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Detection of Virulence Gene in *Aeromonas hydrophila* Isolated from Fish Samples Using PCR Technique

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Abstract: The detection of virulence factors of *Aeromonas hydrophila* is a key component in determining potential pathogenicity because these factors act multifunctionally and multifactorially. In this study fish samples were collected from local market, Chennai, Tamil Nadu, India and totally 15 fish samples were collected, among these only 7 samples namely, Parai, Kadamba, Kelangan, Shankara, Nethili, Prawn and Suthubhai were found to be positive for *Aeromonas hydrophila.* The modified Rimler-Shotts medium and Kaper's multitest medium were used as a selective presumptive isolation medium. For rapid detection of two virulence factors of isolated *Aeromonas hydrophila*, a polymerase chain reaction assay was used. The detected virulence factors include aerolysin (aer A) and haemolysin (hyl H). The band appearance in the amplified virulence genes of screened fish samples shows the molecular weight of aerolysin (aer A-416bp) and haemolysin (hyl H-597bp).

Key words:*Aeromonas hydrophila* · PCR · Modified Rimler shotts agar medium · Virulence factors-aerolysin-haemolysin

INTRODUCTION

In developing countries like India, fishery products contribute a major food item of common man. These products are contaminated by various food borne pathogens. The distribution of *Aeromonas hydrophila* in marine ecosystem including marine fish and retail seafood outlets is well known and this organism is found to be the normal flora of fish variety, under stress condition, this bacterium becomes a pathogen. *Aeromonas hydrophila*, a ubiquitous aquatic microorganism, is an opportunistic pathogen that has been associated to wound infections, gastroenteritis, septicemia and traveler's diarrhea in humans and hemorrhagic septicemia in fish. The main routes of exposure in humans are ingestion of contaminated foods and drinking water, or direct contact with recreational waters. Human exposure to *Aeromonas* has risen due to the increased usage of aquatic recreational sites especially in Southern California with its warm climates throughout most of the year, attracting people to its beaches. Despite the number of surveys on the incidence of *Aeromonas* spp. in food products, there have been few studies on frozen fish. In addition, these studies have used strains which have been inaccurately identified when using traditional biochemical techniques [1], leading to unreliable results [2] The pathogenicity of aeromonads has been linked to exotoxins such as cytolytic enterotoxin, hemolysin/aerolysin, lipases and proteases. The detection method of aerA was recently proposed as a reliable approach by which to identify a potential pathogenic *Aeromonas* strain by using methods involving PCR and restriction fragment length polymorphism analysis, the virulence genes of *Aeromonas* spp. were grouped as aerolysins-hemolysins, cytolytic enterotoxins, or cytotonic enterotoxins [3]. A PCR method for the amplification of the aerolysin gene was shown to detect â-hemolysin-positive *A. hydrophila* isolates from patients with diarrhea [4]. The main objective of this study was to genetically re-identify previously biochemically identified *Aeromonas hydrophila* isolated from frozen fish intended for human consumption and its capability of producing virulence genes of aerolysin and haemolysin were also studied.

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MATERIALS AND METHODS

Bacterial Strains and Growing Conditions: *Aeromonas hydrophila* was isolated from 15 frozen fish purchased in local markets in Chennai City, India. Twenty-five grams of fish flesh were weighed aseptically and homogenized for 2 min in stomacher bags containing 225ml of alkaline peptone water. After 18 h of incubation at 37°C, an aliquot of the enrichment was inoculated in Starch Ambicillin Agar (SAA) and incubated for 18-24 h at 37°C. The plates were flooded with approximately 5 ml of iodine and amylase and oxidase positive colonies were isolated. Isolated cultures were then purified by repeated streaking in nutrient agar and maintained in nutrient agar slants. The isolated colonies were streaked in selective media known modified Rimler shotts agar (mRS) medium. For the confirmation test of *Aeromonas hydrophila* identified using the Kaper's multi test medium [5].

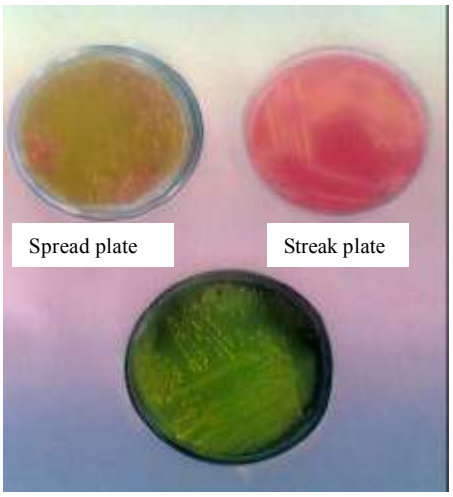
Phenotypic Identification: *Aeromonas hydrophila* were identified biochemically to species level by using 14 tests chosen and were motility, Kovac's oxidase, oxidation and fermentation, catalase, indole, methyl red test, urease test, haemolysin production, sugar fermentation test, voges-proskauer test, reduction of nitrate to nitrite, H_2S production, lysine decarboxylase and arginine dihydrolase and cultures which matched typical reaction of *Aeromonas hydrophila* were confirmed as *Aeromonas hydrophila*..

