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Isolation and Characterization of Multiple Antibiotic Resistant *A. hydrophila* causing Mortality of Indian Major Carps in Ganjam District, Odisha

P. Chandravanshi, S. K. Udgata, J. Kumari, B. Sahu* and N. C. Pashupalaka

College of Fisheries (OUAT), Rangailunda, Berhampur, Odisha, India

**Corresponding author*

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A B S T R A C T

The study was conducted to identify and characterize the etiological agent causing disease and mortality in Indian major carp from fresh water fish farms of Ganjam district in Odisha, India. Diseased fish samples showing symptoms of haemorrhages, red sores, ulcerations, loss of appetite, lethargy, pop-eyes, dropsy and tail or fin rots etc. were collected for the isolation and identification of the pathogenic bacteria using microbiological, biochemical and molecular methods. Out of 65 samples collected, 33 presumptive *Aeromonas* isolates were obtained, which were then pooled to 12 isolates for further analysis based on their source of isolation. The 16S rRNA gene sequencing and phylogenetic analysis of three isolated strains showed close relationship with *Aeromonas hydrophila*. In Antibiogram study, all three strains were found resistant against more than three commonly used antibiotics having MAR index ≥ 0.5. This study highlights the involvement of multiple antibiotic resistant *Aeromonas hydrophila* in the disease outbreak of cultured Indian major carps. The study further affirms the superiority of molecular characterization in identifying *Aeromonas hydrophila* over microbiological and biochemical methods.

Introduction

Aquaculture has been recognized as one of the fastest growing food producing sector in the world. The significant contribution of aquaculture over the past few decades has been able to place India in second position in terms of global inland fish production (FAO, 2018). While aiming at maximizing fish production, fish farms in India are expanding and intensifying their culture system and hence among other reasons, disease outbreak has started to become a disaster in recent days. Disease has emerged out as one of the major limiting factors for development and management of aquaculture in India and many other countries of the world (Kumar *et al.*, 2016).

Out of many diseases, bacterial diseases are the most leading causes of fish mortality in aquaculture farms (Leung and Bates, 2013). Aeromoniasis is an endemic bacterial disease caused by Aeromonads, and is a world-wide

problem affecting many fishes (Robinson *et al.,* 2014). These aeromonads include *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas sobria*, *Aeromonas veronii* etc. *Aeromonas* sp is ubiquitous, free living, gram negative, motile rod that causes multiple infections in fish with varied symptoms including skin haemorrhages, red sores, body ulcerations, loss of appetite, lethargy, pop-eyes, dropsy and tail or fin rots etc. (Hu *et al.,* 2012; Sreedharan *et al.,* 2012).

The disease is often associated with serious damages and economic losses in fish farming industry (Yang *et al.,* 2017). *Aeromonas* sp is also an opportunistic pathogen of a wide variety of hosts including human beings (Samal *et al.,* 2014).

Antimicrobial drug therapy is the most widely used method of control of Aeromoniasis in fish culture farms (Guz and Kozińska, 2004). However, the main problem in the use of antibiotics against Aeromonas infection is the development of resistance by these bacteria to these antibiotics (Sarder *et al.,* 2016). Besides, the massive use of antimicrobials at sub-optimal doses as growth promoters in aquaculture farms encourages the natural emergence of bacterial resistance towards the antimicrobials (Chu and Zhu, 2010; FDA, 1998).

Resistance of *Aeromonas* sp. to commonly used antibiotics is an emerging problem in aquaculture. Repeated reports on multidrug resistance in the genus Aeromonas is a great concern today (Albert *et al.,* 2000; Vivekanandhan *et al.,* 2002; Palu *et al.,* 2006; Lijon *et al.,* 2015; Sarder *et al.,* 2016), since the increase in resistance increases the difficulty in treating such diseases in fishes. The situation becomes grave when such strain is zoonotic in nature and involved in human infections. So, the present study was done to identify the etiological agent in diseased Indian major carps in fresh water fish farms of Ganjam district in Odisha, India and to establish their antibiotic resistance pattern.

