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Phenotypic and genotypic identification of *Aeromonas* species from aquatic environment

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Abstract: A total of 50 strains of *Aeromonas* spp. were isolated from 50 water and fish samples, and identified biochemically and genetically. Biochemical characterization indicated that 20 out of 50 aeromonads were *A. veronii*, 19 were *A. caviae*, 9 were *A. hydrophila* and 2 were *A. trota*. Molecular identification of isolated aeromonads revealed restriction profile of four species i.e. *A. sobria*, *A. media*, *A. allosaccharophila* and *A. schubertii* in addition to *A. veronii, A. caviae, A. hydrophila* and *A. trota* in 16S rDNA RFLP. The 16S rDNA sequencing results for the 13 strains validated the results of PCR-RFLP analysis for *Aeromonas* identification. Most of the isolates identified by 16S rDNA RFLP were positive for virulence factors as revealed by phenotypic tests such as hemolysin production, casein hydrolysis, gelatinase activity, lipase activity, DNase activity, lecithinase production and siderophore production. Further, a variable number of *Aeromonas* spp. was positive for lipase and elastase genes indicating the presence of other genes responsible for these activities observed phenotypically. The present study highlights important incidence of motile *Aeromonas* spp. with virulence potential, from water and fish samples.

Key Words: *Aeromonas*, 16S rDNA, RFLP, virulence

Introduction

Aeromonas spp. are Gram-negative, rod shaped, facultative anaerobic, oxidase positive and glucose fermenting bacteria that belong to the family *Aeromonadaceae* (Ormen *et al*., 2005) and are considered to be emerging bacterial pathogens (Merino *et al*., 1995;

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Kingombe *et al*., 1999; Igbinosa *et al*., 2012). These bacteria have a broad host spectrum, with both cold and warm blooded animals, including humans (Aberoum and Jooyandeh, 2010). *Aeromonas* spp. are universally distributed and widely isolated from clinical (Kuhn *et al*., 1997), environmental (Nakano *et al*., 1990; Kuhn *et al*., 1997) and food samples (Abeyta *et al*., 1986), where they may grow even at low temperatures (Pin *et al*., 1996). There are two phenotypically distinct groups in the genus *Aeromonas*; psychrophilic non-motile *Aeromonas salmonicida* and mesophilic motile aeromonads. *Aeromonas salmonicida* is the causative organism of fish furunculosis, a serious communicable disease affecting primarily Salmonidae whereas, infections caused by motile members of the genus *Aeromonas*, are among the most common and troublesome diseases of fish raised in ponds and recirculating systems. Such infections have been recognized for many years and have been referred to by various names, including motile aeromonad septicemia, motile aeromonad infection, hemorrhagic septicemia, red pest, and red sore and can lead to huge mortality among wild and cultured fishes (Vivekanandhan *et al*., 2005; Rahman *et al*., 2007). Motile *Aeromonas* spp. are also responsible for gastroenteritis and septicemia in human beings (Merino *et al*., 1995). *Aeromonas* virulence is considered to be multi-factorial, and a number of factors such as hemolysins, cytotoxins, enterotoxins, proteases, lipases, DNases and adhesins have all been identified as putative virulence factors in aeromonads (Cascon *et al*., 2000; Rabaan *et al*., 2001). The detection of virulence genes is a crucial step in determining the potential pathogenicity of *Aeromonas* isolates (Yogananth *et al*., 2009).

Generally, the criteria to identify species are primarily based on biochemical tests. However, the conventional microbiological procedures for isolating and identifying *Aeromonas* spp. are laborious and time consuming and none of the methods currently in use is able to accurately discriminate and identify all species and genomic species of *Aeromonas*. The sequencing of the 16S rDNA gene has proven to be valuable in the identification of *Aeromonas* spp. (Demarta *et al*., 1999). The overall sequence similarity between *Aeromonas* spp. is very high, but there is sufficient variability to discriminate different species. PCR-RFLP analysis of 16S rRNA gene is considered to be a rapid and powerful method for identifying isolates of *Aeromonas* to the species level (Borrell *et al*., 1997; Figueras *et al*., 2000; Ghatak *et al*., 2006).

There are numerous reports on isolation of *Aeromonas* spp. from diseased fish (Rathore *et al*., 2005; Citarasu *et al*., 2011; Sahu *et al*., 2012) and water samples in India (Sharma *et al*., 2005; Bhagyalakshmi *et al*., 2009). The present study was undertaken to isolate and characterize the *Aeromonas* spp. from water

and fish by biochemical and molecular methods. In addition, the distribution of putative virulence factors was also studied in the isolated aeromonads.

Materials and Methods

Collection and processing of samples

Bacterial strains were isolated from a total of 50 samples (Tab. 1a,b) which comprised of 12 water samples and 38 freshwater fish (apparently healthy and diseased) samples.

The different tissues including muscle, kidney, gills and intestinal tissues from different fishes were homogenized using a stomacher blender in alkaline peptone water (APW) and incubated at 28ºC for 18 h. The water samples collected from rivers and ponds were also enriched in alkaline peptone water, for 18 h at

Tab. 1a: Details of *Aeromonas* spp. isolated from Water samples.