Polymerase Chain Reaction: The polymerase chain reaction (PCR) was used to detect the presence of the aerolysin and haemolysin genes in all isolates. The primers used (Aer 2F: 5'-agc ggc aga gcc cgt cta tcc a-3' and Aer 2R: 5'-agt tgg tgg cgg tgt cgt agc g-3') and (Hyl 2F: 5' ggc ccg tgg ccc gaa gat gca gg 3' and Hyl 2R: cag tcc cac cca ctt c 3') targeted a 683bp fragment of the aerA and 597bp fragment of Hyl H region of the aerolysin gene and Haemolysin gene. A PCR mix of 25 µl contained 2.5mM $MgCl_2$, 2.5µl 10x reaction buffer, 10nmole each dNTP, 10pmole each primer, 2.5U Taq polymerase (Promega) and 20ng template DNA. PCR was carried out on a Minicycler using the following cycle: preheating 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, followed by 7 min final extension at 72°C. PCR products were examined by electrophoresis in 1.5% agarose gel in TBE buffer. The gel was stained with EtBr and viewed under UV light.

RESULTS AND DISCUSSION

A number of virulence factors derived from *Aeromonas hydrophila* have been explained in an effort to explain the pathogenesis of infections due to this organism. Toxins with haemolytic, cytotoxic and enterotoxic activities have been described in many *Aeromonas* Spp. [6]. Of the 15 fish samples tested, 7 (40%) were positive for *A. hydrophila.* A total of 7 presumptive colonies of *A. hydrophila* on mRS medium were selected for virulence gene identification (Fig. 1).

Aeromonas hydrophila on Starch Ampicillin Agar



Aeromonas hydrophila on Rimler Shotts Mediun

Fig. 1: Growth of *Aeromonas hydrophila* on Starch Ampicillin Agar (SAA) and Rimler Shotts Medium (RS)

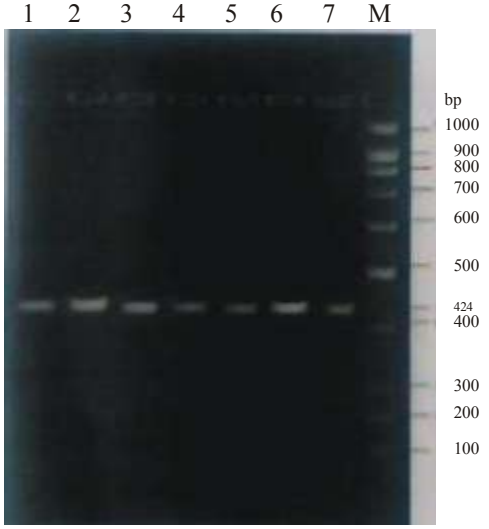


Fig. 2: PCR detection of Aerolysin gene in *Aeromonas hydrophila*

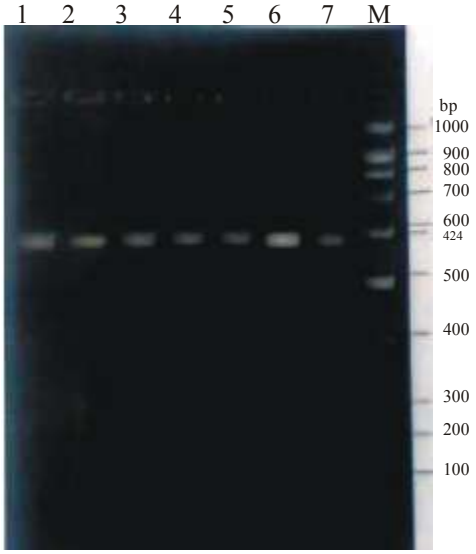


Fig. 3: PCR detection of Haemolysin gene in *Aeromonas hydrophila*
Lane 1. Parai, 2. Kadamba, 3. Kelangan, 4. Shangara, 5. Nethili, 6. Prawn, 7. Suthabai, M. 100bp DNA ladder

The pathogenic and virulence characteristic of *A. hydrophila* are associated with the range of different exotoxins (haemolysin, enterotoxins and cytotoxins) and exoenzymes (eg.., proteases and lipases) [7].The haemolysins produced by *A. hydrophila* are divided into two major groups, such as extracellular haemolysin and aerolysin based on immunological studies [8]. A PCR was done for detecting haemolysin (hyl H) as well as aerolysin (aerA) genes as genetic markers for virulence determinants. Hence for the brief work the Polymerase chain reaction (PCR) application for the aerolysin gene and haemolysin gene were carried out for the detection of pathogenic strains *A. hydrophila.* The role of both genes in *Aeromonas* pathogenicity has already been demonstrated [8, 9]. The PCR was performed in thermocycler using PCR conditions. The final products were resolved in 1.5% agarose gel in TBE buffer and the gel was stained with EtBr and viewed under UV light, when PCR was performed the results clearly indicates that 50% of the isolated organism has both aerolysin and haemolysin genes. The band appearance in the amplified virulent genes of screened fish samples shows the molecular weight of aerolysin (aer gene-416bp) and haemolysin (hyl gene-597bp). Amplification result obtained through the present study also shows the same molecular weight of aerolysin gene (Fig 2) and haemolysin gene (Fig 3), which are the potent virulence genes of *A. hydrophila*. Similar observation was reported in earlier studies on the *A. jandaei* type strain presented the aerolysin/hemolysin gene as stated by Chacón *et al.* [10]. The PCR assay used in this study proved to be a useful tool for the detection of virulent *Aeromonas hydrophila* by detecting haemolysin and aerolysin genes as genetic virulence markers.

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