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Identification of Pathogenic Genes From Marine Fish Using PCR Technique

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Aeromonas hydrophila (*A. hydrophila*) is the opportunistic pathogen, reported to found in air, water, soil, human and animals that infects the soft tissue and immune system that are serve to cause gastroenteritis, bacteremia, cellulitis, septicemia and diarrhea. There are two significant pathogenic genes haemolysin (H) and acrolysin (*aer*H) were the key factor for the medical conditions. In the current study, the fish samples were collected from the marine water, Chennai and the organism (*A. hydrophila*) was isolated, subjected to preliminary test, confirmatory test and biochemical tests were compared to its standard culture. Further, their genomic DNA was isolated and allowed to amplify using a primer and their pathogenic genes were found utilizing the Multiplex PCR approach. In the current study, the amplified PCR product of the pathogenic gene was found as AHH1 gene in Lane 2, 3 and the AH-*aer* gene in Lane 5, 6 after 24 hours and they were compared with 1 kb ladder.

Keywords : Acrolysin, *Aeromonas hydrophila*, haemolysin, Multiplex PCR, pathogenic gene

INTRODUCTION

Aeromonas hydrophila is the resident of aquatic environment, includes clean and contaminated water bodies, fish, birds, reptiles in fresh water, cold blooded marine creatures, meat, dairy products and soil. It was estimated that 7.3/billion people are at the risk of infection because of it (Regmi and Khanal, 2020; Janda and Abbott, 2010; Pessoa *et al*. 2022; Lin *et al*. 2022). It is considered as a significant food borne bacterial zoonotic pathogen, isolated from humans and animals (Ghatak *et al*. 2016; Samayanpaulraj *et al*. 2020). They are heterotropic, gram negative, straight rods with round ends (bacilli to cocci), typically its width and length are 0.3 to 1.0 μm in width, 1.0 to 3.0 μm in length, motile with a polar flagellum, capable of digesting gelatin and hemoglobin (Sawyer, 2020).

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It spreads through fecal-oral transmission during direct ingestion, drinking contaminated water and eating contaminated foods. Moreover, it can also transfer from human-to-human by contact with infected wounds, feces or blood. It was estimated that, 13% of gastroenteritis caused in United States are due to them. Cellulitis is the most frequent soft tissue infection usually accompanied by systemic signs developed within 8 to 48 h (Chou *et al*. 2004). Bacteremia caused, ranges from 1 to 38 days (Hochedez *et al*. 2010). The pathogenic genes responsible for these infections can best be identified using Multiplex PCR, a widely used, very promising, quick, and precise molecular biology approach (Balakrishna *et al*. 2010; Cammas *et al*. 2011; Altinok *et al*. 2008; Zhang *et al*. 2014). Many primers with small lengths, typically between 18 and 22 bases, are designed. Several targets are amplified in a single PCR run.The Primers withstand at 55°C-58°C and sequences with high GC content at 75°C-80°C temperature (Lorenz, 2012). This method also aids in pathogen identification, SNP genotyping (Ye *et al*.2011), RNA detection (Rashid *et al*.,

2023), plate quantification (Han *et al*. 2023), mutation analysis (Morlan *et al*. 2009), gene deletion analysis (Sellner and Taylor, 2004), linkage research and forensic studies (Elkins, 2015). Therefore, the current study aims to identify, isolate, collect its genomic DNA and to identify the pathogenic gene using PCR technique from *A. halophilia* bacteria from marine fishes.

MATERIALS AND METHODS

Sample Collection

Fish samples were collected from the marine water, Chennai they were brought to the lab, washed with sterile distilled water to remove the adherent particles, 25 g of edible flesh portion was macerated and inoculated into 225 ml of alkaline peptone water and incubated for 37˚C for 18-24 hrs.

Preliminary Test
Starch Ampicillin Agar (SAA) Medium

After enrichment, the samples were streaked into starch Ampicillin agar (starch agar containing Ampicillin) using sterile loop, incubated at 37˚C for 18-24 hrs and compared with standard strain *A. hydrophila* (Palumbo *et al*. 1985).

Slant Preparation

The individual colonies were streaked onto the nutrient agar slant. The test tubes were incubated at 37^0 C for 16-24 hrs. After incubation, it was stored under refrigeration.

Gram Staining

The smear preparation was initially done by fixing the material on slide with heat for 1 minute and allow it to cool before gram staining procedure. The procedure was carried out following the protocols of Tripathi and Sapra (2023) and the results were observed under oil Immersion (100x) using a bright field microscope.

Motility Test

Mannitol motility buds were stab inoculated with the test organism, incubated at 37˚C for 24 hrs and examined upto 7 days.

Oxidase Test

This test was performed by soaking the filter paper into 1% phenylene diamine dihydrochloride and kept it on the slide or Petridish, with the help of platinum wire loop 24 hrs grown colony of test organism and its control are picked, they were rubbed in the filter paper.

Confirmation Test (Kaper's Multi Test Medium)

Butt and slants were prepared and the colonies was streaked using sterile loop and incubated at 37˚C for 18 – 24 hrs. (Kaper *et al*. 1980)

Biochemical Test
Indole Test

Peptone broth (pH 7.4) was prepared, sterilized at 121˚C for 15 mins, test sample was inoculated and incubated at 37˚C for 24 – 28 hrs and after incubation, 0.2ml of kovac's reagent was added to the suspension.