Samples	Sampling site	Isolates				
		A1	A2	A3	A4	A5
Pond water [n=4]	NBFGR, Lucknow, Uttar Pradesh; Malda, West Bengal	-	3 (P2,R1,$Po6_B$)	1 ($Po14_B$)	-	-
Ganga river [n=3]	Patna, Bihar; Shivpuri, Uttarakhand	-	1 (MLD)	-	-	2 (GB1,SG1)
Gomti river [n=1]	Lucknow, Uttar Pradesh	-	1 (GMTI1)	-	-	-
Sai river [n=1]	Lucknow, Uttar Pradesh	-	1 (SAI1)	-	-	-
Ken river [n=1]	Panna, Madhya Pradesh	-	1 (KEN1)	-	-	-
Punpun river [n=1]	Patna, Bihar	-	1 (PN1)	-	-	-
Nayar river [n=1]	Pauri, Uttarakhand	-	1 (NYR1)	-	-	-

A1: *A. hydrophila* (n=9); **A2:** *A. caviae* (n=19); **A3:** *A. veronii* bv. *sobria* (n=9); **A4:** *A. veronii* bv. *veronii* (n=11); **A5:** *A. trota* (n=2)

[] = total number of samples, () = isolate number, *= clinical isolates

28ºC. After incubation, 100 µl aliquot of enriched APW was spread over starch ampicillin agar plates and incubated at 28°C for 24 h. Yellow to honey colored colonies surrounded by clear zone, following exposure to iodine vapours were tentatively identified as *Aeromonas*

Tab. 1b: Details of *Aeromonas* spp. isolated from Fish samples.

Samples	Sampling site	Isolates				
		A1	A2	A3	A4	A5
Catla catla [n=8]	Fish farm, Lucknow, Uttar Pradesh	-	3 (CG2,*CT1, AH1)	1 (T2)	4 (C4,Cat,*D1, *E1)	-
Tor tor [n=2]	Srinagar, Uttarakhand	-	-	1 (M2)	1 (TT2)	-
Cyprinus carpio [n=2]	NBFGR, Lucknow, Uttar Pradesh	-	-	1 (L2)	1 (CC2)	-
Clarias batrachus [n=1]	Fish market, Lucknow, Uttar Pradesh	-	-	1 (B3)	-	-
Chitala chitala [n=1]	Malda, West Bengal	-	-	1 (CHTL1)	-	-
Clarias gariepinus [n=7]	Fish farm, Lucknow, Uttar Pradesh	3 (Y3,X6,*CB 2)	1 (*CL1)	1 (11SY4)	2 (*CLR,*GAR)	-
Schizothorax richardsonii [n=1]	Srinagar, Uttarakhand	-	-	-	1 (ST6)	-
Heteropneustes fossilis [n=2]	Fish farm, Lucknow, Uttar Pradesh	-	2 (HF1,HF2)	-	-	-
Cirrhinus mrigala [n=2]	NBFGR, Lucknow, Uttar Pradesh	-	-	1 (*MG7)	1 (*MR6)	-
Labeo rohita [n=4]	Fish farm, Unnao, Uttar Pradesh	2 (*RRMB1, *RU1)	-	1 (*RB1)	1 (*RR1)	-
Channa striatus [n=6]	Fish farm, Lucknow, Uttar Pradesh	4 (*CK1,*J1,* K1, *N1)	2 (*CA1,*CH1)	-	-	-
Helostoma temmincki [n=1]	Fish market, Lucknow, Uttar Pradesh	-	1 (KG2)	-	-	-
Carrasius auratus [n=1]	Fish market, Lucknow, Uttar Pradesh	-	1 (BF3)	-	-	-

A1: *A. hydrophila* (n=9); **A2:** *A. caviae* (n=19); **A3:** *A. veronii* bv. *sobria* (n=9); **A4:** *A. veronii* bv. *veronii* (n=11); **A5:** *A. trota* (n=2)

[] = total number of samples, () = isolate number, *= clinical isolates

species. These colonies were purified on nutrient agar plates and stored at -80ºC for further characterization.

Biochemical identification of *Aeromonas* strains

The yellow colonies were identified to the genus *Aeromonas* by a panel of biochemical tests i.e. Gram staining, motility, oxidase, catalase, fermentation of glucose, resistance to vibriostatic agent O/129 (150 µg) and growth in broth without and with 6% NaCl. All the *Aeromonas* isolates were further confirmed to species level following Carnahan *et al*. (1991) which included esculin hydrolysis, gas production from glucose, acid from arabinose and sucrose, indole production, Voges-Proskauer reaction and resistance to cephalothin (30 µg). Additional tests such as nitrate, citrate, urease and decarboxylase tests were also performed to detect the ability of *Aeromonas* spp. to produce nitrate reductase, citritase, urease and decarboxylase enzymes, respectively.

Molecular identification of biochemically confirmed *Aeromonas* spp.

-Amplification of 16S rDNA by PCR

DNA was extracted using a commercial kit (Chromus Biotech, Bangalore) from isolated *Aeromonas* spp. The oligonucleotides used to amplify the 16S rDNA gene are given in Tab. 2. PCR cycling conditions consisted of denaturation at 93°C for 3 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, and final extension at 72°C was allowed for 10 min. The product was electrophoresed on 1% agarose gel.

