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ORIGINAL ARTICLE

Detection of aerolysin gene in *Aeromonas hydrophila* isolated from fish and pond water

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Abstract Aerolysin is a hemolytic toxin encoded by aerolysin gene (1482 bp) that plays a key role in the pathogenesis of *Aeromonas hydrophila* infection in fish. New species-specific primers were designed to amplify 326 bp conserved region of aerolysin gene for *A. hydrophila*. Twenty-five isolates of *A. hydrophila* recovered from fish and pond water were studied for detection of aerolysin gene. Aerolysin gene was detected in 85% of the isolates during the study. The designed primers were highly specific and showed no cross reactivity with *Escherichia coli, Aeromonas veronii, Vibrio cholerae, Flavobacterium* spp., *Chyseobacterium* spp. and *Staphylococcus aureus*. The sensitivity limit of primers for detection of aerolysin gene in the genomic DNA of *A. hydrophila* was 5 pg.

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Introduction

Aerolysin is a hemolytic toxin protein secreted by *Aeromonas hydrophila.* There are two precursor forms of the toxin. It crosses the inner bacterial membrane as preprotoxin containing signal peptide sequences, which is removed from the N-terminus and activated by proteolytic removal of nearly 25 amino acid from the C-terminus. The active aerolysin protein binds to the specific glycophosphatidylinositol (GPI)-anchored proteins on the target surface of eukaryotic red blood cells and forms pores in the cell membrane causing lysis [1, 2]. Aerolysin is significant and a stable molecular marker to detect the possible virulent *A. hydrophila*.

A. hydrophila is an opportunistic and zoonatically important bacterial fish pathogen belonging to Aeromonadaceae family [3] and is associated with several diseases of fish, such as hemorrhagic septicemia and fin and tail rot [4]. Conventional identification of *A. hydrophila* is achieved through standard biochemical tests that are time consuming, laborious and are not always conclusive. In India, motile aeromonads have been isolated and characterized from aquatic environment [5]. In another study, a total of 36 isolates of *A. hydrophila* were recovered from fish and water samples. All isolates were tested for antibiotic sensitivity and characterized [6].

A. hydrophila secretes many virulence factors such as proteases, elastase, lecithinase, amylase, lipase, gelatinase, chitinase [7, 8] cytotoxic enterotoxins [9], aerolysin

(β-hemolysin) and α-hemolysin [10]. These virulence factors contribute to its pathogenicity and provide the ability to attach to host cells in the development of diseases. These factors can be used for detection and characterization of the bacteria. Polymerase chain reaction (PCR) is a sensitive, specific and rapid molecular tool for the detection of bacteria. PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material usually template genomic DNA. A number of reports are available for PCR amplification of conserved aerolysin gene [11–13] and hemolysin gene of *A. hydrophila* [13–15]. Other virulence genes of *A. hydrophila* have also been detected by PCR. Lipase gene is reported to be present consistently in *A. hydrophila* isolates recovered from human and environmental samples [16]. Similarly, all fish and water isolates of *A. hydrophila* were also positive for lipase gene [17]. The present report describes the development of new species-specific PCR primers for amplification of pore-forming aerolysin gene in fish isolates of *A. hydrophila*. PCR-based techniques will be useful for early detection and control of possibly virulent *A. hydrophila* spreading in new regions and provide support for health management of fish and other animals.

Materials and methods

Bacterial isolates and genomic DNA isolation

All the isolates of *A. hydrophila* were recovered from fish muscle and have been previously characterized by biochemical tests [5] and on the basis of hemolysin gene amplification [14]. Isolates of *A. hydrophila* were tested for β-hemolytic activity on nutrient agar base (HiMedia) supplemented with 5% rabbit erythrocytes with incubation at 37°C for 24 h. The bacterial cultures were revived from –80°C for the isolation of genomic DNA. Genomic DNA of *A. hydrophila* was isolated by method described earlier [18]. Similarly, genomic DNA of other aquatic bacteria viz. *A. veronii, Escherichia coli, Vibrio cholerae, Flavobacterium* spp., *Chyseobacterium* spp. and *Staphylococcus aureus* were also isolated.

