophila* isolated from Nile tilapia (*Oreochromis niloticus*) in Thailand. The presence of integron in wide array of bacteria and in different habitat substantiates the horizontal mobility and stability of this gene capture system (Dubois *et al.*, 2007; Igbinosa *et al.*, 2013).

Conclusion

This study showed a high prevalence of potentially pathogenic *Aeromonas* strains in aquaculture systems that had the risk of infecting humans and other animals in a community. The risk was more serious when the microbes were observed to display resistance to many classes of antibiotics. Hence, periodical surveillance and screening of infected fish samples is highly essential to ensure public health. The potential health risk due to consumption of improperly cooked fish harbouring motile Aeromonads should not be underestimated.

Acknowledgements

The authors like to thank the Director ICAR-Central Institute of Freshwater Aquaculture in Kausalyaganga, Bhubaneswar, India, for

providing the facilities for this study. This study is funded by the NFDB-DAHDF-NBFGR grant under the National Surveillance Programme for Aquatic Animal Diseases.

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