-16S rDNA RFLP

All the phenotypically identified *Aeromonas* spp. were also identified on the basis of restriction fragment length polymorphism patterns obtained from amplified 16S rDNA gene as per Borrell *et al*. (1997). Briefly, 10 µl of the 16S rDNA amplified products were incubated with 5U of each enzyme (*AluI* and *MboI*) and 2 µl of the corresponding 10X buffer (Tango buffer for *AluI* and *MboI*) in a total volume of 20 µl. The reaction mixture was incubated overnight at 37°C. Aliquots of each reaction mixture were electrophoresed on a 4% agarose gel. The gel was stained with ethidium bromide (0.5 µg/ml) and viewed on a UV trans-illuminator. A 100bp DNA ladder was used as a molecular size reference. The dendrogram of the representative isolates based on RFLP banding patterns was constructed using NTSYS software version 2.0.

-16S rDNA gene sequencing

The amplified 16S rDNA gene products of representative isolates (n=13) from each identified group in 16S rDNA RFLP were sequenced using forward primer, from a

commercial sequencing facility (Scigenom Labs). The sequences were aligned independently and phylogenetically analysed using MEGA5 (Fig. 1). The phylogenetic tree was inferred using Neighbor-Joining method (Saitou and Nei, 1987).

Tab. 2: Sequence of the primers used for amplification of different genes of *Aeromonas* isolates

Primer	Primer sequence	Length	Product (bp)	Melting temp. (T_m)	Ref.
16S rRNA F1	5′-AGAGTTTGATCATGGTCAG-3'	19	1500	56ºC	1
16S rRNA F2	5′-GGTTACCTTGTTACGACTT-3'	19			
LipF	5′-ATCTTCTCCGACTGGTTCGG-3′	20	382	58ºC	2
LipR	5′-CCGTGCCAGGACTGGGTCTT-3′	20			
ElaF	5′-ACACGGTCAAGGAGATCAAC-3′	20	513	58ºC	2
ElaR	5′-CGCTGGTGTTGGCCAGCAGG-3′	20			

Ref: (1) Martinez-Murcia et al., 1992; (2) Sen and Rodgers, 2004

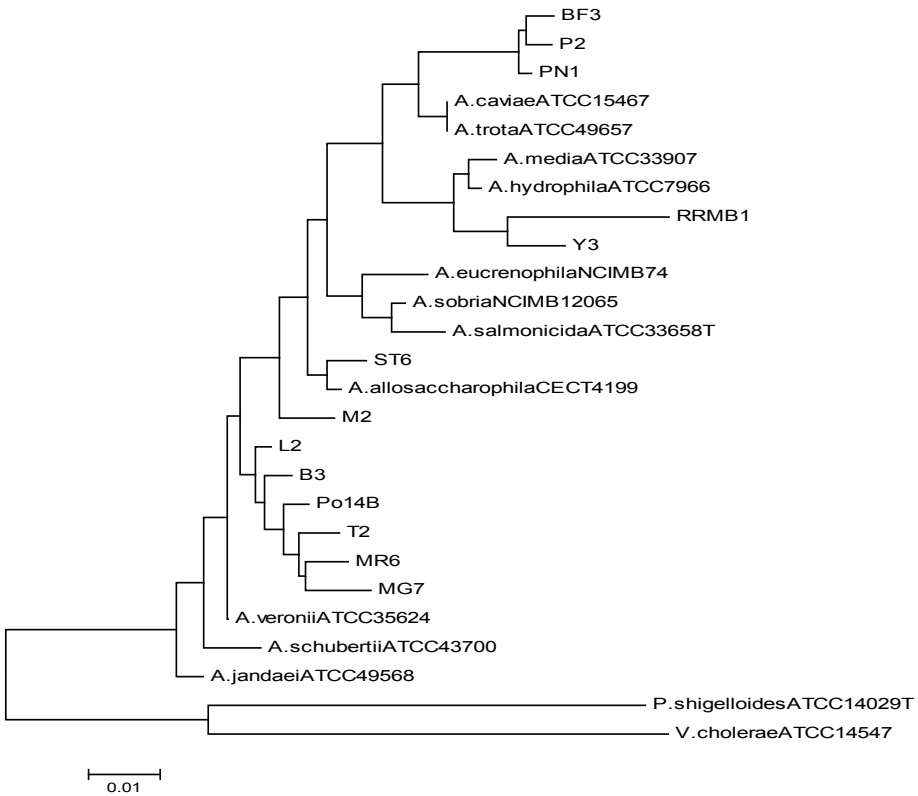

Fig. 1: The phylogenetic tree based on 16S rDNA fragment sequences, showing relationship of the genus *Aeromonas* (constructed by the neighbour-joining method using MEGA5 software); *Plesiomonas shigelloides* and *Vibrio cholerae* were used as outgroups, the bar represents 0.01 nucleotide changes per site.

Characterization of virulence factors

-Phenotypic determination

Purified *Aeromonas* isolates were streaked on different media/substrates for determination of extracellular enzymes produced by *Aeromonas* spp. These tests included hemolytic activity (Santos *et al*., 2008); casein hydrolysis (Koneman *et al*., 2006); gelatinase activity (Isenberg and Garcia, 2004); lipase activity (Anguita *et al*., 1993); DNase activity (Huys *et al*., 1997) and lecithinase activity (Koneman *et al*., 2006). Siderophore test was carried out to detect siderophore production (Barghouthi *et al*., 1989).