Materials and Methods

Collection of diseased fish sample

Randomly selected twelve freshwater fish farms across Ganjam district in Odisha were sampled using cast net for collection of diseased fish samples during January to March 2019. The moribund fish samples of catla (*Catla catla*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), calbasu (*Labeo* calbasu) and common carp (*Cyprinus carpio*) with signs of haemorrhage, ulceration, abdominal swelling, erosion of fin and tail etc. were collected. External sores or lesions were immediately swabbed using sterile cotton swab (Hi-Media) and inoculated into sterile Tryptic Soy Broth (TSB, Hi-Media) aseptically. Tissue samples of external and internal organs like skin, fin, gill, kidney, liver and intestine etc. were collected by dissecting the moribund fish at pond site and inoculated into TSB aseptically following protocols of Pradhan *et al.,* (2014). The samples were then brought to the laboratory under ambient condition for further bacteriological analysis.

Bacteriological examination

Upon arrival at the laboratory, the inoculated broths were incubated at 30°C. Next day, the overnight cultures were serially diluted upto 10^{-6} and 100 µl each from last three dilutions were spread plated on Rimler Shotts (RS) Agar medium in duplicate and incubated at 30°C for 24 h.

Isolated colonies appearing slightly convex, mucoid and yellowish in colour were presumed to be *Aeromonas hydrophila* (Sahoo and Das, 2014), and hence sub-cultured onto Tryptic Soy Agar (Hi-Media) slants for further analysis.

Biochemical characterization

All the presumptive isolates were characterised morphologically (Gram's staining, shape and motility) and subjected to set of biochemical test meant for *A. hydrophila* such as, Indole, Methyl red (MR), Voges Proskauer (VP), Citrate utilization (IMViC reaction), Oxidase, Catalase, Oxidation-Fermentation, Triple Sugar Iron (TSI) and Amino Acid Decarboxylase test for Arginine, Lysine and Ornithine (Khuntia, 2011; Sahoo and Das, 2014; Monir *et al.*, 2016). The Reference strain *A. hydrophilla* (MTCC 1739) was used as positive control while testing the presumptive isolates.

Molecular identification

Presumptive isolates showing deviation to some of the biochemical tests as compared against standard reference strain were subjected to molecular identification. The genomic DNA from these selected isolates were extracted using the DNeasy UltraClean Microbial Kit (QIAGEN, Germany) as per manufacturer's instruction. The 16S rRNA genes from the pathogenic isolate was then amplified by PCR using a pair of universal primers, 27f (5'-AGAGTTTGATCCTG GCTCAG-3') and 1492r (5'-CGGTTACCT TGTTACGACTT-3') according to Cao *et al.*, (2010) and Kupfer *et al.*, (2006). Sequencing was performed by the fluorescent-labeled dideoxynucleotides termination method (with a BigDye terminator) on ABI 3730 XL DNA Sequencer (Applied Biosystems, Waltham, MA, USA) using BDT v3.1 cycle sequencing kit and another pair of primers, forward (5'-GGATTAGATACCCTGGTA-3') and reverse (5'-CCGTCAATTCMTTTRAGTTT-3'). Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. Homology searches for 16S rRNA gene sequences were performed using the Basic Local Alignment Search Tool (BLAST) version 5 software (National Library of Medicine, Bethesda, MD, USA) available at the National Center for Biotechnology Information (NCBI).

Based on maximum identity score first ten sequences were selected and few distantly related sequences were aligned using multiple alignment software program Clustal W in MEGAx (Kumar *et al.*, 2018) and the phylogenetic analysis was performed by using the Maximum likelihood method based on the Kimura 2-parameter model.

Antibiotic sensitivity study of the *Aeromonas hydrophila* isolates

Antibiogram profile of the confirmed *Aeromonas hydrophila* isolates was done using ten commonly used antibiotics. The details of antibiotics used in the present study have been given in the Table 1.

