SCREENING OF PATHOGENIC AEROMONAS SPECIES FROM MARKETED FISH SAMPLES

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ABSTRACT

Aeromonas spp. have received increasing attention as opportunistic as well as primary pathogens in humans, aquatic and terrestrial animals. Aeromonads are common contaminants in foods such as fish and other sea foods, raw and cooked meat, poultry, vegetables, milk and milk products. Factors contributing to virulence include toxins, haemolysins, adhesins and various hydrolytic enzymes. The present study was aimed to screen the pathogenic Aeromonas sp. in the marketed fish samples. About 20 fish samples were processed, in which 15 samples (75%) were found to be contaminated with Aeromonas. Conventional biochemical identification may lead to miss identification of Aeromonas isolates, so molecular based identification-16S rRNA gene was used for the identification of Aeromonas isolates. Further haemolytic activity of the Aeromonas isolates were performed on 5% blood agar plates and about 20% of the isolates showed α-haemolysis, 60% showed β-haemolysis and 20% showed γ-haemolysis. As proteolysis was reported as a virulent trait, the isolates were subjected to proteolytic activity on skim milk agar plates. About 73% of the isolates showed proteolysis. About 40% of the isolates possessed both the β-haemolytic and proteolytic activity. The presence of β-haemolytic and proteolytic activity among the Aeromonas isolates revealed the existence of pathogenic Aeromonas isolates in marketed fish.

Keywords: Aeromonas, Virulence, Haemolysis, Proteolysis.

INTRODUCTION

Fish and fishery products are of great importance worldwide due to their nutritional value, clear health benefits and wholesome properties [1]. In developing countries like India, fishery products contribute a major food item of common man. These products are contaminated by various food-borne pathogens [2]. Fish gastrointestinal tract is one of the major infection tracts. It presents a favourable medium for bacterial multiplication [3, 4].

Aeromonas spp. are ubiquitous bacteria found in a variety of aquatic environments worldwide, including bottled water, chlorinated water, well water and heavily polluted waters. Aeromonas species are Gram-negative, facultative anaerobic rod. They cause infections in invertebrates and vertebrates, such as frogs, birds and domestic animals. Various fish species develop haemorrhagic disease and furunculosis resulting from infections by Aeromonas spp. It was thought to be an opportunistic pathogen in immunocompromised humans. A variety of extracellular virulence factors such as enterotoxins, cytotoxins, haemolysins, aerolysins, proteases, hagglagglutinins produced by A. hydrophila are associated in their epidemiological associations [5]. The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in Aeromonads [6, 7]. β-hemolysin has been reported as a virulence factor in motile Aeromonas [8]. Aerolysin and hemolysin genes are reported to be the putative virulence genes of A. hydrophila [9].

Microbial proteases play an important role in the use of proteins or peptides as a nutrient source for bacterial growth, and in the pathogenesis and virulence of disease development [10]. Extracellular proteases have also been suggested as a virulence factor of Aeromonads [11] and both temperature-labile serine proteases and temperature-stable metalloproteases have been characterized in A. hydrophila. Aeromonas isolates secrete at least four or five different proteases, as determined of pH optima and substrate specificities [12]. The present study was aimed to determine the prevalence of pathogenic Aeromonas isolates in marketed fish samples.

MATERIALS AND METHODS

Bacterial source and maintenance of cultures

Fish specimens were randomly collected from Układam fish market, Coimbatore in sterile polyethylene bags and brought to the laboratory using an ice chest in less than an hour.

The intestine of the fish was aseptically removed and enriched in alkaline peptone water (APW) for overnight. The enriched cultures were streaked on starch ampicillin agar medium (SAA) and incubated for 24hrs at 28°C. A characteristic yellow to honey coloured colonies were selected and used for further testing. After enrichment and streaking onto SAA, honey coloured colonies were subjected to Gram staining as well as enzymatic tests such as oxidase and catalase were also performed. The oxidase and catalase positive colonies were then purified by repeated streaking on the nutrient agar and were maintained in the nutrient agar slants.

Genotypic identification of isolates

DNA extraction

A single bacterial colony was inoculated in 5 mL of Luria-Bertani broth and incubated overnight at 37°C, 120 rpm in a shaker incubator (REM). Overnight cultures (1.5 mL) were transferred to microfuge tubes (appendorf) and centrifuged (REMI) at 8,000 rpm for 5 minutes at 4°C. Supernatant was removed and cells were washed with 400 μL STE buffer and centrifuged for 8,000 rpm for 5 minutes at 4°C. The pellets were re-suspended in 200 μL TE buffer and 100 μL Tris-saturated phenol (Rankem, India), followed by a vortex mixing for 60 seconds and centrifuged at 13,000 rpm for 5 minutes at 4°C to separate the aqueous phase and organic phase.