-Genetic detection of virulence genes

The presence of genes encoding for extacellular lipase (*pla/lip/lipH3/alp-1*) and elastase (*ahyB*) was determined by PCR using primers (Tab. 2) and conditions already published (Sen and Rodgers, 2004). In brief, 25 µl of the reaction mix contained 2.5 µl of 10X buffer, 1.5 µl of 25mM $MgCl_2$, 0.5 µl of 10mM dNTP, 1U of *Taq* polymerase, 10 pmol/µl of each primer, 0.5 µl of the template and 18.75 µl of sterile water. Same PCR conditions were used for both the genes and consisted of an initial denaturation at 95°C for 5 min, followed by 25 cycles of melting at 95°C for 25 sec, annealing at 58°C for 30 sec, elongation at 72°C for 1 min and a final single cycle at 70°C for 5 min. The PCR products were detected by subjecting the samples to 1% agarose gel electrophoresis, stained with ethidium bromide and photographed.

Results

A total of 50 isolates exhibiting typical aeromonad morphological properties such as Gram-negative, rod shaped, motile bacteria, which were oxidase positive and resistant to vibriostatic agent O/129 (150 µg), were isolated from 50 samples. The results of the biochemical tests are given in Tab. 3. The isolates were categorized in four different species using Aerokey II. From the biochemical tests, the most prevalent species in the present study were *A. veronii* (n=20) exhibiting two biovars, namely bv. *sobria* (n=9) and bv. *veronii* (n=11), *A. caviae* (n=19), *A. hydrophila* (n=9) and *A. trota* (n=2).

All the biochemically identified *Aeromonas* spp. were also analysed by RFLP of PCR amplified 16S rRNA gene, with selected endonucleases *AluI* and *MboI*. The resulting fragments ranging from 69 to 346 bp were taken into account for identification of *Aeromonas* spp. These RFLP patterns of *Aeromonas* spp. are presented in Fig. 2. Though most of the results were in agreement with biochemical tests but some discrepancies were observed. Four additional species viz., *A. sobria, A. media*, *A. allosaccharophila* and *A. schubertii* were identified on the basis of restriction

Tab. 3: Biochemical profile of *Aeromonas* species isolated from water and fish samples.

Tests	A1	*A2*	A3	A4	A5
Motility	100	100	100	100	100
Oxidase	100	100	100	100	100
Catalase	100	100	100	100	100
Oxidation/Fermentation	100	100	100	100	100
Glucose(gas)	100	0	89	82	50
Lysine decarboxylase	100	0	89	100	100
Ornithine decarboxylase	0	0	0	100	0
Arginine decarboxylase	100	89	100	0	50
Esculin hydrolysis	100	100	0	100	0
Indole	100	100	100	100	100
Nitrate reduction	100	100	100	100	100
Citrate	89	84	67	91	50
Urease	0	21	0	0	0
Methyl red	100	100	100	100	100
Voges-proskauer	89	63	78	73	0
0% NaCl	100	100	100	100	100
6% NaCl	0	0	0	0	0
Acid from					
Glucose	100	100	100	100	100
Sucrose	100	79	100	100	50
Arabinose	78	100	22	18	0
Resistance to					
Cephalothin	100	100	33	0	50
O/129(150µg)	100	100	100	100	100

A1: *A. hydrophila* (n=9); **A2:** *A. caviae* (n=19); **A3:** *A. veronii* bv. *sobria* (n=9); **A4:** *A. veronii* bv. *veronii* (n=11); **A5:** *A. trota* (n=2)
-Values in the table are indicating the percentage of isolates showing positive reaction

patterns (Tab. 4). Four presumed *A. caviae* isolates were classified as *A. trota* by RFLP analysis. Two *A. sobria* strains, one *A. media* strain, single strain of *A. allosaccharophila* and a strain of *A. schubertii*, as shown by the 16S rRNA gene restriction patterns were biochemically characterized as *A. veronii* bv. *veronii* and *A. veronii* bv. *sobria* strains, respectively. Similarly, two strains that were identified biochemically as *A. trota*, were classified by RFLP analysis as *A. caviae*. The dendrogram based on RFLP patterns of the representative isolates (Fig. 3) depicts the categorization and relatedness between *Aeromonas* spp. Dendrogram depicts major groups; strains RRMB1, J1, K1, N1, CB2, RU1, Y3, X6 and CK1 belong to *A. hydrophila*, strains T2, CLR, M2, GAR, CHTL1, D1, B3, $Po14_B$, Cat, CC2, RB1, 11SY4, C4, MR6 and MG7 belong to *A. veronii*, strains CA1, P2, CH1, SAI1, KEN1, KG2, BF3, NYR1, PN1, GMTI1, CG2, GB1, HF1, CL1, SG1, AH1 and HF2 belong to *A. caviae* and strains MLD, $Po6_B$, R1 and CT1 belong to *A. trota*. In addition, strain TT2 and RR1 belong to *A. sobria*, strain L2 belongs to *A. schubertii* and strain ST6 and E1 belong to *A. allosaccharophila* and *A. media*, respectively.

Thirteen strains identified by PCR-RFLP analysis were sequenced using primer 16S rRNA F1. Species identification of the 13 strains and the similarity values with the type strains are shown in Tab. 5. The identification by sequence analysis corresponded to the identification by PCR-RFLP analysis. The sequenced regions of 13 strains were identical to the regions in the 16S rDNA gene of their type strains. The sequences of three isolates i.e. M2, L2 and ST6 identified as *A. veronii*, *A. schubertii* and *A. allosaccharophila* differed from the type strain (*A. veronii* strain ATCC 35624, *A. schubertii*

strain ATCC 43700, *A. allosaccharophila* strain CECT 4199) by 1, 5 and 3 bases, respectively. However, each sequence was closely related to the respective type strains (Fig. 1) and showed the highest similarity value in the available *Aeromonas* sequences of the GenBank.