The antibiotic sensitivity test was performed using Kirby Bauer Disc Diffusion Method (Bauer *et al.*, 1966; Jorgensen and Ferraro, 2009) following Clinical and Laboratory Standards Institute guidelines (CLSI, 2018). Briefly, the test was performed on Mueller Hinton Agar plates for all the *Aeromonas* isolates against the selected antibiotics. Zone of inhibition was measured, recorded and interpreted as Susceptible (S), Intermediate (I) and Resistant (R) by considering Enterobacteriaceae breakpoints (Lamy *et al.*, 2012). MAR index was calculated as the ratio of number of resistant phenotype to total number of antimicrobials to which the strains were exposed (Hossain *et al.*, 2017).

Results and Discussion

Isolation of *Aeromonas hydrophila* from diseased fish

Sample collection of diseased fishes was done from 12 fish farms of Ganjam district, Odisha.

Out of which disease outbreak was seen in 3 farms located at Aska, Duba and Chatrapur, from which a total of 65 samples were collected from gill, skin, fin, liver, kidney and intestine of live and moribund fishes of Indian major carps (Fig. 2). Out of which, 33 isolates were able to grow on Rimler Shott's Agar medium producing slightly convex, mucoid and yellowish colonies. Different researchers have used different media for presumptive isolation of *Aeromonas hydrophila* from aquatic animals, aquatic environment, seafoods, animal products, fishes and humans like Rimler-Shotts medium (RS), Starch Ampicillin agar (SA), Starch-Glutamate-Ampicillin-Penicillin-based medium (SGAP-10C) and Pseudomonas Aeromonas selective agar base (GSP) (Shotts and Rimler, 1973; Jenkins and Taylor, 1995; Singh, 1997). In this study, we have used RS medium as selective medium for presumptive isolation of *Aeromonas hydrophila* by following methods of Sahoo and Das (2014). All the 33 presumptive *Aeromonas* isolates were pooled, considering single isolate responsible for manifestation of disease in a fish affecting multiple organs, to 12 isolates based on their source of isolation for further analysis.

Biochemical characterization of presumptive *Aeromonas hydrophila* isolates

All the 12 pooled isolates were subjected to different biochemical tests besides Gram staining and motility test along with the reference strain of *Aeromonas hydrophila* (MTCC 1739) as positive control. The isolate wise detailed result of biochemical tests has been depicted in Table 2. In this study, majority of the isolates were found to be Gram negative, motile, rod shaped, able to produce indole from tryptophan, utilised glucose to produce different acid by mixed acid pathway, but not able to follow butylene glycol pathway, utilized citrate as sole carbon source, produced cytochrome oxidase, were fermentative, able to utilize glucose, had lysine and arginine decarboxylase enzyme but not ornithine decarboxylase enzyme (Table 2). Many authors have performed similar biochemical test for Aeromonads along with many other tests like acid production from maltose, manitol, esculin, starch and gelatin hydrolysis, sensitivity to vibrostatic agent O/129, Nitrate reduction, growth at different temperature and salt concentration etc. and have got almost similar results like the present study with minor variations (Ashiru *et al.,* 2011; Jeeva *et al.,* 2013; Ali *et al.,* 2014; Laith and Najiah, 2014; Samal *et al.,* 2014; Thiyagarajan *et al.,* 2014; Lijon *et al.,* 2015; Monir *et al.,* 2016; Nahar *et al.,* 2016).

Few of the isolates showed deviations in some of the characteristic reactions compared to the reference strain *A hydrophila* (MTCC 1739), which has been described below. Almost all the isolates showed positive reaction towards MR test as evidenced by red coloration in culture tube after addition of methyl red reagent that showed production of mixed acid. However, only one isolate *i.e*., *Ah*7 showed negative reaction to this test. Our findings are in agreement with Jeeva *et al.,* (2013); Samal *et al.,* (2014); Thiyagarajan *et al.,* (2014); Lijon *et al.,* (2015), who have reported positive reaction towards MR test, while Ali *et al.,* (2014); Monir *et al.,* (2016); Rawal *et al.,* (2016), very few isolates of Laith and Najiah, (2014) and Samal *et al.,* (2014) have reported negative reaction to MR test as the characteristic reactions for *Aeromonas hydrohila*. Similarly, VP test was performed to detect acetoin production following butylene glycol pathway by addition of Barritt's reagent A and B. While majority of our isolates have shown negative reaction towards VP test, similar to that has been reported only by Samal *et al.,* (2014), few of our *A hydrophila* isolates namely *Ah*2, *Ah*7 and *Ah*9 have shown positive reaction as well. Positive reactions of *A hydrophila* towards

