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DETECTION OF THE HOLE FORMING TOXIN HEMOLYSIN GENE IN AEROMONAS HYDROPHILA ISOLATES

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Aeromonas hydrophila is a ubiquitous important bacterial pathogen, which is Gram-negative, motile and rod shaped. It has been associated with several diseases in fish. *A. hydrophila* secretes many virulence factors. These virulence can be used for detection and characterization of the bacteria. Hemolysin is a significant virulence factor of *A. hydrophila*. Isolation of *A. hydrophila* from aquatic environment has been reported by biochemical tests (Rathore *et al.*, 2005). Previously, the detection of *A. hydrophila* by amplification of hemolysin gene through PCR has been done (Xia *et al.*, 2004) and lipase H3 gene (Swaminathan *et al.*, 2004). The present report describes the development of new species - specific PCR primers for amplification of the hole forming hemolysin gene of *A. hydrophila* isolates.

Materials and Methods

Bacterial isolates and Genomic DNA isolation : All isolates of *A. hydrophila* was recovered from *Ophiocephalus striatus*. All isolates of *A. hydrophila* used in this study have been previously described (Rathore *et al.*, 2005). Bacterial genomic DNA of *A. hydrophila* was isolated as per described method of Hiney *et al.*, (1992).

Designing of specific primers, PCR conditions and amplification : *A. hydrophila* hemolysin gene sequence was taken from NCBI (Accession no. AY442273) and the nucleotide sequences were aligned (ClustalW). Primers were designed for hemolysin gene amplification with DNAstar software (Lasergene 6.0). The highly conserved region (nucleotide 1168 - 1364 bp), forward primer AHF13 (5' TGGCCTTCTACCTCAACGTC 3') and reverse primer AHR13 (5' ATCCGCACTATCTTGGCATC 3') that are specific to the hole forming hemolysin gene of *A. hydrophila* were synthesized commercially. The PCR reaction mixture (50 µl) consisted of 10 ng of genomic DNA, 2.5 units of Taq polymerase, 5µl of 10X PCR amplification buffer (100 mM Tris - HCl, 25 mM $MgCl_2$, 500 mM KCl pH-8.3), 200 μM dNTP and 10 pmoles of each primer. Amplification included initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 min., annealing of primers at 54.7°C for 1 min., and primer extension at 72°C for 1 min., A final extension at 72°C for 10 minutes was used. Ten µl of the reaction mixture was then analyzed by submarine gel electrophoresis in 1.4% agarose with ethidium bromide at 8V/cm and the reaction products were visualized under UV light.

Sensitivity of PCR and cross-

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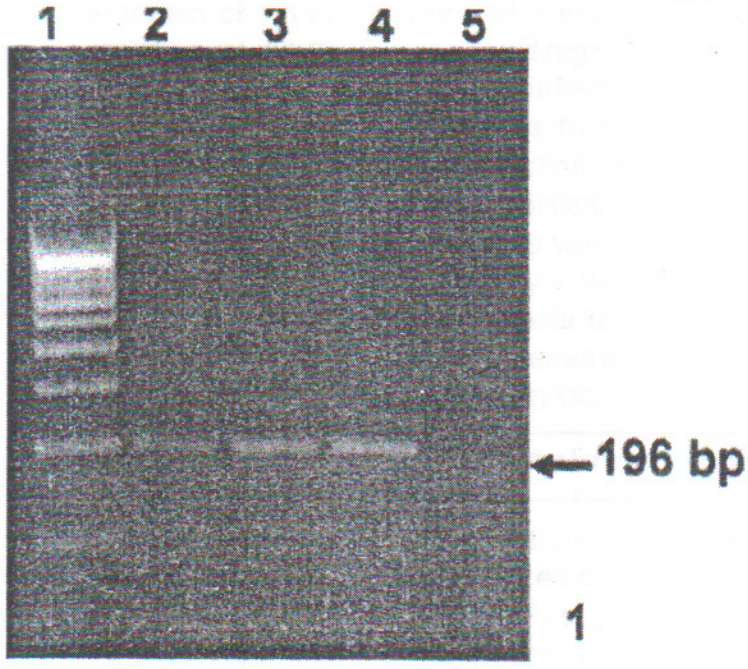