Tab. 4: Comparison of the biochemical and genetic identification (16S rDNA-RFLP) of *Aeromonas* species from water and fish samples.

	Biochemical identification					
Genetic identification	*A. hydrophila* (n=9)	*A. caviae* (n=19)	*A. veronii* bv. *sobria* (n=9)	*A. veronii* bv. *veronii* (n=11)	*A. trota* (n=2)	*A. schubertii* (n=0)
A. hydrophila (n=9)	9					
A. caviae (n=17)		15			2	
A. veronii (n=15)			8	7		
A. trota (n=4)		4				
A. schubertii (n=1)			1			
A. sobria (n=2)				2		
A. media (n=1)				1		
A. allosaccharophila (n=1)				1		

Results of the phenotypic activity of the selected enzymes encoding putative virulence factors and the detection of lipase and elastase genes by PCR are shown in Tab. 6. All the *A. hydrophila* and *A. veronii* strains displayed hemolytic activity, while only 65% of *A. caviae* and 50% of *A. trota* strains were positive for hemolytic activity. Protease activity was evaluated with caseinase and gelatin liquefaction tests. Both the tests were positive in all the strains and therefore, seemed to be useful for demonstrating protease activity. All the isolated *Aeromonas* strains also showed lipase activity. All the strains of *A. hydrophila*, *A. caviae* and *A. trota* were DNase positive, while only 67% of *A. veronii* strains were positive for this enzyme. Lecithinase activity was observed in 100% of *A. veronii*,

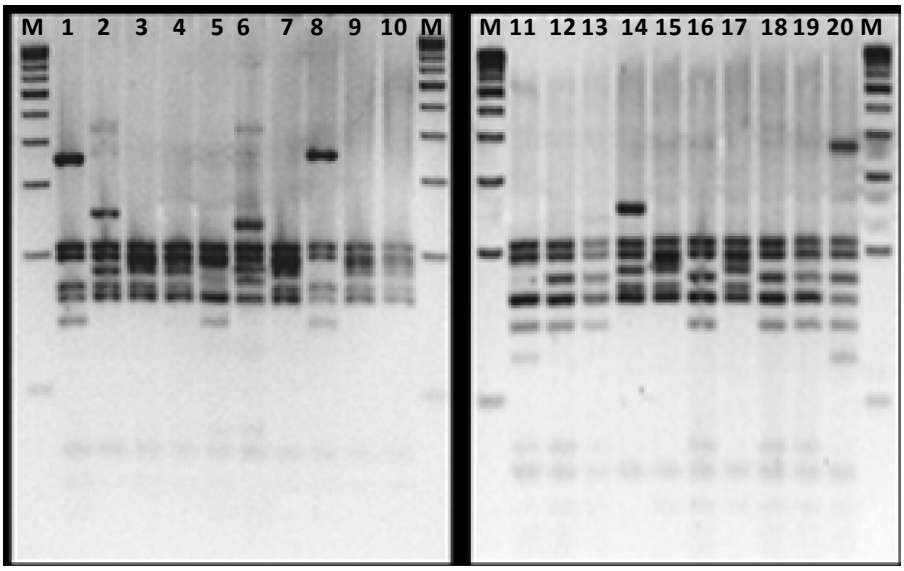

Fig. 2: Agarose gel showing the RFLP patterns of isolated *Aeromonas* species obtained by digestion of the 16S rRNA amplified PCR product with *AluI* and *MboI* restriction enzymes. Lane M: Ladder 100bp, Lane 1 and 8: *A. hydrophila*, Lane 2 and 14: *A. trota*, Lane 3, 4, 7, 9, 10, 15 and 17: *A. caviae*, Lane 5, 12, 13, 16, 18 and 19: *A. veronii*, Lane 20: *A. allosaccharophila*, Lane 6: *A. schubertii*, Lane 11: *A. sobria*.

Tab. 5: Sequencing results of *Aeromonas* species (n=13) identified by PCR-RFLP analysis.

Isolates	Identification by PCR-RFLP	Identification by sequencing	Accession no. of type strain used	Similarity
RRMB1	*A. hydrophila*	*A. hydrophila*	X60404	1.000
T2	*A. veronii*	*A. veronii*	X60414	1.000
P2	*A. caviae*	*A. caviae*	X60409	1.000
M2	*A. veronii*	*A. veronii*	X60414	0.998
L2	*A. schubertii*	*A. schubertii*	X60416	0.990
BF3	*A. caviae*	*A. caviae*	X60409	1.000
B3	*A. veronii*	*A. veronii*	X60414	1.000
$Po14_B$	*A. veronii*	*A. veronii*	X60414	1.000
ST6	*A. allosaccharophila*	*A. allosaccharophila*	S39232	0.995
PN1	*A. caviae*	*A. caviae*	X60409	1.000
Y3	*A. hydrophila*	*A. hydrophila*	X60404	1.000
MR6	*A. veronii*	*A. veronii*	X60414	1.000
MG7	*A. veronii*	*A. veronii*	X60414	1.000