The 160 μL upper aqueous phase was transferred to a clean microfuge tube and 40 μL TE buffer was added to make 200 μL and mixed with 100 μL chloroform (Rankem, India) and centrifuged at 13,000 rpm for 5 minutes at 4°C. The lysate was purified by chloroform extraction until a white interface was no longer present. This step was repeated for 2-3 times. To the upper aqueous phase, 40 μL TE and 5 μL RNase were added and incubated at 37°C for 10 minutes to digest the RNA. Then, 100 μL chloroform was added to the tube, mixed well and centrifuged at 13,000 rpm for 5 minutes at 4°C. The upper aqueous phases which contain purified DNA was transferred to clean microfuge tube and stored at -20°C.

Amplification of 16S rRNA gene of the isolates

Polymerase chain reaction (PCR) was carried out for 16S rRNA gene of about 1050 bp, by using genus specific primers. The PCR conditions used were 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute, 72°C for 5 minutes.
Haemolysis assay

The haemolytic activity was determined for all the isolates by blood agar plate method. Haemolytic activity was determined as a zone of haemolysis around the colonies on blood agar plates containing 5% human blood after 24hrs of incubation at 37°C. Blood agar base was prepared, sterilized and was cooled to 48-50°C. With a sterile pipette, 5 mL of blood was added to 95 mL of the blood agar base aseptically. The content of the flask was mixed well and poured into the sterile petriplates with care to avoid air bubbles. Aeromonas isolates were simple streaked on blood agar plates and incubated at 37°C for 24hrs and were observed for haemolytic activity. Haemolytic positive isolates were identified by the presence of clear (β-haemolysis) or diffuse (α-haemolysis) halos around the colonies [13].

Proteolytic activity

Presence of protease enzymes from producer bacteria was carried out using skim milk agar. About 52 grams of skim milk agar (Himedia, India) was suspended in 1000 mL of distilled water. The media was dissolved completely by heating and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Later, the media was cooled, mixed well and poured onto sterile petriplates. Producer bacteria were spot inoculated into the plates and media was cooled, mixed well and poured onto sterile petriplates. Plates were observed after 18-24hrs of incubation for possible clear zones.

RESULTS AND DISCUSSION

Majority of the studies showed the occurrence of A. hydrophila in aquatic environments such as estuarine, fresh drinking water [14], fresh water fish [15]. Fish may also be a vehicle for pathogenic bacteria naturally occurring in aquatic environments referred to as indigenous or derived from polluted waters and or from postcapture contamination, storage and handling. The incidence of microbial pathogens, especially those of bacterial origin is one of the most significant factors affecting fish culture [16].

Yellow to honey coloured Gram-negative colonies were further subjected to enzymatic tests. Aeromonas isolates were positive for oxidase and catalase tests. Based on the above results and predictions, all the yellow colonies were subjected for the identification of signature regions. The presumptive identification of Aeromonas isolates were further confirmed on the basis of amplification of 16S rRNA genes using genus specific primer. The expected amplicon size for 16S rRNA was 1050 bp was observed in all the isolates that showed positive for Aeromonas in the presumptive identification tests (Figure 1). In the present investigation, prevalence of Aeromonas spp. in various fish intestine samples was analyzed and the results revealed that the significant level was recorded in all the sampling areas. During the period of sampling, about 20 fish samples were processed and 15 isolates i.e. 75% showed positive for Aeromonas.

A. hydrophila is reported in fresh fishes sold in retail outlets of Switzerland [20]. Conventional biochemical identification may lead to miss identification of Aeromonas isolates. So 16S rRNA gene based identification can be a suitable method to characterize Aeromonas isolates.

![Image](image-url)

Lane M- Marker 100-1000 bp ampiclon - 1050bp
Note: Distinct bands indicate amplification of 16S rRNA gene of Aeromonas isolates

Figure 1: Amplification of 16s rRNA gene

Table 1: Haemolytic and proteolytic activity of the Aeromonas isolates

<table>
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<tr>
<th>Isolates</th>
<th>Haemolytic activity</th>
<th>Proteolytic activity</th>
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<tbody>
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<td>1</td>
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<tr>
<td>2</td>
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<td>3</td>
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Previous studies have indicated that the pathogenic nature of Aeromonas spp. is in part, associated with the production of exoenzymes, such as proteases and lipases. In this regard, all the Aeromonas isolates used in this study were screened for protease producing ability on skim milk agar. In the present study about 15 Aeromonas isolates were screened and of which 11 isolates i.e. 73% showed proteolysis. About 94.8% of Aeromonas isolates from fish possessed protease activity. Specifically, 100% of A. hydrophila and
A. veronii bv. sobria were producers of protease, followed by 81.8% of A. caviae [23]. In this study, among the 15 isolates screened for both β-haemolysis and proteolytic activity, 40% of the isolates possessed both these activities, which was an indicative of pathogenic isolates in the marketed fish samples.

CONCLUSION

In this study about 40% of the isolates possessed both the β-haemolytic and proteolytic activity, while the presence of β-haemolytic and proteolytic activity among the Aeromonas isolates infers the existence of pathogenic isolates in marketed fish. This would pose serious public health concern. The existence of pathogenic Aeromonas isolates in marketed fish may lead to disease outbreaks possibly through cross contamination.

CONFLICT OF INTERESTS

Declared None

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