Fig. 1 : PCR amplification of 196 - bp hemolysin gene of *A. hydropila* : 1.4% Agarose gel electrophoresis. Lane 1 : 100 bp Marker Bangalore Genei, Lane 2 : Isolate No. AH 14, Lane 3 : Isolate No. AHTK5, Lane 4 : Isolate No. AH25, Lane 5 : blank

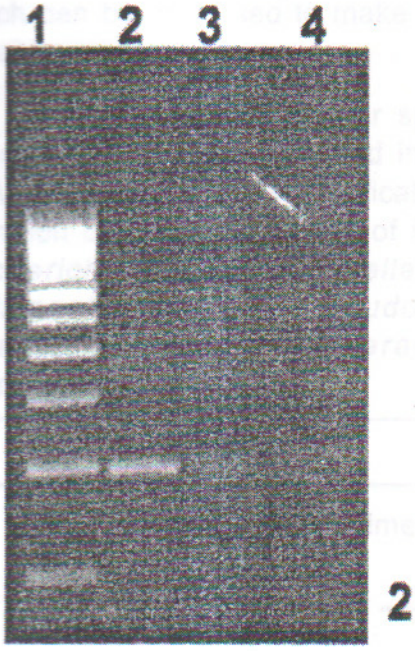

Fig. 1 : Sensitivity of PCR amplification for hemolysin gene of *A. hydrophila;* 1.4% Agarose gel Electrophoresis. Lane 1 : 100 bp Marker Bangalore Genei, Lane 2 : 20 ng Genomic DNA of *A. hydrophila,* Lane 3 : 2ng Genomic DNA of *A. hydrophila,* Lane 4 : 0.2 ng Genomic DNA of *A. hydrophila.*

reactivity of primers : The genomic DNA (20ng) of *A. hydrophila* was diluted from 10^{-2} to 10^{-4} in ten-fold dilutions and amplification by PCR was done using the diluted DNA template with conditions as described above. The cross-reactivity of *A. hydrophila* hemolysin gene primers were checked by NCBI-BLAST and by genomic DNA amplification of other bacteria, i.e. *Escherichia coli, Edwardsiella tarda, Salmonella arizonae, Pseudomonas alcaligenes, Vibrio cholerae* and *Staphylococcus aureus.*

Results and Discussion

PCR Product amplification : One pair of synthetic hemolysin gene specific oligonucleotide primers targeting a 196 bp conserved region of the gene encoding for the hole-forming toxin was used in PCR. Twenty fish isolates of *A. hydrophila*

hemolysin gene (196 bp) were amplified by PCR on optimal concentration of 1.5 mM $MgCl_2$ and primer annealing at 54.7°C (Figure 1).

Specific and sensitivity of primers: Both primers were 100% homologous to the gene bank sequences of hemolysin of *A. hydrophila* accession number AB206039, AB206040, AB206041, AHU81555 and AY442273. In this present study, hemolysin gene amplification 196 bp fragments were observed in the template dilutions 10^{-1} and 10^{-2}. Above these dilutions, PCR product was not obtained. Therefore, the PCR detection sensitivity of hemolysin gene was calculated to be 2ng of genomic DNA of *A. hydrophila* (Figure 2). The PCR sensitivity limit for hemolysin gene was 1 ng (Pollard *et al.*, 1990), lipase gene 0.89 pg (Cascon *et al.*, 1996) and b-hemolysin gene 10 pg (Xia *et al.*, 2004), Low sensitivity of our primer may be due to short length of primer which can be increased to make it more sensitive.

No cross-activity of our species-specific primers was observed in NCBI-BLAST as well as in PCR amplification with common bacterial pathogens of fish viz. *Escherichia coli, Edwardsiella tarda, Salmonella arizonae, Pseudomonas alcaligenes, Vibrio cholerae* and *Staphylococcus aureus.*

Summary

New species-specific primers were developed to detect the virulence hemolysin gene of *Aeromonas hydrophila*. The conserved region of hemolysin gene 196bp was amplified in all 20 isolates of *A. hydrophila* from *Ophiocephalus striatus*. The detection limit for hemolysin gene by PCR amplification of genomic DNA of *A. hydrophila* was 2 ng. There was no cross reactivity with *Escherichia coli, Edwardsiella tarda, Salmonella arizonae, Pseudomonas alcaligenes, Vibrio cholerae and Staphylococcus aureus.*

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