VP test has been reported by the previous researchers like Jeeva *et al.,* (2013); Ali *et al.,* (2014); Laith and Najiah, (2014); Thiyagarajan *et al.,* (2014); Lijon *et al.,* (2015); Monir *et al.,* (2016); Nahar *et al.,* (2016); Rawal *et al.,* (2016). In the case of Citrate utilization, majority of the isolates showed positive reaction, as has been reported by Laith and Najiah, (2014); Samal *et al.,* (2014); Thiyagarajan *et al.,* (2014); Lijon *et al.,* (2015); Monir *et al.,* (2016); Nahar *et al.,* (2016), while three isolates namely *Ah*2, *Ah*4 and *Ah*7 were unable to utilize citrate as sole carbon source. Similar to this finding Rawal *et al.,* (2016), few isolates of Awan *et al.,* (2005) and Samal *et al.,* (2014) have reported negative reaction towards citrate utilization test.

Aeromonas isolates are capable of utilising glucose as shown by colour changes in TSI slant as red for alkaline nature and yellow for acidic nature in the butt due to anaerobic oxidation. Positive result for TSI is interpreted as K/A for alkaline slant and acidic butt and negative result as A/A for acidic slant and acidic butt.

Majority of the isolates in this study have shown positive result for TSI in agreement with the findings of Nahar *et al.,* (2016), Monir *et al.,* (2016) and Samal *et al.,* (2014). However, three isolates namely *Ah*2, *Ah*7 and *Ah*9 showed negative result for TSI test, which has also been reported by Samal *et al.,* (2014), Laith and Najiah (2014).

Aeromonas group of microorganism can be identified and characterized up to genus level on the basis of amino acid utilization (Janda and Abbott, 2010). Majority of our isolates were capable of utilizing lysine and arginine due to presence of specific decarboxylase enzyme as has been reported by Abbott *et al.,* (2003); Jeeva *et al.,* (2013); Samal *et al.,* (2014); Nahar *et al.,* (2016).

On the other hand, Monir *et al.,* (2016), few isolates of Samal *et al.,* (2014) have reported absence of lysine decarboxylase enzyme. Absence of arginine decarboxylase enzyme was observed for three of our *A hydrophila* isolates namely, *Ah*3, *Ah*7 and *Ah*9, that corroborates the findings for few isolates by Samal *et al.,* (2014). To summarise, all presumptive isolates; except three namely, *Ah*2, *Ah*7 and *Ah*9; have shown the characteristic biochemical reactions similar to the standard reference strain *A hydrophila* MTCC 1739, and hence can be confirmed as *A hydrophila.*

Molecular identification

The PCR amplified 16S rRNA gene sequence of pathogenic isolates *Ah*2, *Ah*7 and *Ah*9; that were showing many deviations in biochemical tests from the standard reference strain; were sequenced, analyzed and submitted to GenBank (GenBank Accession No MT974689.1, MT974690.1 and MT974691.1). The BLAST analysis of the 16S rRNA gene sequence of *Ah*2 showed 100% similarity with *Aeromonas hydrophila* strain CCM 7232 (GenBank Accession No. NR_043638.1) and JCM 1027 (GenBank accession no. NR_113342.1). At the same time, that of *Ah*7 and *Ah*9 showed 100% and 99.73% sequence similarities with *Aeromonas hydrophila* strain ATCC 7966 (GenBank Accession No. NR_118944.1) respectively. Accordingly, the molecular identification results confirmed that the isolated bacterial fish pathogen are *A. hydrophila.*