Strategies for designing specific primers for amplification of aerolysin gene

A. hydrophila aerolysin gene sequences were collected from National Center for Biotechnology Information (NCBI) with different accession no. AF485766, AF485767, AF485769, M84709, M16495, DQ186611, AF410466 and AY136943. These nucleotide sequences were aligned by ClustalX 1.83 software and the open reading frame of aerolysin gene was selected. Primers were designed with Oligo 4.0 software and synthesized from Integrated DNA Technology (IDT, USA). The highly conserved region (nucleotide 463–788 bp) was selected for designing specific primers (Table 1). The designed primers were then simultaneously compared to the other sequences in the GenBank database to verify their identity and similarity to *A. hydrophila* aerolysin gene and with those of other bacterial species.

Table 1 Primers used for the detection of aerolysin gene in *Aeromonas hydrophila*

Primer	Position within gene	Sequences (5′-3′)	Product length (bp)
AHAF2 Forward	463-484	5-CACAGCCAATATGT-CGGTGAAG-3	326
AHAR2 Reverse	770-788	5-GTCACCTTCTCGCT-CAGGC-3	

PCR conditions and amplification

The PCR reaction mixture (50 µl) consisted of 10 ng of bacterial genomic DNA, 1.5 units of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl. pH 8.3), 200 µM deoxynucleotide triphosphate (dNTP) and 5 pmoles of each primer. Amplification included initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing of primers at 52°C for 30 s and extension at 72°C for 30 s. A final extension at 72^0C for 10 min was used. Ten µl of the reaction mixture was then analyzed by submarine gel electrophoresis in 1.2% agarose with ethidium bromide run at 8 V/cm. The PCR products were visualized under gel documentation system.

Sensitivity and specificity of primers

Genomic DNA (50 ng/µl) of *A. hydrophila* was diluted from 10^{-2} to 10^{-8} in ten-fold dilutions and amplification by PCR was done using the diluted DNA template with conditions as described above. The specificity of the aerolysin gene primers was checked by genomic DNA amplification of *E. coli, A. veronii, V. cholerae, Flavobacterium spp, Chyseobacterium spp.* and *S. aureus*.

Results and discussion

The sources of isolates of *A. hydrophila* used in this study are given in Table 2. Many of the sampled fish showed signs of hemorrhage on the body surface, while some

Table 2 Relation of hemolytic activity on blood agar and aerolysin gene in isolates of *Aeromonas hydrophila*

Isolates name	Sources of isolates	Location	Aerolysin production	PCR product 326 bp
A. hydrophila MTCC 1739 strain	IMTECH repository	IMTECH	–	–
A. hydrophila TK4	Water sample	NBFGR, Quarantine Wet Lab	–	–
A. hydrophila S9-6	Diseased *Channa punctatus*	NBFGR, Quarantine Wet Lab	–	+
A. hydrophila S10-8	*Channa punctatus*	NBFGR, Quarantine Wet Lab	–	+
A. hydrophila S7-1	*Channa punctatus*	Telibagh fish market, Lucknow	–	+
A. hydrophila PN3-4	Water sample	NBFGR Pond no. 3	+	+
A. hydrophila GFG 5	Gold fish gill	Alambagh Aquarium, Lucknow	–	+
A. hydrophila PN3-5	Water sample	NBFGR Pond no. 3	+	+
A. hydrophila TK5	Water sample	NBFGR, Quarantine Wet Lab	–	–
A. hydrophila S10-1	*Channa punctatus*	NBFGR, Quarantine Wet Lab	+	+
A. hydrophila LR3	*Labeo rohita*	Telibagh fish market, Lucknow	+	+
A. hydrophila AH11	Diseased *Channa punctatus*	Kalli fish market, Lucknow	+	+
A. hydrophila AH12	Diseased *Channa punctatus*	Nimoha, Lucknow	+	+
A. hydrophila AH13	Diseased *Channa punctatus*	Nimoha, Lucknow	+	+
A. hydrophila AH14	Diseased *Channa punctatus*	Nimoha, Lucknow	+	+
A. hydrophila AH15	*Channa punctatus*	Nimoha, Lucknow	+	+
A. hydrophila AH16	*Channa punctatus*	Nimoha, Lucknow	+	+
A. hydrophila AH17	*Channa punctatus*	Nimoha, Lucknow	+	+
A. hydrophila AH18	*Labeo rohita*	Karora, Lucknow	–	+
A. hydrophila AH19	*Labeo rohita*	Pachhu Parau, Lucknow	+	+
A. hydrophila AH20	Diseased *Channa punctatus*	Pachhu Parau, Lucknow	–	+
A. hydrophila AH21	Diseased *Channa punctatus*	Pachhu Parau, Lucknow	–	–
A. hydrophila AH22	Diseased *Labeo rohita*	Mohaan, Lucknow	+	+
A. hydrophila AH23	Diseased *Labeo rohita*	Mohaan, Lucknow	+	+
A. hydrophila AH24	Diseased *Labeo rohita*	Mohaan, Lucknow	+	+
A. hydrophila AH25	Diseased *Labeo rohita*	Mohaan, Lucknow	–	+

