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Detection of 232bp Virulent Gene of Pathogenic *Aeromonas hydrophila* through PCR Based Technique: (*A Rapid Molecular Diagnostic Approach*)

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ABSTRACT

The pathogenicity of aeromonads produces due to exotoxins such as cytolytic enterotoxin, hemolysin or aerolysin, lipases and proteases. Rapid detection of *A. hydrophila* cytolytic enterotoxin (AHCYTONE) gene and their characterization has proven importance so that proper and rapid preventive and control measures could be taken up to reduce mortality and loss in fish culture. The main objective of the present study is to genetic identification of AHCYTONE positive *Aeromonas hydrophila.* Strains were isolated from fishes from different fish market in West Bengal and water samples from different river and ponds. Initially strains were identified by their phenotypic and biochemical characterization. Due to contradiction of those results, molecular characterization was done by polymerase chain reaction, which is proved a suitable and rapid diagnostic tool for identification and characterization of *A. hydrophila*. We have also evaluated the potential risk to human health that this finding can represent by determining the presence of the cytolytic enterotoxin gene in such isolates.

Keywords: *Aeromonas* Spp.; Hemolysin Gene; AHCYTONE; Molecular Diagnosis; PCR

1. Introduction

In India and especially in West Bengal, Fish and fishery products are major food products to attain the protein requirements. But, these food products are contaminated by various food borne pathogens. *Aeromonas* species are becoming dangerous in recent years in fish pathology [1]. This is because of increased incidence of diseases in man and animals by these agents, as they can often act as opportunistic agents. *Aeromonas hydrophila* is a ubiquitous and heterogeneous organism and that has been associated to wound infections [2]. This pathogens are mesophilic motile and psychrophilic nonmotile gram-negative ubiquitous bacteria [3] and causes gastroenteritis, septicemia and traveler's diarrhea in humans and hemorrhagic septicemia in fish [4,5] also associated by *Aeromonas hydrophila*. Disease produces by ingestion of contaminated fishes, fish foods and drinking water, or direct contact with recreational waters. Aeromonads can also play a role as primary pathogens, being responsible for enteric or, less often, extra-enteric infections. In humans, *A. hydrophila* has been reported as the causative agent of numerous manifestations associated with gastroenteritis, systemic infections, bacterial endocarditis, wound infections and localized infections of eyes and throat [6-8].

The pathogenicity of aeromonads is caused by exotoxins such as cytolytic enterotoxin, hemolysin or aerolysin, lipases and proteases [9,10]. Hemolysins are exotoxins and the lytic activities on red blood cells are reported to be important for nutrient acquisition or for causing anemia [11]. Haemolytic toxins as haemolysin and aerolysin released by *A. hydrophila* [12] and pathogenicity has been linked to hemagglutinins, adhesins, several hydrolytic enzymes and enter tissue in culture [13] and play significant role in pathogenesis. EPS, (exopolysaccharides) are importantly play very important role in the bacteria and their environment interactions as they are organic molecules formed by polymerization of organic fractions, carbohydrates, proteins, and humic substances [14-16].

Mutagenesis studies reported that both the hemolysin and the aerolysin genes are involved in hemolytic activity of *A. hydrophila*. However, the pathogenesis of *A. hydrophila* infection is complex and multi-factorial with the involvement of a number of virulence factors [17-19].

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Aerolysin and hemolysin genes are reported to be the putative virulence genes. Aerolysin, produced by some strains of *A. hydrophila*, is extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic activities [20]. Hirono and Aoki (1991) [21] reported, discussed and described the AHHl haemolysin. That consists of 577 amino acid residues with a molecular mass of 63.7 kDa. The gene encoding this haemolysin was cloned and sequenced from *A. hydrophila* ATCC 7966 [21]. The gene encoding a similar haemolysin, designated ASH4, was cloned from *Aeromonas salmonicida* by Hirono and Aoki (1993) [22]. The ASH4 haemolysin gene encodes a 578 amino acid polypeptide with a molecular mass of 64.4 kDa which was 84% homologous to the AHH1 haemolysin.

