Isolation and Identification of Multi-antibiotic Resistant *Aeromonas veronii* by 16s rDNA Gene Sequencing from the Gut of Fresh Water Loach, *Lepidocephalycthys guntea* (Hamilton Buchanan)

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Abstract

An investigation was conducted to study the isolates of multiple antibiotic resistant *Aeromonas* sp. from the skin, gill and gut of fresh water loach, *Lepidocephalichthys guntea* (Hamilton Buchanan) from four different sites of river Lotchka in Darjeeling District, West Bengal, India. Isolated bacteria were identified by different biochemical procedures and Polymerase Chain Reaction (PCR) was performed using genus specific 16S rDNA primers for confirmation of identification of *Aeromonas* sp. Antibiotic susceptibility test of bacterial isolates was also done by Disc Diffusion method. A total of 18 *Aeromonas* sp. were isolated from skin, gill and gut of fish. Maximum resistance was exhibited for Penicillin-G, Ampicillin and Cephalothin (100%, 92% and 84% resistant in the gut of fish). In Ciprofloxacin and Tetracycline resistance was almost nil. Among all the isolates, one particular strain showed resistance against eight antibiotics. The bacterial strain was subjected to the PCR with *Aeromonas* genus specific 16S rRNA primers and the PCR product was sequenced. The sequence was performed by comparative analysis with the Genbank and maximum similarity was found with *Aeromonas veronii.* Presence of *Aeromonas veronii* resistance to multiple antibiotics indicated that the loach were contaminated with multi-antibiotic resistant enteric pathogenic bacteria.

1. Introduction

Lepidocephalicthys guntea, a fresh water loach, is having both ornamental and edible value (Mondal et al., 2007). The fish is readily available in the local markets of Siliguri city (Jha et al., 2010) and is commonly found in the rivers of Terai region of West Bengal. It is widely distributed in the region of Northern India, Pakistan, Nepal, Myanmar, Thailand and Bangladesh (Talwar and Jhingran, 1991). In early studies have shown that fresh water fishes were contaminated with many opportunistic pathogenic bacterial population which were influenced by their surrounding environment (Roy and Barat, 2011; Al-Harbi, 2003; Apun et al., 1999). Among the opportunistic pathogen *Aeromonas* sp. are most common (Cahill, 1990).

Members of the genus *Aeromonas* are found in a wide variety of ecological niches. They are able to inhabit surface water (rivers, lakes), sewage, drinking water (tap and bottled mineral), thermal water and sea water (Figueras, 2005, Beaz-Hidalgo et al., 2010). Antimicrobial resistance in bacteria associated with different ecological niches has been a global concern. The emergence of antimicrobial resistant strains of pathogenic bacteria has become a great threat to the public health (Sudha, 2001). The impact of antibiotics on the resident microflora is difficult to assess because of the complexity of the aquatic environment, whilst the resistance patterns of bacterial fish pathogens often reflect an intensive use of antimicrobial substances (Smith et al., 2007).

The present study was, aimed to investigate the antibiotic resistance among isolates of *Aeromonas* sp. from the fish, *Lepidocephalicthys guntea* and further identified any particular multi-antibiotic resistant strain through cultivation and 16s rDNA gene sequencing based techniques.

2. Materials and Methods

2.1. Study area

Live and healthy loach, *Lepidocephalicthys guntea* were collected from river Lotchka at monthly intervals during March 2009 to February 2011.

2.2. Bacterial isolation and biochemical characterization

During each sampling, 10-12 freshly caught fishes, *Lepidocephalicthys guntea*, from all the sites of the river were

brought to the laboratory in sterile plastic bags containing the river water and processed within 2 hours of their collection. Through dissection, skin, gill and gut samples of each fish were removed aseptically and collected separately in sterile containers. Skin, gill and gut had been particularly selected because these organs are readily associated with their surrounding environment. The organs were then homogenized in a sterile glass tissue homogenizer. Serial dilutions of homogenized organs samples were prepared in sterile physiological saline and plated onto *Aeromonas* Starch DNA Agar Base (HiMedia laboratories Ltd, Mumbai, India) for recovery of the selective organisms using Spread Plate technique (Okpokwasili and Alapiki, 1990). All the plates were incubated at 37±1°C for 24-48 hours. After incubation, the selective media plates were examined and the growth of colonies on the plates were recorded for count of *Aeromonas* spp. Growing colonies of selective media were followed by the streaking method for isolation of pure cultures. The bacterial isolates were then used for observation of cell morphology, followed by Gram Staining procedure. Biochemical tests were then performed involving IMvic tests, H_2S production, Urease, Oxidase, Citrate, Aesculine hydrolysis, Arginine utilization, Acid and Gas production from different carbohydrates (Cheesbrough, 2000, Barrow and Feltham, 1993 and Holt et al., 1994).