IMTECH: Institute of Microbial Technology, NBFGR: National Bureau of Fish Genetic Resources

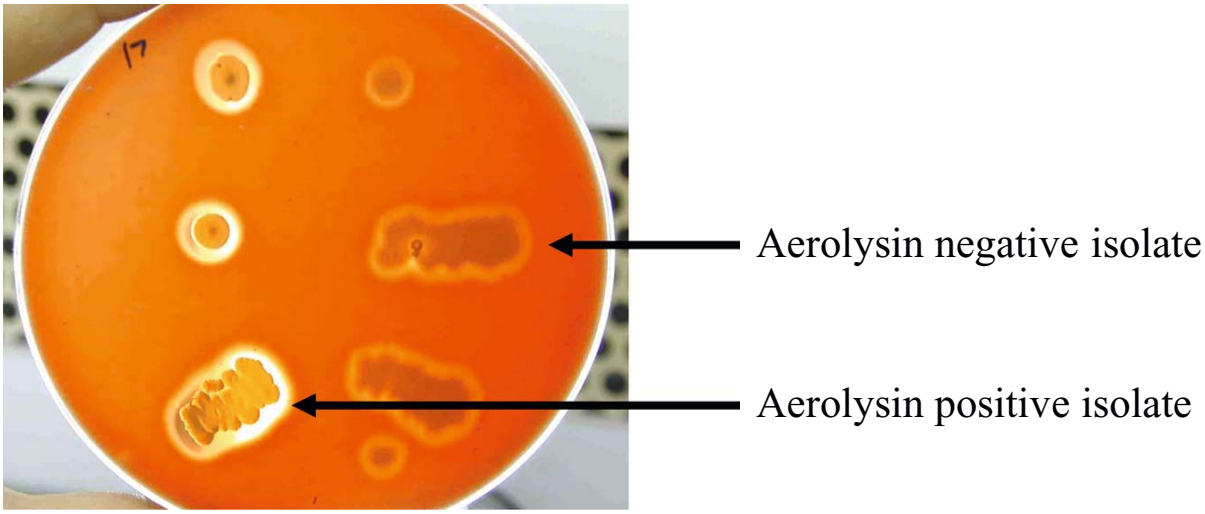

Fig. 1. *In vitro* demonstration of aerolysin activity of *Aeromonas hydrophila* on 5% rabbit blood agar. Clear zone indicates the positive production of aerolysin and translucent zone indicates negative aerolysin producing *A. hydrophila* isolates.