Methyl Red Test

Glucose phosphate broth (pH 6.9) was prepared, sterilized at 121˚C for 15 mins, test culture inoculated and incubated at 37˚C for 24hrs. After incubation, 5-6 drops of methyl red indicator were added and mixed thoroughly.

Voges-Proskauer Test

Glucose phosphate broth (pH 6.9) was prepared, sterilized at 121˚C for 15 mins, the test culture is inoculated and incubated at 37˚C for 24 – 48 hrs. After incubation, 3 ml of barris reagent A and 1 ml of barris reagent B was added, the test tube was shaken at regular interval to ensure maximum aeration.

Simmon Citrate Agar

The suspected culture was streaked onto Simmon Citrate Agar and incubated at 37˚C for 24 hrs.

Urease Test

Urease broth is a differential medium that contains two pH buffers, phenol red was added to it.

Triple Sugar Iron Test (TSI)

TSI slant was first stabbed through the center of the medium to the bottom of the tube and then streaking was done on the surface of the agar slant. During the incubation period of 24 hrs at 37˚C, the cap is to be left on the tube loosely.

Detection of pathogenic genes by PCR Technique

Genomic DNA Extraction from Bacteria

The cultures of *A.hydophila* was centrifuged at 8000 rpm for 5 mins, suspended in 500 µl of 10% Sodium Dodecyl Sulphate (SDS) and vortexed. The mixture was incubated in water broth for 20 minutes by 60˚C, cooled at room temperature and 30 µl of phenol, chloroform, isoamyl alcohol was added;centrifuged at 1000 rpm for 10 minutes, the pellet collected and 200 ml of ethyl alcohol was added and centrifuged again at 1000 rpm for 10 minutes. The pellet was collected after centrifugation and TE was added and mixed by tapping till the solution settled at the bottom of the tubes mixed thoroughly, finally it was incubated at 37^{p} C, DNA was isolated.

Agarose Gel Electrophoresis

Agarose was dissolved in 100 ml of 1xTAE buffer, 5 µl of ethidium bromide was added and dissolved and the mixture was spread onto a gel tray for electrophoresis. A well was created using a comb. 20 µl of digested DNA was loaded along with 3 µl of gel loading buffer.

Amplification

Unique primers were designed for the amplification of the genes (Wang and Seed, 2003), AHH1F (GCCGA GCGCCCA GAA GGT GAGTT), AHH1R (AGCGGCTGGATGCGG TTGT),AH-*aer*AF (CAAGAACAAGTTC AAGT GGCCA), AH-*aer*AR (ACGAAG GTGTG GTTC CAGT), AHH1F (GCCGAGCGCCCAGA AGGTGAGTT). DNA samples (5 mg per reaction mixture) were amplified in a 25 µl reaction mixture. Each contained: 50 mM potassium chloride,10 mM Tris chloride (pH 8.3), 1.25 mM magnesium chloride, 200 µM (each) dATP, dCTP, dGTP and dTTP, 2.0 µM (each) AHH1 primers, 1.5 µM AH-*aer* A, 1.25 U of Fast Start *Taq* DNA polymerase (Roche Diagnostic, Germany). The PCR was performed with initial denaturation at 95˚C for 5min (50 cycles), Annealing 59˚C (5 min), Primer extension 72˚C (5min), Final extension 72 ˚C (7min). The Amplifications were performed with a model 2400 DNA thermal cycler. The Post PCR analysis were detected by subjecting a sample from each reaction tube to 2 % agarose gel electrophoresis.

RESULTS AND DISCUSSION

Fish samples were taken from marine water, Chennai and a preliminary test was used to check for *A. hydrophila*. The results were collated in Table 1.The table reveals that- SSA medium produced colonies that were honey yellow in color; gram staining revealed gram negative cocci; the organism was motile by turning the medium opaque in the motility test; in the oxidase test, the filter paper turned purple; in the citrate test, the organism turned blue from green; and in the TSI agar test, the colonies showed butt and slant with gas production. These observations were compared to standard strains of *A. hydrophila*. Finally, the color changed, indicating positive confirmation for *A. hydrophila* according to Kaper's Multi test.

The exotoxins, endotoxins, cytotoxins and exoenzymes (lipases and proteases) are produced by *A. hydrophila* are associated with pathogenicity. Based on immunological studies, the exotoxins of *A. hydrophila* is classified into two types haemolysin and aerolysin. From the previous literature studies, it was reported that the genes that code for haemolysin is H and acrolysin (*aer* H), used as a molecular marker to find the unknown desired pathogenic strains using Multiplex PCR (Yoganath *et al*. 2009) In the current study, the detection of genomic DNA with amplified PCR product of pathogenic gene is found as AHH gene in Lane 2 and 3; AH-*aer*A gene was observed in Lane 5 and 6, after 24 hours viewed in UV transilluminator (Fig.1).

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Table 1: Preliminary and biochemical test for identification of *A. hydrophila*

PRELIMINARY TESTS	RESULTS
Starch ampicillin agar medium	Positive
Gram staining	Gram negative
Motility	Motile
Oxidase	Positive
Confirmatory test-Kaper's Multi test	Postive
Biochemical Tests	
Indole test	Positive
Methyl red test	Positive
Voges proskauer test	Positive
Urease test	Negative
Triple sugar iron	Positive

Fig.1: Detection of Genomic DNA with PCR Reaction after 24 hours

Lane 1 : 1kb ladder
Lane 2,3 : Amplified PCR Product of AHH1
Lane 5,6 : Amplified PCR Product of AH-aerA

DECLARATION

Conflict of Interest. Authors declare no conflict of interest

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