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Molecular Identification and Characterisation of *Aeromonas Hydrophila*isolated From Infected Fish Rohu

KEYWORDS: Aeromonas hydrophila –pathogen identification –virulence genes- bio film formation.

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ABSTRACT *The present investigation has been carried out to isolate A.hydrophila andto identify at molecular level and characterise it.In this study, presence of virulence factors which is considered as gold standard in pathogen identification has been performed and been successful also. The study reveals the presence of 3 virulence genes of A.hydrophila which contributes the pathogencity of the organism.The biofilm forming capability of the organism was also checked and it was found that it is moderate biofilm producer.*

MATERIALS AND METHODS:

1. Molecular identification of Aeromonas hydrophila:

Aeromonas hydrophila was isolated from Infected fish with local hemorrhagic lesions and abdominal swelling Rohu , and grown in sterile alkaline peptone water for enrichment and was inoculated into Sterile Aeromonas selective agar base medium and the plates were incubated at 30°C for 24-48h for colony growth.

0.1. 16s rRNA identification of Aeromonas hydrophila:

➢ **Extraction of genomic DNA from the isolates:**

About 1.5 mL of overnight culture was centrifuged and to the pellet 1.5 mL of culture was added and again centrifuged at 10,000 rpm for 10 min at 4°C. To the pellet, 467 µl of TE buffer, 50 µL of 0.5 % lysosyme, 30 µl of 10 % SDS was added. Then 3 µl of Proteinase - K (20 mg/mL) was added, mixed well and incubated for 1 hour at 37°C. After incubation, equal volume of phenol: chloroform: isoamyl alcohol mixture (25:24:1) was added and mixed well by inverting the tube until the phases are completely mixed. The tubes were again centrifuged at 10, 000 rpm for 10 minutes at 4°C.The upper aqueous phase was collected in a new tube and equal volume of chloroform / isoamylalcohol (24:1) was added and mixed well. The mixture was centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube and 1/10th volume of sodium acetate was added to the tube. Then, 0.6 volume of isopropanol was added and mixed gently to precipitate DNA. The tube then centrifuged at 8000 rpm for 10minutes at 4°C. To the pellet, 400µl of ethanol was added and centrifuged at 8,000rpm for 10mins. The supernatant was discarded and the pellet was air dried completely. To the pellet, 30µl of TE buffer was added and stored in ice cold condition at 4°C .

➢ **PCR amplification of 16s rRNA gene:**

(i) Primer used: Aero16S F - 5'-CAGAAGAAGCACCGGCTAAC-3'

Aero16S R- 5'-TTACCTTGTTACGACTTCAC-3'

Unique primers were designed using Primer Premier V 5 and Genetyx-version 7 for amplification of the genes coding for 16S rRNA of A. hydrophila. Primers were designed from the conserved regions. The primers were then searched for their uniqueness and specificity to the respective 16S rRNA gene from different A. hydrophila isolates. The primers target 16S rRNA with the size of 1050bp.

(ii) PCR conditions: The initial Denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min.

(iii) PCR conditions mixture: PCR of 15µL reaction was carried out using 7µL of 2X master mix (Fermentas, USA), 3µL of nuclease free water, 1.5µL of each forward and reverse primers 119 (10pM) and 2µL of template DNA (50ng).

➢ **Identification of Virulent Genes by PCR Amplification**

Unique primers were designed for the amplification of the genes coding for hlyA, aexT and ascU of A. hydrophila. Primers were designed from the conserved regions. The primers were then searched for their uniqueness and specificity to the respective gene from different A. hydrophila isolates.

Table-1 showing primers used for virulent genes amplication and identification for the study.

Table: 1 Primer sequence for virulence genes amplification

Primers	Sequence	Size
hlyA	F 5'GGC CGG TGG CCC GAA GAT GCA GG 3'	597 bp
	R 5'GGC GGC GCC GGA CGA GAC GGG TA 3'	
aexT	F-5'TCAAACCTCGACCGAGGTGA 3'	502bp
	R-5'CCTCAGGACTGCTTTCGCTA 3'	
ascU	F-5'TGGTGATCGCCATCGCCGA 3'	772 bp
	R-5'GACGGCGCTTGCTCTTGAT 3'	

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Table:2 PCR Conditions for amplification of virulent genes

No.	Gene	Initial Denaturation (°C)/time	Denaturation (°C)/ time	Anealing (°C)/time	Extension (°C)/ time	Final Extension (°C)/time	Cycles (No.)
1.	hlyA	94/5 min	95/2 min	56/30sec	72	72/10min	30
2.	aexT	95/5 min	94/60 sec	55.2/30sec	72	72/7min	30
3.	ascU	95/5 min	94/60 sec	45.8/45sec	72	72/5min	30

Reaction mix (25.0 µL):
Template DNA, 2.5µl; Primer F1, 1.5µl; Primer R1, 1.5µl; 2X PCR master mix (Fermentas), 12.5µl; Nuclease free water (Promega), 7.0µl.