Table.1 Particulars of antibiotics used in the present study

Sl. No.	Antibiotics	Disc content (mcg)	Class
01	Ciprofloxacin	5 mcg	Fluroquinolones 2G
02	Ofloxacin	5 mcg	Fluroquinolones 2G
03	Norfloxacin	10 mcg	Fluroquinolones 2G
04	Streptomycin	10 mcg	Aminoglycosides
05	Gentamicin	10 mcg	Aminoglycosides
06	Tetracycline	30 mcg	Tetracyclines
07	Azithromycin	15 mcg	Macrolides
08	Penicillin	10 units	Penicillin
09	Ampicillin	10 mcg	Aminopenicillin
10	Amoxyclav (Amoxycillin/ Clavulanic acid)	30 mcg (20/10)	Aminopenicillin+β-lactam inhibitor

Table.2 Biochemical characterization of presumptive *Aeromonas* isolates from diseased fish samples

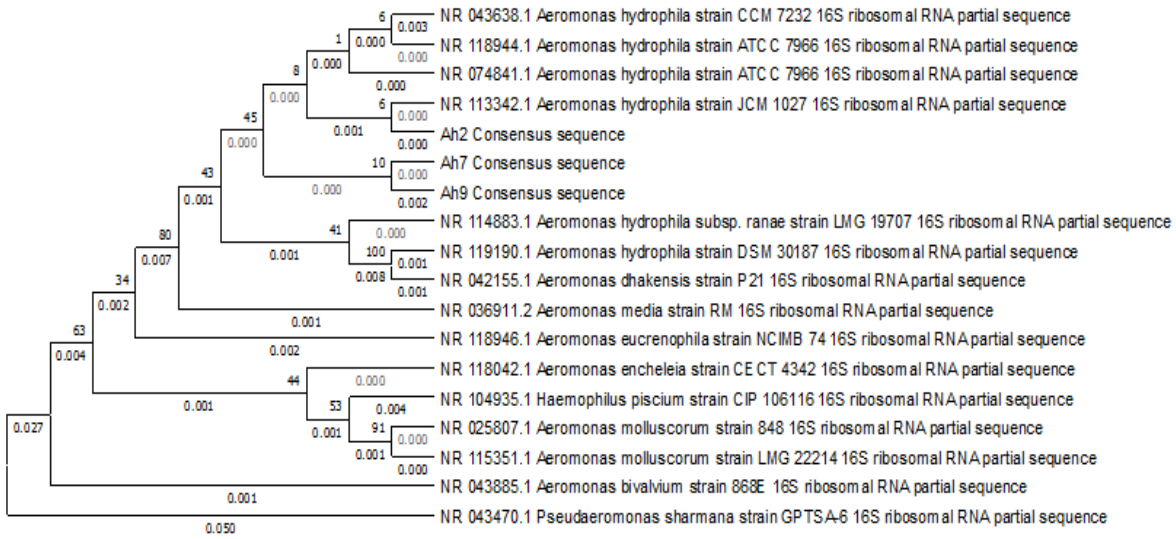
Sl. No	Test	*Aeromonas* Isolates												*A.h.* MTCC 1739
		Ah1	***Ah2***	***Ah3***	***Ah4***	***Ah5***	***Ah6***	***Ah7***	***Ah8***	***Ah9***	***Ah10***	***Ah11***	***Ah12***	
1.	Motility	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
2.	Gram staining	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3.	Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
4.	Indole	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
5.	Methyl Red	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
6.	Voges – Proskauer	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve
7.	Citrate utilization	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
8.	Oxidase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
9.	Catalase	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
10.	Oxidation / Fermentation	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
11.	Triple sugar iron	K/A	K/K	K/A	K/A	K/A	K/A	K/K	K/A	K/K	K/A	K/A	K/A	K/A
12.	Arginine	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
13.	Lysine	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
14.	Ornithine	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

* - K/A Alkaline slant and acid butt, A/A Acid slant and acid butt, K/K Alkaline slant and alkaline butt