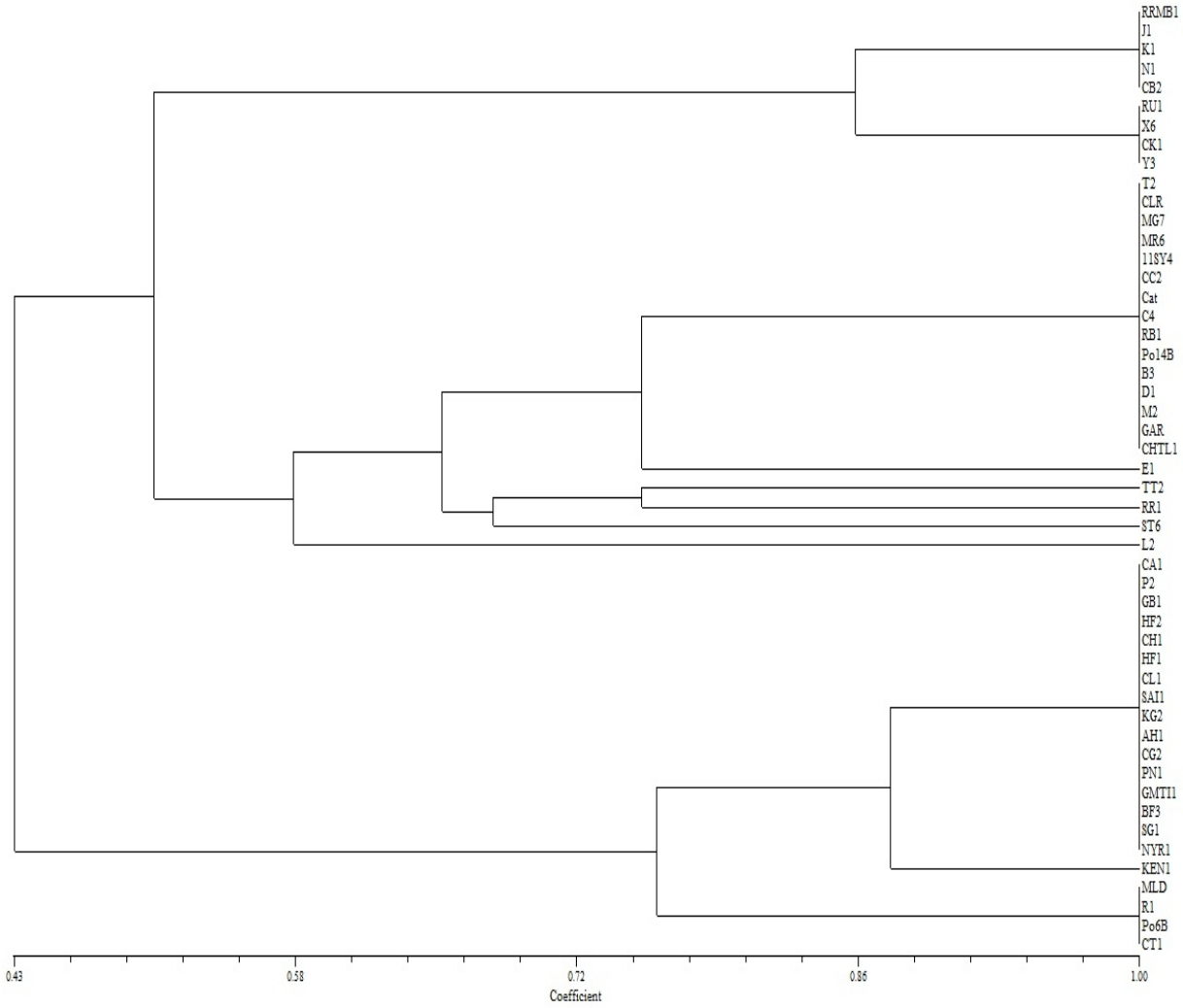

Fig. 3: The dendrogram of *Aeromonas* strains based on RFLP patterns using *AluI* and *MboI* restriction enzymes (NTSYS version 2.0). Coefficient in the dendrogram represents percentage of similarity between *Aeromonas* species

56% of *A. hydrophila*, 82% of *A. caviae* and 50% of *A. trota* strains, respectively. Similarly, siderophore activity was detected in all the strains of *A. hydrophila* and *A. veronii* while only 53% and 25% of *A. caviae* and *A. trota* strains, respectively were positive for siderophore test.

Genotypically, results for elastase (*ahyB*) and lipase (*pla/lip/lipH3/alp-1*) genes are given in Tab. 6. The primers ElaF and ElaR gave an amplification of 513 bp in 33% of the *A. hydrophila*, 71% of *A. caviae* and 80% of *A. veronii* strains (Fig. 4a), but amplification was not observed in *A. trota*, *A. schubertii*, *A. media* and *A. sobria* strains. Similarly, a 382 bp product was amplified using LipF and LipR primer set in 66% of *A. hydrophila*, 76% of *A.*

caviae, 60% of *A. veronii* and 25% of *A. trota* strains whereas, no amplification was observed in *A. schubertii* and *A. media* strains (Fig. 4b).