In the present paper, Rapid detection of cytolytic enterotoxin (AHCYTONE) gene of *A. hydrophila* and their characterization has assumed prime importance so that suitable preventive and control measures could be taken up to reduce mortality and loss in fish culture. In view of this economic importance to aquatic animals and human health hazard problems, it would be of prime importance for rapid detection of this pathogen and characterization of isolates using molecular techniques. With this in view, The main objective of this study was to genetically re-identify previously biochemically identified *Aeromonas hydrophila* isolated from different fishes and its capability of producing. We have also evaluated the potential risk to human health that this finding can represent by determining the presence of the cytolytic enterotoxin gene (AHCYTOEN) in such isolates [3,23].

2. Materials and Methods

2.1. Collection of Bacterial Isolates and Phenotypic Identification

Aeromonas hydrophila were isolated from different fishes, purchased from different local fish market of West Bengal. 25 ml fish fleshes were aseptically homogenized for in stomacher with 225 ml alkaline peptone solution. After 18 h of incubation at 37˚C, aliquots were inoculated in Rimler Shotts (RS) agar medium for 24 h at 37˚C.

Initially colonies were identified by their morphological characteristics as round, cream to light yellow. Gram staining was confirmed the bacterial colonies were gram negative. Biochemical tests were done in all strains by "*Áutomated Microbial Analyzer*" (Biolog, US). The metabolic profiles were compared automatically using the Biolog microtiter plates with the MicroLog GN database. Biolog identifications were reported if the similarity index of the genus or species was 0.5 or greater after 24 hr of incubation. Test samples were incubated under the same conditions as used [24].

Aeromonas hydrophila were also identified by different biochemical tests to species level by using motility, oxidation and fermentation, catalase, indole, methyl red test, urease test, haemolysin production, sugar fermentation test, reduction of nitrate to nitrite, H_2S production and etc. were confirmed as *Aeromonas hydrophila*.

2.2. Extraction of Genomic DNA

DNA was isolated by modification of the protocol of Sambrook *et al.*, 1889 [25]. Overnight grown bacterial culture in 5ml Tryptic Soya Broth (Hi media) and centrifuge at 12,000 g × 5 min at 4˚C. Resuspended pellet in 0.2 ml of TE-1 buffer and added 50 µl lysozyme (3 mg/ml) and keep at 37˚C × 15 min then 50 µl lysozyme was added with 50 µl proteinase K (15 mg/ml) and incubated at 56˚C for 3 - 4 hrs in a water bath. DNA were extracted by adding equal volume of TE saturated phenol by slow mixing and centrifugation 12,000 g × 5 min at 4˚C. With the supernatant, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and again centrifuged. To the supernatant, 200 µl chloroform was added and supernatant was collected after centrifugation and 25 µl of 5 M NaCl. 250 µl of chilled absolute alcohol was added and DNA was observed as bunch of threads. Then DNA was collected after wash with 70% alcohol and dried it. Pure DNA was dissolved in TE buffer and stored at 4˚C for further use [26].

2.3. Polymerase Chain Reaction

Polymerase chain reaction was used to detect 232 bp gene in isolates. Primers specific for hemolysin gene (232 bp product) [24] were used as the target genes for PCR amplification. Primer used as AHCF1: 5'-gag aag ctc acc acc aag aac a-3' and AHCR2: 5'-aac tga cat cgg cct tga act c-3'.

A 25 µl PCR mixture contained 2.5 mM $MgCl_2$, 2.5 µl 10 X reaction buffers, 10 nmole each 200 µM dNTPs, 10 pmole each primer, 2 units of Taq DNA polymerase (Promega) and 20 ng template DNA. PCR was carried out on a Thermo Electron PCR system using the cycle as initial denaturation at 95˚C for 5 min followed by 30 cycles at 95˚C for 2 min, 55˚C for 1 min and 72˚C for 1 min. 7 min final extension at 72˚C. Amplicons were examined and visualized by electrophoresis in 1.5% agarose gel in TBE buffer. The gel was stained with EtBr (Sigma) and viewed in Gel Doc System.