Table.3 Antibiogram profile of *A. hydrophila* (n=3) isolates against different antibiotics

Sl. No.	Antibiotics	Disk content (mcg)	*Ah2*	*Ah7*	*Ah9*	Percentage		
						Sensitive	Intermediate	Resistant
1	Ciprofloxacin	5	S	S	S	100	0	0
2	Ofloxacin	5	S	S	S	100	0	0
3	Norfloxacin	10	S	S	S	100	0	0
4	Streptomycin	10	I	S	I	33.3	66.67	0
5	Gentamicin	10	S	S	S	100	0	0
6	Tetracycline	30	R	R	R	0	0	100
7	Azithromycin	15	R	I	I	33.3	66.67	0
8	Penicillin	10 units	R	R	R	0	0	100
9	Ampicillin	10	R	R	R	0	0	100
10	Amoxyclav	20/10	R	R	R	0	0	100
	R / I / S		**5/1/4**	**4/1/5**	**4/2/4**			
	MAR index		**0.5**	**0.4**	**0.4**			

Fig.1 Evolutionary analysis by Maximum Likelihood method



The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-2862.32) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.0646)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 51.44% sites). This analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1545 positions in the final dataset.

Fig.2 Fishes infected with *A. hydrophila*

Phylogenetic analysis

The phylogenetic cladogram was constructed using maximum likelihood algorithm. It revealed the relationship of the isolate *Ah*2, *Ah*7 and *Ah*9 with other *Aeromonas* species. The fifteen other 16S rRNA gene sequences homologous to *A. hydrophila* were obtained from NCBI GenBank. The phylogenetic cladogram revealed that 16S rRNA gene sequence of *Ah*2, *Ah*7 and *Ah*9 are evolutionarily very close to 16S rRNA gene of *A. hydrophila* with highest bootstrap value (Fig. 1).

Antibiotic sensitivity study of *A. hydrophila* isolates

The antibiogram analysis of three isolates namely *Ah*2, *Ah*7 and *Ah*9; that were confirmed as *A. hydrophila* through molecular methods; showed 100% susceptibility to Fluroquinolone class of antibiotics namely, Ciprofloxacin, Ofloxacin, Norfloxacin and Gentamicin (Aminoglycosides). Intermediate response was observed towards Streptomycin (Aminoglycosides) and Azithromycin (Macrolide). On the other hand, the isolates were found to be 100% resistant to Tetracycline and Penicillin class of antibiotics namely, Penicillin, Ampicillin and Amoxyclav. Multiple antibiotic resistance (MAR) index for different *A. hydrohila* isolates namely, *Ah*2, *Ah*7 and *Ah*9 were found to be 0.5, 0.4 and 0.4 respectively.

In the present study, it is evident from Table 3 that all the *A. hydrophila* isolates were 100% susceptible to Fluroquinolone class of antibiotics comprising of Ciprofloxacin, Ofloxacin and Norfloxacin. The findings of this study match well with the previous reports that *Aeromonas hydrophila* isolated from various fresh water fishes from Syria, Bangladesh and Rajasthan were 100% susceptible to Ciprofloxacin (Daood, 2011; Lijon *et al.*, 2015; Monir *et al.*, 2016; Rawal *et al.*, 2016). However, few researchers of different regions have reported varying percentage of resistance for *A. hydrophilla* isolates towards Ciprofloxacin *i.e.*, 31.75% in South China (Yang *et al.*, 2017), 6.25% at Dhaka in Bangladesh (Sarder *et al.*, 2016) and 30.8% at Odisha and Andhra Pradesh in India (Samal *et al.*, 2014). A report by Samal *et al.*, (*2014*) on 100% susceptibility to Ofloxacin and only 15.4% resistance to Norfloxacin corroborates our findings.