Discussion

All the *Aeromonas* isolates were identified up to species level using Aerokey II (Carnahan *et al*., 1991) which is based on esculin hydrolysis, gas from glucose, acid from arabinose and sucrose, indole production, Voges-Proskauer reaction and resistance to cephalothin (30 µg). On the basis of biochemical tests, the most prevalent species in the present study was identified as *A. veronii* followed by *A. caviae*, *A. hydrophila* and *A. trota*. In previous studies, *A. hydrophila* and *A. sobria* were the most frequently isolated species from apparently healthy fishes (Santos *et al*., 1988) whereas, Minana-Galbis *et al*. (2002) reported that the predominant species in freshwater was *A. bestiarum* (74.3%), followed by *A. caviae* (18.8%) and *A. salmonicida* (5%). Similarly, Rathore *et al*. (2005) reported a high prevalence of *A. hydrophila* followed by *A. sobria*, *A. veronii*, *A. schubertii* and *A. caviae* from water and fish samples. The reasons for the disagreement could be that most studies on fish rely on biochemical tests which are not as accurate as genetic identification systems (Borrell *et al*., 1997; Figueras *et al*., 2000).

Tab. 6: Phenotypic expression of virulence factors and incidence of virulence genes in *Aeromonas* species isolated from fish and water samples.

Identification 16S rDNA-RFLP	Hemolysin test	Lipase test	Protease test	Gelatinase test	Lecithinase test	Nuclease test	Siderophore test	Lipase gene	Elastase gene
A. hydrophila (n=9)	100	100	100	100	56	100	100	66	33
A. caviae (n=17)	65	100	100	100	82	100	53	76	71
A. veronii (n=15)	100	100	100	100	100	67	100	60	80
A. trota (n=4)	50	100	100	100	50	100	25	25	0
A. schubertii (n=1)	0	100	100	100	100	0	100	0	0
A. media (n=1)	0	100	100	100	100	0	100	0	0
A. sobria (n=2)	0	100	100	100	50	100	50	50	0
A. allosaccharophila (n=1)	100	100	100	100	0	100	100	0	100

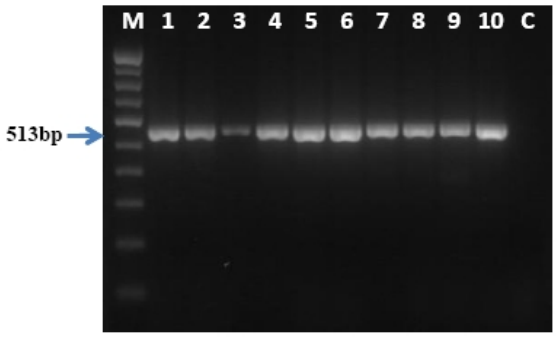

Fig. 4a: Amplification of the 513 bp region of the elastase gene. Lane M: 100 bp DNA marker, Lanes 1-10: elastase genes amplified from *Aeromonas* strains, Lane C: negative control.

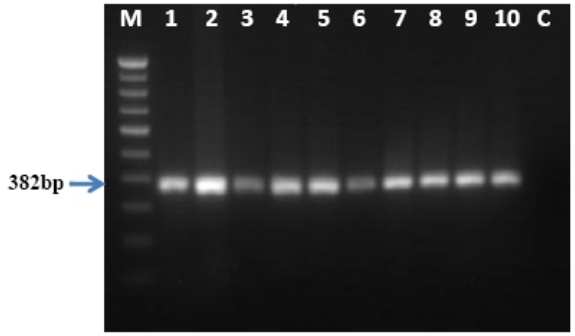

Fig. 4b: Amplification of the 382 bp region of the lipase gene, Lane M: 100 bp DNA marker, Lanes 1-10: lipase gene amplified from *Aeromonas* strains, Lane C: negative control.

16S rDNA RFLP is considered to be a simple and reliable method for discrimination of *Aeromonas* spp. without the need of sequencing and has been used to study the distribution of *Aeromonas* spp. in fish farms (Lee *et al*., 2002). Borrell *et al*. (1997) developed a method for identification of all known *Aeromonas* species on the basis of restriction patterns of the PCR-amplified 16S rRNA gene, using *AluI-MboI* restriction enzymes. These enzymes produce species-specific profile for *A. hydrophila*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. jandaei*, *A. trota, A. sobria, A. veronii* bv. *sobria, A. allosaccharophila* and *A. schubertii*. In the present study, the RFLP analysis of the biochemically identified *Aeromonas* species with a combination of *AluI-MboI* endonucleases showed the characteristic pattern as reported earlier (Borrell *et al*., 1997). However, there were some discrepancies in identification results from biochemical tests and those obtained by genetic methods. In PCR-RFLP, restriction profile of four species i.e. *A. sobria*, *A. media*, *A. allosaccharophila* and *A. schubertii* was observed in addition to *A. veronii, A. caviae, A. hydrophila* and *A. trota.* In the present study, *A. veronii* isolates were grouped in two biovars namely biovar *sobria* and biovar *veronii* on the basis of biochemical tests (Carnahan *et al*., 1991). However, the restriction profile for the two *A. veronii* biovars was found to be similar, in accordance with earlier reports (Popoff *et al*., 1981; Borrell *et al*., 1997). Such discrepancies in identification results between biochemical and molecular methods have been reported earlier as well (Borrell *et al*., 1997; Castro-Escarpulli *et al*., 2003).