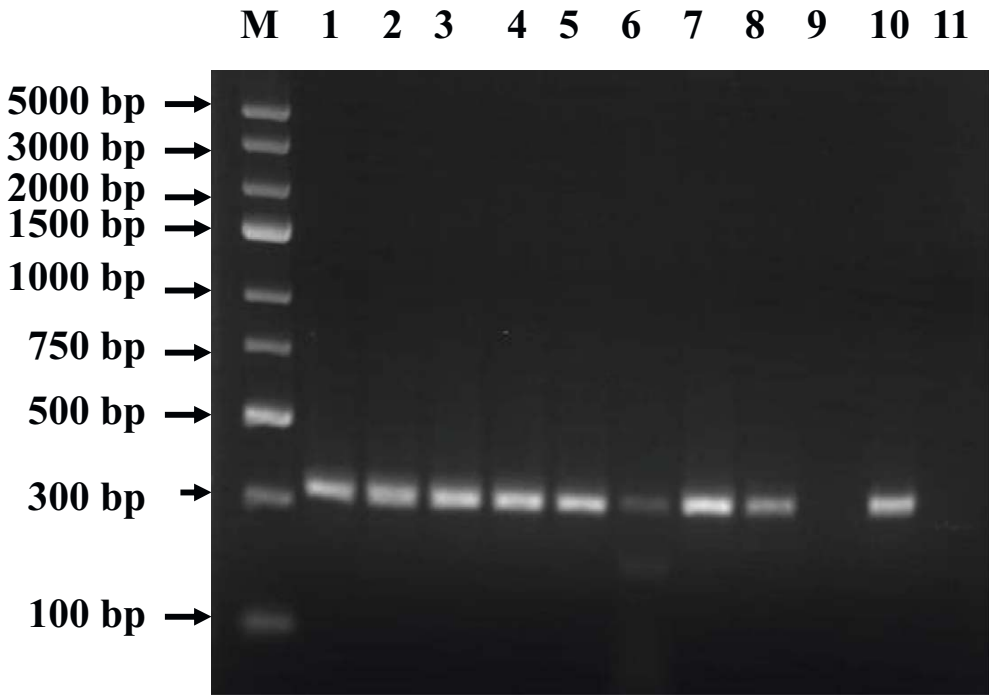

Fig. 2. PCR amplification of 326-bp aerolysin gene of different *A. hydrophila* isolates on 1.2% agarose gel. Lane M: Express DNA 100-bp ladder (Fermentas), Lane 1: AH14, Lane 2: AH15, Lane 3: AH16, Lane 4: AH17, Lane 5: AH20, Lane 6: AH23, Lane 7: AH24, Lane 8: AH25, Lane 9: AH MTCC 1739, Lane 10: AH GFG5 and Lane 11: without template (negative control).

fish appeared apparently healthy, but still tested positive for *A. hydrophila.* It is known that pathogenic isolates of *A. hydrophila* secrete aerolysin toxin that causes the lysis of the RBCs and results in hemorrhagic signs on the skin and internal organs of fish. In present study, the hemolytic activity of aerolysin toxin was observed on blood agar medium (Fig. 1). Our results showed that out of the 25 isolates, only 15 were positive for aerolysin production on blood agar. In support of the phenotypic characterization of aerolysin toxin, the detection of aerolysin gene of *A. hydrophila* was done by PCR.

A pair of specific primers targeting a 326 bp conserved region of the aerolysin gene was used in PCR. A total of 25 isolates of *A. hydrophila* from fish were tested for detection of aerolysin gene (326 bp) by PCR at an optimal concentration of 1.5 mM $MgCl_2$ and primer annealing at 52°C (Fig. 2). Out of the 25 isolates, only 15 isolates were phenotypically positive for aerolysin on the blood agar medium. However, 22 isolates were positive for presence of 326 bp aerolysin gene by PCR amplification. It is possible that some of the isolates though positive for aerolysin gene did not cause hemolysis on blood agar either due to disruption of gene or due to mutation in gene [13]. Another interesting finding is that there appears a positive correlation between the phenotypic production of aerolysin toxin and the pathogenicity of *A. hydrophila*. The presence of aerolysin toxin was demonstrated in all the isolates recovered from hemorrhagic fish, whereas 60% of *A. hydrophila* isolates recovered from apparently normal fish showed the production of aerolysin toxin. Therefore it can be safely assumed that aerolysin plays a role in the bacterial pathogenicity along with other virulence factors.

Phenotypic detection of the aerolysin toxin has been reported from different species of *Aeromonas*, such as *A. hydrophila*, *A. caviae, A. sobria, and A. veronii*. A 209 bp fragment of the aerolysin gene was detected in PCR using specific primers in hemolytic strains of *A. hydrophila.* However, aerolysin gene was not detected in non-hemolytic *A. hydrophila* and even in hemolytic *A. caviae* and *A. sobria* [11]. PCR assay has also been used to detect the aerolysin gene in *A. hydrophila* and *A. sobria* strains isolated from drinking water, fish and foods. These strains were also characterized for the production of virulence factors such as hemolysin, protease and cytotoxin. In this study also, the primers used in the PCR targeted a 209 bp fragment of the aerolysin gene coding for β-hemolysin. The aerolysin gene was detected only in hemolytic *A. hydrophila* strains. The hemolytic *A. sobria* and non-hemolytic *A. hydrophila* were consistently PCR negative [12]. Detection of hemolysin gene and aerolysin gene has been also done by multiplex PCR in Aeromonads. Out of 82 hemolytic isolates of *A. hydrophila*, hemolysin gene was present