Electrophoresis
Following amplification, the products (10 µL, along with 5 µL of loading buffer) were separated by horizontal electrophoresis through 1.5 % agarose (low EEO grade, HiMedia, India) gel supplemented with ethidium bromide (final conc. of 0.5mg mL^{-1}) for 2 h at 50 V in tris-acetate -EDTA buffer (Sambrook et al., 1989). The bands were visualized using a UV- Transilluminator. The sizes of the amplified products were determined by comparison with 100 bp DNA ladder.

1.2 . Microtitre plate biofilm assay:
Bacterial attachment to an abiotic surface is assessed by measuring the stain taken up by adherent biomass in a 96-well plate format by means of microtitre biofilm assay. The test organisms were grown in 96-well microtitre plate for 48 h. Cells remaining adhered to the wells were subsequently stained with a dye that allowed visualization of the attachment pattern.

About 20mL of 12 to 18 h culture (A10) is selected for the test. From the culture broth, about 100 µl was pipetted into eight wells in a fresh microtitre plate. The plate was covered and incubated at optimal growth temperature for 24-48 h. Four small trays were set up in a series and 1 to 2 inches of tap water was added to the last three. The first tray was used to collect waste, while the others were used to wash the assay plates. Unbound bacteria if any were removed from each microtitre dish by briskly shaking the dish out over the waste tray.

About 125 µl of 0.1% crystal violet solution was added to each well and incubated for 10 min at room temperature. The crystal violet solution was removed by shaking each microtitre dish out over the waste tray. The dishes were washed successively in each of the next two water trays and as much liquid as possible was shaken out after each wash. To remove any excess liquid, each microtitre dish was inverted and vigorously tapped on paper towels. The plates were allowed to air-dry. Added 200 µl of 95% ethanol to each stained well. The plates were covered to allow solubilisation by incubating for 10 to 15 min at room temperature. The contents of each well were briefly mixed by pipetting.

The standard classification of biofilm formation is tabulated below(Table.3).

Table: 3 Classification of biofilm formation

Mean OD values	Biofilm formation	Biofilm index
<0.120	Nil	Non / weak
0.120-0.240	Moderately	Moderate
>0.240	Strong	High

1. Results:
PCR technique has been used to amplify genes of 16Sr RNA gene from genomic DNA of all A. hydrophila isolates. The results of diagnosis using PCR technique for 16SrRNA gene detection clarify that 10 isolates of A. hydrophila carry 16SrRNA gene that is characteristic of A. hydrophila. In this study, all the 10 strains which were isolated from fish were phenotypically identified as Aeromonas spp. Furthermore, all Aeromonas isolates gave the specific 1502 bp band by amplification of 16S rDNA. 16SrRNA gene sequence analysis has become the "goldstandard" method for definitive species identification. Primers targeting the conservative regions of the rDNAs were used to generate amplicons of variant regions that are informative in taxonomic assignment. In this study, PCR assay, using primer H1, showed that 6 (A1,A2,A3,A4,A5 and A10) (60%) of the isolated strains were positive for hemolysin genes. All of the hemolytic strains on blood agar were PCR positive for hlyA gene. PCR assay, using primer aex T and asc U, showed that 6 (A1, A2, A3, A4, A5, and A10) (60%) strains were positive for TSS genes. All the positive strains were positioned at 597bp by targeting the presence of hly A gene, at 502 bp by targeting the presence of aexT gene, at 772 bp by targeting the presence of asc U gene. In this study the presence of virulence genes (HlyA, Aex T and AscU) confirms that the isolated Aeromonas hydrophila is a pathogen which can cause Hemolysis and toxic Shock Syndrome. The presence of aerolysin and hemolysin genes in isolates shows that it is a virulent strain and transmission of such virulent strains through contacts during handling of fish results in infection to human. The organism present in fish wounds and infections may enter the water bodies and cause water pollution. Humans are directly affected by the pollution impact. They enter the human body through daily activities in poor hygiene conditions. They are opportunistic pathogen and cause gastroenteritis infection in humans.

Biofilm formation of Aeromonas sps was also assessed. Various volumes of Aeromonas sps grown in LB broth (0-100µL) were used. From this study the biofilm formation was assessed by microtitre plate assay and it was noted that the isolate was a moderate biofilm producer.