The strains under this study were also found to be 100% sensitive towards Gentamycin and 33% sensitive and 67% intermediate towards Streptomycin, although both of them belong to Aminoglycosides class of antibiotics. Varying level of resistance of *A. hydrophila* has been reported towards Streptomycin *i.e*. 8.7% resistance in Southern India (Vivekanandhan *et al.,* 2002), 18.1% in Turkey (Yucel *et al.,* 2005), 7.7% at Odisha and Andhra Pradesh in India (Samal *et al.,* 2014), 25% in Bangladesh (Sarder *et al.,* 2016), 87.5% at Rajasthan in India (Rawal *et al.,* 2016) and 79.37% in South China (Yang *et al.,* 2017). On the other hand, our findings as 100% sensitive towards Gentamycin corroborates with many others earlier reports (Lijon *et al.,* 2015; Monir *et al.,* 2016; Rawal *et al.,* 2016; Sarder *et al.,* 2016; Yang *et al.,* 2017). However, there are certain deviations such as 7.5% resistance reported in Southern India (Vivekanandhan *et al.,* 2002), 54% in Turkey (Yucel *et al.,* 2005), 4.69% in Syria (Daood, 2011) and 30.8% at Odisha and Andhra Pradesh in India (Samal *et al.,* 2014).

It is clear from the Table 3 that the *A. hydrophila* isolates used in this study were 100% resistant towards Tetracycline. Varying level of resistance have been reported for Tetracycline elsewhere across the globe *i.e*. 51.4% in Southern India (Vivekanandhan *et al.,* 2002), 36.3% in Turkey (Yucel *et al.,* 2005), 53.12% in Syria (Daood, 2011), 38.5% in Odisha and Andhra Pradesh (Samal *et al.,* 2014), 36% and 37.5% in Bangladesh (Monir *et al.,* 2016; Sarder *et al.,* 2016) respectively, 88.89% in South China (Yang *et al.,* 2017). On the other hand, 100% susceptibility has been reported towards Tetracycline for *A hydrophilla* isolated from water samples of Lake Fateh Sagar and Lake Pichhola of Udaipur, Rajasthan in India (Rawal *et al.,* 2016) and catfish at Marang River in Malaysia (Laith and Najiah, 2014).

In accordance to the findings of this study, very low or almost zero resistance of *A hydrophila* isolates has been reported for Azithromycin by (Samal *et al.,* 2014; Lijon *et al.,* 2015; Monir *et al.,* 2016; Yang *et al.,* 2017). Similarly, many researchers (Yucel *et al.,* 2005; Daood, 2011; Laith and Najiah, 2014; Samal *et al.,* 2014; Lijon *et al.,* 2015; Monir *et al.,* 2016; Rawal *et al.,* 2016; Sarder *et al.,* 2016; Yang *et al.,* 2017) have reported 100% resistance for Penicillin class of antibiotics namely, Penicillin, Ampicillin and Amoxyclav which are also similar to the findings of this study.

Out of 10 antibiotics tested in this study, all strains were found to be resistant to more than 3 antibiotics. That's why the MAR index of all these strains were found to be more than 0.2, which indicates the elevated rate of use or the high-risk exposure of antibiotics for animal treatment (Laith and Najiah, 2014). Bacteria become resistant to multiple antibiotics, may be due to presence of antibiotic resistant genes, that may be chromosomal or extrachromosomal (Subashkumar *et al.,* 2007; Lukkana *et al.,* 2012). However, if it is extrachromosomal and mobile in nature like plasmid mediated then there is every possibility of its transmission to other dreaded pathogens in the environment and cause serious menace (Sahu *et al.,* 2010). The extensive and misuse of antimicrobial drugs accelerates the emergence of drug-resistant strains. Resistance of a microorganism to an antimicrobial drug is a global concern, because this resistance threatens the effective prevention and treatment of the infections caused by the pathogens both in human and animals.

To summarize, the findings of the study indicate the involvement of multiple antibiotic resistant *Aeromonas hydrophila* as one of the major pathogen in the disease outbreak of

cultured Indian major carps. The study further affirms the superiority of molecular characterization in precise identification of *Aeromonas hydrophila* over the conventional microbiological and biochemical methods.

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