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Detection of serine protease gene (ser-p) in marine isolates of *aeromonas hydrophila.*

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ABSTRACT

Marine water samples were collected from marina beach in Chennai for screening the isolation of serine protease gene from *Aeromonas hydrophila* by using PCR analsis. The Samples were collected and analysed the genomic DNA of *Aeromonas hydrophila* was isolated. Before analysing polymerase chain reaction with the selected isolates each primer set was tested. Polymerase chain reaction was performed to screen the ser-P genes. This study showed that PCR detection may prove to be an vital tool for the detection, identification, differentiation and distribution of serine protease genes in organisms. This polymerase chain reaction technique will give scientists an alternative way to find out the nature of pathogenicity in *Aeromonas* species and their distribution in isolates from different sources .Our present study clearly indicate that the PCR analysis detects serine protease genes in *Aeromonas hydrophila* species by using a pair of primers for gene.

KEY WORDS: Serine protease, Gene, *Aeromonas hydrophila,* PCR, Environment.

1. INTRODUCTION

Aeromonas hydrophila is a gram-negative that morphologically resembles members of the family. Fourteen species of *Aeromonas* have been identified, most of which have been associated with human diseases. The most important pathogens are *A. hydrophila, A. caviae* and *A. veronii* biovar sobria. The organisms are ubiquitous in fresh and brackish water. They group with the gamma subclass of the Proteo bacteria. Major diseases were associated with *Aeromonas* species are gastroenteritis and wound infections, with or without bacterium. Gastroenteritis typically occurs after the ingestion of contaminated water or food, whereas wound infections result from exposure to contaminated water. Although some potential pathological factors such as endotoxins, hemolysins, enterotoxins and adherence factors have been identified, their precise role is still unclear. *Aeromonas* species cause highly opportunistic and systemic diseases in immune compromised patients such as diarrheal disease and wound infections.

The presence of virulence or disease causing genes and integrons was determined in 81 strains of *Aeromonas species* was isolated from farm-raised catfish. Polymerase chain reaction (PCR) protocols were used to determine the presence of genes for cytotoxic enterotoxin (act), aerolysin (ser-P) two cytotonic enterotoxins (AST and ALT), glycerophospholipid:cholesterol acyltransferase (gcaT), DNases (exu), elastase (ahyB) serine protease (ser), lipase (lip) and the structural gene flagellin (fla) in the template DNA. Oligonucleotide primers amplified a 231-bp region of the act gene from the template DNA of 97.0% of the isolates. Oligonucleotide primers specific for the amplification of lip, gcaT, ser and fla genes, amplified their respective amplicons from 85.0, 78.0, 82.0 and 80.0% of the isolates. Several of the isolates (48.0%) contained class I integrons that confer resistance to multiple antibiotics; various sizes between 0.6 and 3.1 kb were found.

2. MATERIALS AND METHODS

Sample Collection: Marine water samples was collected from near Marina beach in Chennai. The samples were collected in a sterile tubes.

Isolation and Identification of *A.hydrophila*

Spread Plate Technique (SA Agar media): SA agar was prepared and sterilized. At bearable warmth, Ampicillin was added and plated then, 1 ml of the samples were transferred in Starch Ampicillin agar plates and spreaded evenly by using the L-rod. The inoculated SA agar plates were incubated at 37^{o}C for 24 hrs. The yellow colored colonies were scored as *Aeromonas hydrophila* and were sub cultured into the nutrient agar slants and stored at 4^{o}C for further studies.

Confirmation Test: The was prepared and sterilized for confirmation of the yellow color colonies from nutrient agar were taken and stabbed and streaked onto Kaper's multi test media tubes. The tubes were incubated at 37^{0}C for 24 hours. Appearance of yellow colour colonies after 24 hours incubation at 37 ^{o}C was considered confirmation of *Aeromonas hydrophila*. The organisms isolated were subcultured on nutrient agar and used for Gram's staining and biochemical tests.

Test for Indole: The isolated cultures were inoculated in peptone broth and incubated at 37^{0}C for 24 hours. After incubation few drops Kovac's reagent was added, a pinkish red ring is formed at the surface of the medium. This indicates positive reaction. The isolated cultures were inoculated in Glucose phosphate broth (MR-VP broth) and incubated at 37^{0}C for 48 hours. After incubation few drops of methyl red indicator was added and shake well. MR test is employed to detect the production of acid and maintenance of low pH.