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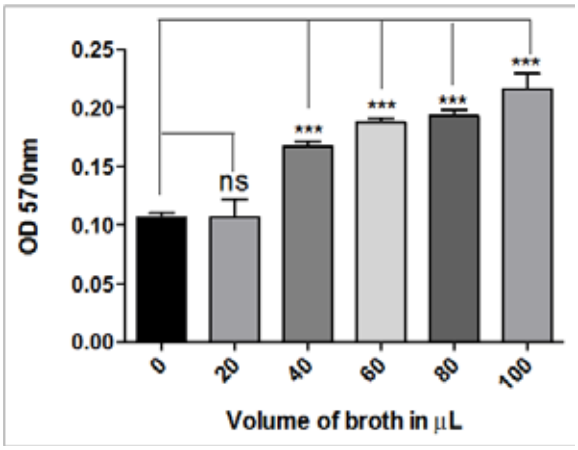


Fig:1 Each of them were compared with the control (0 uL) using Student t-test. Except the low volume of 20uL (ns= not significant), all other dilutions were found to be statistically significant. P < 0.0001 (***). Each bar represents the average of three replicates, and vertical lines represent standard errors.

Fig:2 AGAROSE GEL ELECTROPHORESIS for *hly* A gene

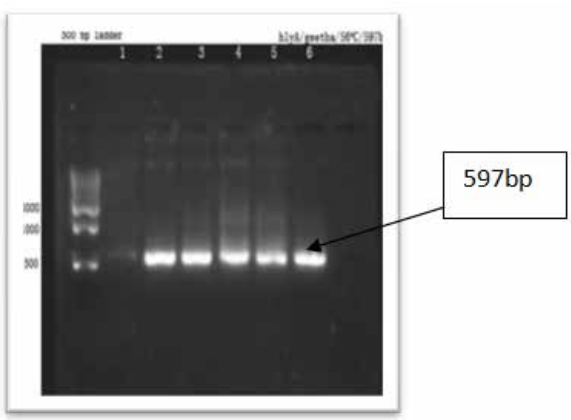


Fig:3 AGAROSE GEL ELECTROPHORESIS for *aex* T gene

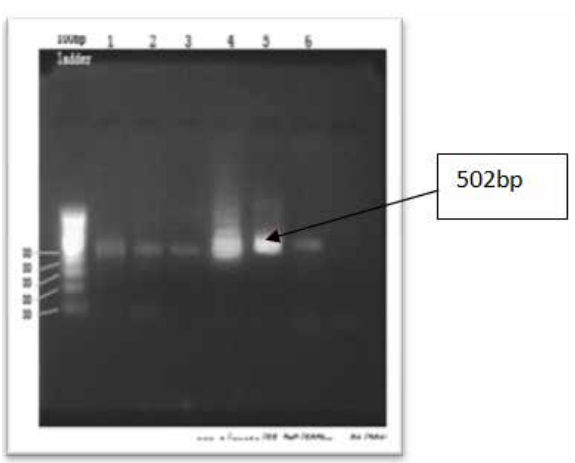
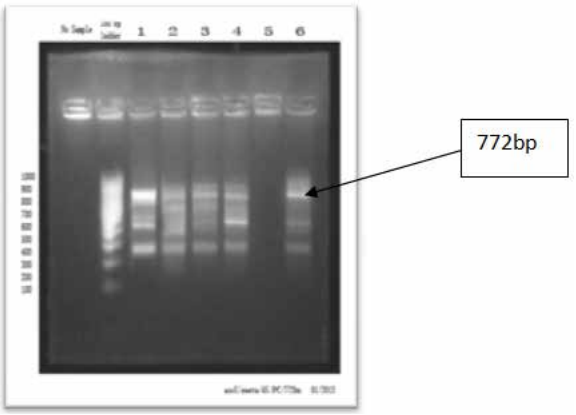


Fig:4 AGAROSE GEL ELECTROPHORESIS for *asc U* gene



2. Discussion:

In this study,16SrRNA gene sequence analysis has become the "goldstandard" method for definitive species identification. Primers targeting the conservative regions of the rDNAs were used to generate amplicons of variant regions that are informative in taxonomic assignment. In this study the presence of virulence genes (HlyA, Aex T and AscU) confirms that the isolated Aeromonas hydrophila is a pathogen which can cause Hemolysis and toxic Shock Syndrome. The presence of aerolysin and hemolysin genes in isolates shows that it is a virulent strain and transmission of such virulent strains through contacts during handling of fish results in infection to human. The source of the organism may be ambient environment, secondary contamination due to catching, transporting, handling and etc. may also contribute for its distribution. Suitable management measures like maintaining good water quality in the rearing systems, proper feed management and use of approved antibiotics in proper levels for control would help to prevent and control the A. hydrophila infection in fish but also its spread to humans. The method described here may be a useful detection tool to assist in further investigation of aero and hlyA genes in the genus Aeromonas, especially for food microbiologist.