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Original Article

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, haemagglutinins produced by *A. hydrophila* support 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 Aeromonads [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 Ukkadam 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 (REMI). Overnight cultures (1.5 mL) were transferred to microfuge tubes (eppendorf) 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 8000 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].

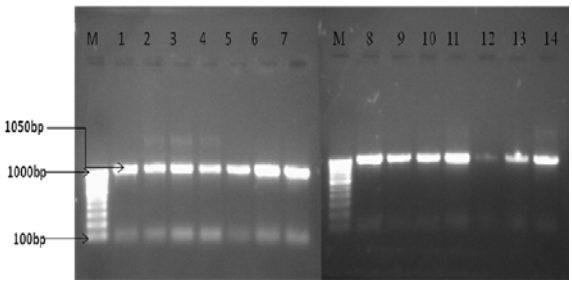
Proteolytic activity

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 incubated at 30°C. 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 *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 1050bp 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 research on the screening of *Aeromonas* in fish revealed that 22.3% of fishes were contaminated with *A. hydrophila* [17]. In the United Kingdom, the fish specimen from retail outlets exhibited 19.4% incidence of this organisms. About 28% of incidence of *Aeromonas* in fishes was recorded from retail outlets of New Zealand [18]. Maximum of incidence (89.8%) of *A. hydrophila* was recorded in marketed fish [19]. An extremely high prevalence (95.06%) of *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.



Lane M- Marker 100-1000 bp amplicon - 1050bp

Note: Distinct bands indicate amplification of 16S rRNA gene of *Aeromonas* isolates

Fig. 1: Amplification of 16s rRNA gene

Aeromonas spp. secretes several extracellular proteins including enterotoxin, haemolysin and aerolysin that are associated with the bacterial virulence. Previous studies have shown that two hemolytic toxins, haemolysin A and aerolysin A contribute to the virulence of *A. hydrophila* [21]. The production of haemolysins by the tested *Aeromonas* isolates indicates its pathogenicity. All the isolates were subjected to haemolytic activity on 5% blood agar plates. All the isolates showed varying haemolysis (α, β, γ) production. About 20% of the isolates showed α-haemolysis, 60% showed β-haemolysis and 20% showed γ-haemolysis (Table 1). β-haemolysis, complete lysis of the blood cells was found to be more prevalent among the isolates. This infers the presence of pathogenic *Aeromonas* isolates in the marketed fish samples. The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in Aeromonads [22].

Table 1: Haemolytic and proteolytic activity of the *Aeromonas* isolates

Isolates	Haemolytic activity	Proteolytic activity
1	β	+
2	α	+
3	γ	+
4	γ	+
5	β	+
6	β	+
7	β	-
8	β	+
9	β	-
10	β	-
11	α	+
12	β	+
13	γ	-
14	β	+
15	α	+

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, where 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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