Test for Voges Proskauer: The isolated cultures were inoculated in Glucose phosphate broth (MR-VP broth) and incubated at 37^0C for 48 hours. After incubation few drops of Barrit's reagent was added and shake well and give red colour.
Test for Citrate: Simmons Citrate agar was prepared, dispensed in tubes and then sterilized. Later, slants were prepared, by allowing it to solidify in a slanting position. The organisms were inoculated and incubated overnight 37ºC. Colour change was observed. The colour of the medium changes from green to blue shows the positive result.
Test for Urease: The isolated cultures were inoculated in the slants and incubated at 37°C for 24 hours. Change in colour of the medium to bright pink shows positive result.
Test for Lysine Arginine Ornithine: Isolated cultures were inoculated on LAO slants and incubated at 37^0C for 24 hours. Purple colour indicates positive result.
Activity of Protease Activity: The skimmed milk powder was dissolved by boiling it and allowed to cool. Then the skimmed milk preparation and nutrient agar were mixed and poured in petri plates. The isolated cultures were inoculated on Milk agar plate and incubated at 37ºC for 24 – 48 hours. Positive result shows clear zone formation around the bacterial colony as the organism produced protease enzyme which hydrolysed the protein casein present in the milk.
***A.hydrophila* genomic DNA isolation:** The isolated cultures were inoculated in Luria Britani (LB) broth and incubate at 37° C for 24 hours and the culture was found to be turbid.
Analysis of PCR: The polymerase chain reaction (PCR) is used to amplify a region of DNA that lies between two regions of known sequence. This reaction requires two short single stranded DNA primers that anneal to opposite ends of the template DNA and flank the region of the amplification (ROA). In addition to the template DNA and the DNA primers, two other types of biological molecules are required for this reaction: DNA polymerase and the dNTP's. Amplification results from repeated cycles of the following three steps**.** Denaturation of the template DNA, Annealing of the primers, Extension of the complementary DNA.
Serine Protease gene detection by PCR: The PCR mix was prepared in the thin walled PCR tubes in a sterile laminar hood.
***Ser-p gene*:** Forward: 5' CAGTCCCACCCACTTC 3' Reverse: 5' GCCTGAGCGAGAAGGT 3'

3. RESULTS AND DISCUSION

The enriched culture was found to be turbid showed that there was increased recovery of the organisms in the broth.

Table.1. shows the Colony on Starch Ampicillin Agar.

Sl.No.	Samples	Colour of Colony
1.	Marina Beach(MB1-7)	Yellow

The yellow colour colonies were convex, circular, regular, smooth and 2mm in size.

Figure.1. shows that the isolation of A. hydrophila in SA agar

Isolation in Kaper's Multitest Media: After 24 hours of incubation the tubes showed yellow colour colonies confirming *Aeromonas hydrophila*.
Identification of *A.hydrohila*
Gram's staining: Gram-negative straight rods with rounded ends such as bacilli to coccibacilli were observed.
Biochemical Tests

Table.2. shows the Biochemical test for *Aeromonas hydophila.*

Sl.No.	Tests	Results
1.	Indole test	+
2.	Methyl red test	+
3.	Voges – Proskauer test	+
4.	Citrate test	+
5.	Urease test	-

6.	LAO-test Lysine test Arginine test Ornithine test	 + + -

To detect the presence of serine protease gene in *Aeromonas hydrophila*, primers were annealed with the template DNA (isolated genomic DNA of *Aeromonas hydrophila*) and caused amplification. Totally seven isolates were subjected to PCR. In gene amplification, the isolates were confirmed of this gene with 416 bp using the 1000-100 bp marker DNA. The organism was isolated through selective media from various food and water samples and identified by Gram's staining, biochemical tests and confirmatory test.

The genomic DNA was isolated and bands were observed by performing agaroes gel electrophoresis using one percent agarose gel containing EtBr. The isolated DNA was used as template in the PCR study. From the 7 DNA isolate were selected from different sources. Before performing PCR with the selected isolates, each primer set was tested with MTCC control strain no.646 to confirm the production of an amplicon of predicted size. PCR were performed in PCR Thermocycler under the PCR conditions of the genes.

PCR technique clearly identified ser-P gene in Aeromonas hydrophila. The PCR products were mixed with gel loading buffer and loading in 1% agarose gel containing Ethidium bromide using 1X TBE buffer. Results clearly indicates the presence or absence of the ser-P gene in the isolated organisms by observing the bands at the level 416 bp for ser-P gene.

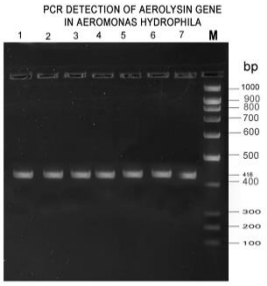

Figure.2. PCR detection of serine protease gene in aeromonas hydrophila

A total number of 7 isolates obtained from marine samples were determined. All the isolates were identified containing *A. hydrophila* by cultural and biochemical characteristics. Overall 35% of samples examine were positive for *A.hydrophila* .The density of A. hydrophila was in the range of 2.2x10-2-9.4x10-5CFU. High level of incidence of *A.hydrophila* in marine water was recorded. On Starch Ampicillin Agar *Aeromonas hydrophila* produced yellow color colonies. On kaper's multi test medium appearance of alkaline slant and acid butt after 24 hours incubation *A. hydrophila* was identified using conventional methods. The presence of virulence gene (serine protease) was detected using PCR technique. *A.hydrophila* isolated from marine samples were studied to harvoring the virulent gene. Ser - p gene fragment (416bp) amplified from *A.hydrophila* isolates reported that 91.67% of the A. hydrophila isolates from mineral and thermal water in Italy were ser-p. Aslani and Hamzeh (2004) showed that 56% of the *A. hydrophila* isolates from diarrhoeal and healthy asymptomatic controls were positive for ser-p gene.

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