3. Results

Aeromonas hydrophila produced yellow colonies in the RS-medium. Gram staining of these colonies gives gram negative reaction, microscopically analysis gives rod shaped and motile, biochemical tests gives oxidase positive, fermentative and antibiotic resistance tests con-

cluded as novobiocin resisitant, primarily indicated that colonies are aeromonads (**Table 1**). All isolates were con-firmed to the species level *Aeromonas hydrophila* by *Automated Microbial Analyzer* (Biolog, US) [24].

The pathogenicity and virulence characteristics of *A. hydrophila* are associated with presence of different exotoxins e.g., haemolysin, enterotoxins and cytotoxins and exoenzymes e.g., proteases and lipases [27]. The two major groups of haemolysins like extracellular haemolysin and aerolysin [28] produced by *A. hydrophila*. PCR amplification result showed by this study that the same molecular weight of the potent virulent gene of *A. hydrophila* as haemolysin gene. Chacón *et al.* stated in 2003 [29] the similar observation was in earlier studies on the *A. jandaei* type strain presented the aerolysin/hemolysin gene. Pathogenicity of enteric bacteria is produced by cytotoxin [30].

PCR amplification of DNA from pathogenic *A. hydrophila* isolates using specific primers for hemolysin genes resulted in the expected PCR products of 232 bp sizes in lane one to five, lane eight and nine (**Figure 1**), whereas no amplification products were found in lane six and seven due to non-pathogenic nature of aeromonads and does not have AHCYTOEN gene. AH1 isolates have been classified as pathogenic strain due to presence of 232 bp gene in 2% agarose gel (**Figure 2**).

Table 1. Physiological and biochemical characteristics of isolates in the study.

Tests	Reaction of isolates						
	AH1	AH2	AH5	AH6	AH7	AH8	AH9
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
O/F test	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Fermentation of	+	+	+	+	+	+	+
Sugar glucose	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Moltose	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+
Indole							
Decarboxylase reaction	+	+	+	+	+	+	+
Arginine	-	-	-	-	-	-	-
Lysine	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	-

4. Discussion

Screening of 232 bp gene has been reported to be the most effective way of diagnosis of Aeromonas virulence factors [31]. Previous clinical studies [32,33] reported that the expression of multiple biological activities, as in the case of the AHCYTOEN gene, is necessary for the expression of microbial pathogenicity. This cytolytic enterotoxin gene is considered a characteristic virulence trait in *Aeromonas* [3,34] and its general presence in *A. culicicola*, indicates that this species may have public health significance. The presence of hemolysin genes in isolates shows that it is a virulent strain and as a result infection in human by contacts during handling of fish. Secondary contamination also may be due to catching, transporting, handling and etc. and may also contribute for its distribution. 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 fishes but also its spread to humans. The PCR assay used in this study proved to be a useful diagnostic tool for the

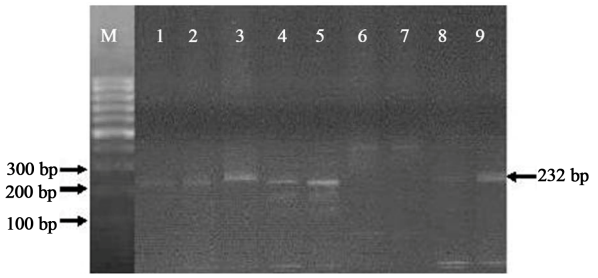

Figure 1. Detection of *Aeromonas* sp. using PCR and amplification of 232 bp DNA bands visualized on 2% agarose gel. M: Indicates the 100 bp ladder.

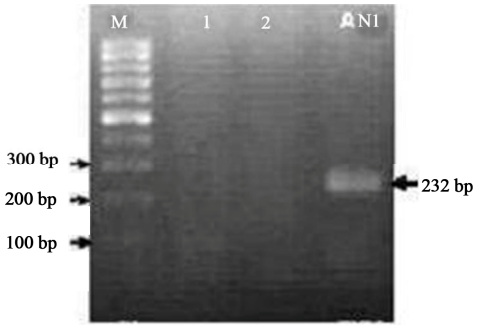

Figure 2. Detection of 232 bp amplicons of pathogenic *Aeromonas hydrophila* (AH1) on 2% agarose gel. M: Indicates the 100 bp ladder.

detection of virulent *Aeromonas hydrophila* by detecting AHCYTOEN gene as genetic virulence markers.

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