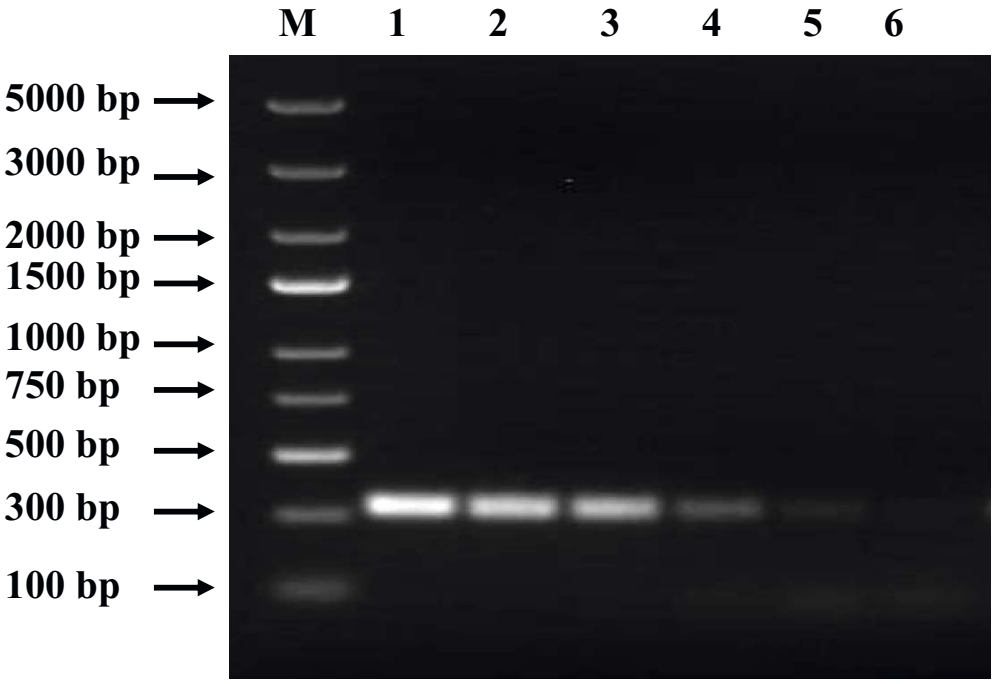

Fig. 3. PCR sensitivity for aerolysin gene amplification of genomic DNA of *A. hydrophila* AH14 isolate on 1.2% agarose gel. Lane M: Express DNA 100 bp ladder (Fermentas), Lane 1: 50 ng, Lane 2: 5 ng, Lane 3: 0.5 ng, Lane 4: 50 pg, Lane 5: 5 pg and Lane 6: 0.5 pg.

in 35 isolates, whereas 46 isolates were positive for both hemolysin and aerolysin gene. In non-hemolytic *A. hydrophila* isolates (5 no.), three were positive for hemolysin and remaining two for both genes [13]. Recently, specific primers based on β-hemolysin gene (208 bp) have been designed for the detection of pathogenic isolates of *A. hydrophila* of fish from China [15].

Our primers were designed to specifically detect aerolysin gene of *A. hydrophila.* Comparison of these primers to the sequences in NCBI GenBank showed 100% sequence identity to aerolysin gene of *A. hydrophila* (AF485766, AF485767, AF485769, M84709, M16495, DQ186611, AF410466, AY136943 X65045, DQ302123, AF539467). No cross-reactivity of our species–specific primers was observed in NCBI-BLAST as well as in PCR amplification with other aquatic bacteria viz. *E. coli, A. veronii, V. cholerae, Flavobacterium spp, Chyseobacterium spp.* and *S. aureus.* This indicates that the designed primers were highly specific for detection of *A. hydrophila* by PCR.