2.3. Genomic DNA extraction and PCR identification

Genomic DNA was extracted from each bacterial isolates by lysozymes, freeze-thawing and phenol-chloroform method (Lee et al., 1996) and DNA was stored at -20˚C for further use. Genus specific 16S rDNA primers (Table 1) were used for the confirmation of identification of *Aeromonas* sp.

2.4. Antibiotic susceptibility test

Table 1: Genus specific 16S rDNA primers

Primers	Sequence (5'-3')	Length	Annealing Temp	References
Ar-F Ar-R	CTACTTTTGCCGGC GAGCGG TGATTCCCGAAGG CACTCCC	953bp	68°C	Lee et al. 2002

Fresh culture of *Aeromonas* spp. isolates in nutrient broth was inoculated on a Mueller-Hinton Agar (HiMedia Laboratories Ltd, Mumbai, India) plate for determination of antibiotic resistance by a Disc Diffusion Method (Bauer et al., 1966) for the ten antibiotics (µg ml^{-1}) namely Penicillin (10), Ampicilliin (10), Streptomycin (10), Gentamycin (10), Tetracycline (30), Kanamycin (30), Ciprofloxacin (5), Moxifloxacin (5), Erythromycin (15), Cephalothin (30) (HiMedia Laboratories Ltd, Mumbai, India). Antibiotic discs were placed on inoculated agar plates and after overnight incubation at 37˚C, resistance was estimated by measuring the inhibition zone as per standards given in the manufacturer's manual.

3. Results and Discussion

Antibiotic resistant 18 *Aeromonas* sp. were isolated from skin, gill and gut of fish. All the isolates gave result i.e., Gram negative, motile, rod shaped and thus, produced oxidase, catalase, acid and gas from glucose (Table 2). The antibiotic resistance of *Aeromonas* sp. has shown full range of resistance

Table 2: Biochemical profile of *Aeromonas* sp.

Gram stain	-ve	Lactose	nd
MR	-ve	Sorbitol	nd
VP	+ve	Mannitol	nd
Indole	+ve	Raffinose	nd
H_2S Production	nd	Sucrose	+ve
Aesculine Hydrolysis	+ve	Salicin	+ve
Arginine utilization	+ve	Maltose	+ve
Citrate Utilization	nd	Arabinose	-ve
Glucose	+ve	Inositol	+ve

+ve: Positive; -ve: Negative; nd: Not done

(0-100%) for ten antibiotics (Table 3), which are commonly used in human beings. Maximum resistance was exhibited for Penicillin-G, Ampicillin and Cephalothin (100%, 92% and 84% resistant in the gut of fish). In Ciprofloxacin and Tetracycline, resistance was almost nil in skin and gill. Percentage of multi -antibiotic resistant pattern were higher in the gut, compared to than skin and gill this could be due to bacterial load being maximum in the gut of the fish, *L. guntea* (Roy and Barat, 2011). Many researchers mentioned that gut of many fishes contained wide variety of bacteria (Sugita et al., 1982, Sakata et al., 1984).