The 16S rDNA sequences of *Aeromonas* have proven to be valuable in the identification

of *Aeromonas* spp. (Demarta *et al*., 1999). The sequence similarity between *Aeromonas* spp. is very high (ca 98-100%), but there is enough variability to discriminate among species. In the present study, the amplified 16S rDNA gene of representative isolates of *Aeromonas* identified by PCR-RFLP analysis was also sequenced using 16S rRNA F1 primer. The sequencing results for the 13 strains validated the results of PCR-RFLP analysis for *Aeromonas* identification. Ten strains had the same 16S rDNA sequences as their reference strains, and only 3 strains identified by PCR-RFLP analysis as *A. veronii*, *A. schubertii* and *A. allosaccharophila* had one, five and three bases difference, respectively. The above differences did not affect the identification by 16S rDNA sequencing and PCR-RFLP analysis.

It is widely accepted that the pathogenesis of *Aeromonas* infections is multi-factorial and it is virtually impossible to establish a hierarchical classification of virulence factors according to their role in the disease-causing process. In addition, no single virulence factor or defined combinations of these factors has been unequivocally correlated to virulence in the different *Aeromonas* species. Therefore, there is a need to continuously survey the presence of several accepted virulence factors in *Aeromonas* isolates for better understanding of pathogenesis and epidemiology of *Aeromonas* infections (Sen and Rodgers, 2004). In the present study, *Aeromonas* spp. produced putative virulence factors i.e. hemolysins, lipases, proteases, DNases as well as for siderophores in varying proportion and this reflects the ability of these isolates to cause disease in fishes and humans. All the *A. hydrophila* and *A. veronii* strains produced hemolysins. The hemolytic activity has been reported in *Aeromonas* spp. in a number of earlier studies (Kingombe *et al*., 1999; Santos *et al*., 2008) and regarded as a strong evidence of pathogenic potential in aeromonads. All the *Aeromonas* spp. isolated from water and fish samples showed lipase activity. Lipolytic and/or phospholipolytic genetic determinants are widely present in different *Aeromonas* spp. (Sen and Rodgers, 2004). The lipases are considered important for bacterial nutrition (Pemberton *et al*., 1997) and they also have a role in virulence since insertion mutants for the lipase gene *plc* reduces the lethal dose (LD_{50}) in mice and fish (Merino *et al*., 1999). It has been reported that lipases play an important role in invasiveness and establishment of infections, while secreted phospholipases act as both hemolysins and glycerophospholipid cholesterol acyl-transferases (Scoaris *et al*., 2008). Similarly, all the *A. veronii*, *A. schubertii* and *A. media* strains showed lecithinase activity while only 56% of *A. hydrophila*, 82% of *A. caviae* and 50% of *A. trota* and *A. sobria* strains, respectively were positive for this enzyme.

In this study, protease activity was determined with caseinase and gelatinase test,

and all the tested strains were positive for these tests. Castro-Escarpulli *et al*. (2003) recommended the use of gelatinase for evaluating protease activity and reported that caseinase test seemed to be the least useful for evaluating protease activity. It has been reported that proteases produced by *Aeromonas* cause tissue damage, aid invasiveness and the establishment of infection by overcoming host defences and provide nutrients for cell proliferation (Leung and Stevenson, 1988). These proteases are also considered to be important factors in the spoilage of foods, and the presence of proteases and hemolysins is used as an indicator of potential pathogenicity (McMahon, 2000). Austin and Adams (1996) correlated the proteolytic activity of *A. hydrophila* with its ability to induce pathology in fishes. The role of DNases is unknown in *Aeromonas* pathogenicity but is considered important for bacterial nutrition (Pemberton *et al*., 1997). Extracellular nucleases are reported to be important virulence factors for establishing and maintaining the infection, such as *Streptomyces* infection (Podbielski *et al*., 1996). All the strains of *A. hydrophila*, *A. caviae* and *A. trota* produced DNase enzyme, while only 67% of *A. veronii* strains were positive for this enzyme. Siderophore production was reported in all the strains of *A. hydrophila, A. veronii, A. schubertii*, while *A. caviae* and *A. trota* showed 53% and 25% siderophore production, respectively. This virulence factor has the ability to scavenge required nutrients such as iron. Many pathogenic bacteria utilize iron uptake pathways, such as the production of siderophores, to access iron for growth and subsequent production of other virulence factors. Earlier studies have reported that the ability to produce siderophores correlated with higher virulence in *Aeromonas* species (Lee *et al*., 2000). In this study, the isolated *Aeromonas* spp. were capable of producing wide array of enzymes which have been correlated with bacterial virulence and therefore is indicative of potential of these isolates to cause disease in fishes as well as humans.

The lipase gene was amplified in varying proportion of isolated *Aeromonas* spp. though the lipase activity was detected phenotypically in all the isolates. Similarly, elastase gene was also amplified in varying number of *Aeromonas* spp. This implies that there may be other genes coding elastase and lipase which were not targeted by the primers used in the present study. Genes encoding lipase and elastase are commonly found in *Aeromonas* spp. and were amplified in 88% of *Aeromonas* isolates (Sen and Rodgers, 2004). Song *et al*. (2004) demonstrated the potential of association of elastase with the damage caused by aerolysin in cell cultures. It has also been reported that the capacity of extracellular enzymes to cause lysis to feed the bacterial cells is very important for *Aeromonas* spp. (Cascon *et al*., 2000).

The present study highlights that water and fish samples are potential sources of motile aeromonads with virulence potential and can cause disease in fish and humans whenever, the conditions are favourable.

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