Besides specificity, sensitivity of these primers was also checked by PCR. The amplification of aerolysin gene was observed when the template genomic DNA used ranged from 50 ng to 5 pg. The PCR product was obtained only between these concentrations. Above these concentrations, PCR product was not obtained. Therefore, the PCR sensitivity limit of specific primers for detection of aerolysin gene was calculated to be 5 pg of genomic DNA of *A. hydrophila* (Fig. 3). This sensitivity limit was better as compared to earlier reports of 2 ng for hemolysin gene [14] and 1 ng for aerolysin gene [11]. A sensitivity limit of 10 pg for detection of β-hemolysin gene using specific primers has also been reported previously [15]. Higher sensitivity of our primer may be due to its high GC content (56%), which creates strong hydrogen bonds during annealing of primers with the template in PCR. In conclusion, new specific and sensitive primers for detection of conserved region of aerolysin gene of *A. hydrophila* isolated from fish and water samples were developed.

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References

1. Howard SP, Garlnd WJ, Green JM and Buckley JT (1987) Nucleotide sequences of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. J Bacteriol 169: 2869–2871
2. Parker MW, Buckley JT, Postma JPM, Tucker AD, Leonard K, Pattus F and Tsernoglou D (1994) Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. Nature 367:292–295

3. Colwell RR, MacDonell MT and Deley J (1986) Proposal to recognize the family Aeromonadaceae fam. nov. Int J Syst Bacteriol 36:473–477
4. Austin B and Austin DA (1999) Bacterial fish pathogens: Diseases in farmed and wild fish. Praxis Publishing, Chichester, UK
5. Rathore G, Swaminathan TR, Abidi R, Mahanta PC and Kapoor D (2005) Isolation and characterization of motile Aeromonads from aquatic environment. Indian J Fish 52: 241–248
6. Rathore G, Singh V, Kumar G, Swaminathan TR and Mahanta PC (2006) Antibiotic sensitivity and characterization of *Aeromonas hydrophila* isolated from fish and water samples. J Ecophysiol Occup Hlth 6:41–43
7. Merino S, Rubires X, Knochel S and Tomas JM (1995) Emerging pathogens: *Aeromonas* spp. Int J Food Microbiol 28:157–168
8. Pemberton JM, Kidd SP and Schmidt R (1997) Secreted enzymes of *Aeromonas.* FEMS Microbiol Lett 152: 1–10
9. Chopra AK, Peterson CW and Jin GF (1993) Cloning, expression and sequence analysis of cytolytic enterotoxin gene in *Aeromonas hydrophila.* Can J Microbiol 39: 513–523
10. Howard SP and Buckley JT (1985) Activation of the hole forming toxin aerolysin by extracellular processing. J Bacteriol 163:336–340
11. Pollard DR, Johnson WM, Lior H, Tyler SD and Rozee RK (1990) Detection of aerolysin gene in *Aeromonas hydrophila* by the polymerase chain reaction. J Clin Microbiol 28:2477–2481
12. Baloda SB, Krovacek K, Eriksson L, Linne T and Mansson I (1995) Detection of aerolysin gene in *Aeromonas* strains isolated from drinking water, fish and foods by the PCR. Comp Immunol Microbiol Infect Dis 18:17–26
13. Wang G, Clifford CG, Liu C, Pucknell C, Munro CK, Kruk TM, Caldeira R, Woodward DL and Rodgers FG (2003) Detection and characterization of a hemolysin gene in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. J Clin Microbiol 41:1048–1054
14. Singh V, Rathore G, Kumar G, Swaminathan TR, Sood N, Kapoor D and Mishra BN (2007) Detection of the hole forming toxin hemolysin gene of *Aeromonas hydrophila* isolates. Indian Vet J 84:900–902
15. Xia JA, Ma ZH, Rahman MH and Wu ZG (2004) PCR cloning and identification of the β-hemolysin gene of *Aeromonas hydrophila* from fresh water fishes in China. Aquaculture 229:45–53
16. Cascon A, Anguita J, Hernnz G, Sanchez M, Fernandez M and Naharro G (1996) Identification of *Aeromonas hydrophila* hybridization group I by PCR assays. Appl Environ Microbiol 62:1167–1170
17. Swaminathan TR, Rathore G, Abidi A and Kapoor D (2004) Detection of *Aeromonas hydrophila* by polymerase chain reaction. Indian J Fish 51(2):251–254
18. Hiney M, Dawson MT, Heery DM, Smith RP, Gannon F and Powell R (1992) DNA probe for *Aeromonas salmonicida*. Appl Environ Microbiol 58:1039–1042