Among the isolates one particular strain from the gut had shown resistance to eight antibiotics namely Penecillin-G, Ampicillin, Chephalothin, Streptomycine, Gentamycine, Kanamycine, Cephalothin, Ciprofloxacine, Erythromycine. The particular bacterial strain was subjected to the PCR with *Aeromonas* genus specific 16S rRNA primers and the PCR product was sequenced. The 16S rRNA sequence (Accession No JN796925) was compared and aligned with sequence deposited in the NCBI-Gen Bank database using BLAST (Altschul et al., 1997) for identification of bacteria. The sequences were aligned in CLUSTAL X (Thompson et al., 1997). The computed alignment was then manually checked and corrected. Pair

Table 3: Antibiotics resistance (%) among bacterial isolates from different body parts of fish, *Lepidocephalicthys guntea*

SI	AMP			PEN			CEPHA			ERY			KANA			STREPTO			CIPRO			MOXI			TETRA			GENTA		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Skin	76	7	7	100	0	0	75	0	25	12	38	50	25	0	75	0	12	88	0	0	100	12	13	75	0	0	100	0	12	88
Gill	82	9	9	90	10	0	64	18	18	54	10	36	54	10	36	27	9	64	9	19	72	9	28	63	0	0	100	0	18	82
Gut	84	8	8	100	0	0	92	0	8	84	8	8	8	42	50	16	9	75	8	16	74	8	26	66	0	25	75	34	0	66

SI: Source of Isolates; R:Resistance; I-Intermediate; S-Sensitive (%) for *Aeromonas* sp.; AMP: Ampicillin; PEN: Penicillin; C: PHA: Cephalothin; ERY: Erythromycin; KANA: Kanamycin; STREPTO: Streptomycin; CIPRO: Ciprofloxacin; MOXI: Moxifloxacin; TETRA: Tetracycline; GENTA: Gentamycin

wise evolutionary distances were computed using the MEGA 4 program and a phylogenetic tree was constructed by the neighbour-joining method program available online (Tamura et al., 2007) and maximum similarity was found with *Aeromonas veronii*. The 16S rRNA sequences of *Aeromonas veronii* were used for constructing phylogenetic tree (Figure 1).

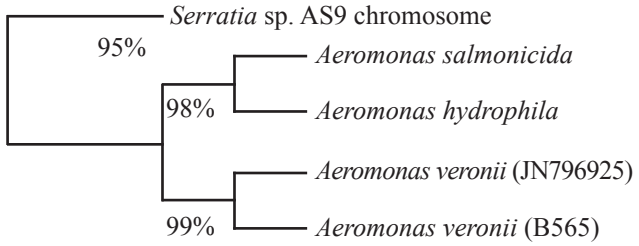

Figure 1: Phylogenetic tree based on 16S rDNA sequences. The evolutionary distance was inferred using the neighbour-joining method

The resistance exhibited for Erythromycin, Kanamycin, Ciprofloxacin and Gentamycin is a signal of the ineffectiveness of broad spectrum antibiotics of the present generation. It appears that the source of the problem of antibiotic resistance in riverine ecosystems is due to faecal contamination water and fish population in them (Pathak and Gopal, 2005). Several molecules belonging to the same chemical family are usually affected by a single mechanism of resistance (cross-resistance). Both ampicillin and cephalothin, for example, are inactivated by chromosomal beta lactamases produced by many Aeromonas species (Walsh et al., 1995). Similar to our study antibiotic resistance motile Aeromonad were also found in fresh water fishes (Hatha et al., 2005) and in Danish rainbow trout fish (Schmidt et al., 2000). Saha and Pal (2002) reported on the multiple antibiotic resistant *Aeromonas hydrophilla*, isolated from Epizootic Ulcerative Syndrome (EUS) affected fish in India.

Aeromonas veronii is present widely in aquatic environment and known to pose an aquatic animal health risk. *A. veronii* may spread in one environment to other by infections and causes serious problems as it is also one of the most important pathogen of humans as well. *Aeromonas veronii*, is a food borne bacterial pathogen causing infections in fish, animals and humans. It is Gram-negative, rod-shaped bacteria found in fresh water and have been associated with animal diseases. *A. veronii* is an opportunistic human pathogen that causes diarrhea and extra intestinal infections, i.e., wound infection and septicaemia. *A. veronii* causes various diseases ranging from wound infections and diarrhea to septicemia in immune compromised patients (Janda and Abbott, 1996).

4. Conclusion

Presence of *Aeromonas* veronii and its resistance to multiple antibiotics therefore indicates, that the fish was contaminated with multi-antibiotic resistant enteric pathogenic bacteria. This study, evidently provides valuable information for making policy decisions aimed at reducing microbial contamination of fish and the indiscriminate use of antibiotics. Further research on antibiotic susceptibility in the aquatic environments of northern region of West Bengal India is needed.

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