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RESEARCH ARTICLE

SCREENING OF HAEMOLYTIC AEROMONAS SP. ISOLATED FROM MARINE FISH SAMPLES

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Abstract

Aeromonas species are facultatively anaerobic Gram negative bacterium that belongs to the family *Aeromonadaceae*. In the present study, 350 marine fish gut samples were collected and analyzed among which 200 samples showed the presence of *Aeromonas.* Starch ampicillin agar was used for *Aeromonas* isolation, and the colonies were identified phenotypicaly and genotypicaly through biochemical characterization and *rnp*B amplification respectively. Conventional biochemical identification is time consuming and may lead to misidentification of *Aeromonas* isolates, so molecular based identification- *rnp*B gene was used for the identification of *Aeromonas* isolates. Further haemolytic activity of the *Aeromonas* isolates were performed on 5% blood agar plates in which some revealed 44% of β haemolysis and over 8.4% showed α haemolytic activity but some isolates did not have any haemolytic activity i.e., 47% showed γ haemolysis. The presence of *hyl* gene which is responsible for haemolytic activity was also confirmed using PCR and 51.5% of the isolates were positive for *hyl* gene (550 bp). Thus the *Aeromonas* sp. is considered as an emerging pathogen and identified as a high-risk carrier.

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INTRODUCTION

Aeromonas is a gram-negative, facultative anaerobic rod that morphologically resembles members of the family Enterobacteriaceae. They are widely distributed in the environment, commonly inhabit aquatic environment and are also part of the normal intestinal microflora of healthy fish (Trust and Sparrow, 1974). Aeromonads are more commonly isolated from wide variety of sources including seafood (Snieszko, and Bullock, 1976; Vivekanandhan *et al*., 2005) and associated with economic loss in fish culture worldwide.Some *Aeromonas* species are recognized as pathogens to poikilothermic animals and humans. They are isolated from patients with diarrhoea, soft tissue infections, otitis, cystitis, septicaemia and extraintestinal diseases. The pathogenesis of *Aeromonas* infections is therefore complex and multifactorial. *Aeromonas* species produce an array of virulence factors including enterotoxins, hemolysin, exoenzymes, siderophores, flagella and secretion mechanisms (Castilho *et al*., 2009).

Housekeeping genes are considered to be better molecular markers than the 16S rRNA gene for the study of phylogenetic and taxonomic relationships at the species level. Differentiation of *chlamydial* strains and species has been shown to be possible using the RNase P RNA gene as a marker (Herrmann *et al*., 1996). Haemolysins are certain proteins and lipids that cause lysis of red blood cells by damaging their cell membrane. A series of bacteria are able to lyse erythrocytes, based on the activity of heterogeneous group of toxins, the Haemolysin (Scheffer *et al*., 1988). Three types of haemolysis can occur and are classified as α, β and γ haemolysis. α haemolysis, referred to as partial or incomplete haemolysis results due to incomplete lysis of the blood cells. β haemolysis refers to the complete lysis of the blood cells .γ haemolytic organisms are referred to as non-haemolytic and are identifiable based on a lack of change in the colour of the medium. The production of haemolytic toxins has been regarded as

strong evidence of pathogenic potential in aeromonads (Santos *et al.,* 1999; Turnbull *et al*., 1984). β hemolysin has been reported as a virulence factor in motile aeromonads (Majeed and MacRae, 1993).

MATERIALS AND METHODS

Bacterial source and maintenance of cultures

Fish specimens were randomly collected from fish retail outlets in sterile polyethylene bags and brought to the laboratory using an ice chest. Sample processing was done aseptically within 2 - 5 h of collection. 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

Polymerase Chain Reaction was done for screening of the *rnp*B gene by using genus specific primer with the expected size of 400 bp amplicon. Each reaction was carried out using 7µL of PCR master mix (Fermentas, USA), 3 µL of nuclease free water, 1.5µL of each forward and reverse primers (10pM) and 2 µL of template DNA (50ng). The PCR conditions used were 94°C for 30 seconds, 54.5°C for 30 seconds, 72°C for 1 minute, 72°C for 5 minutes and 30 cycles.

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% sheep 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.

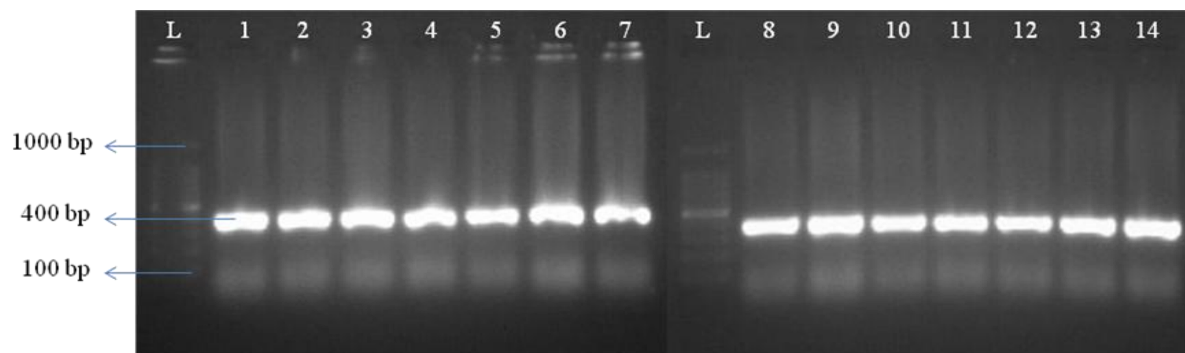
Detection of the *hyl* encoding haemolysins by PCR

The primers designed according to the conserved region of *A. veronii* biovar *sobria* and *A. hydrophila* by using Primer Premier Version 3 software. The reaction volume for this study was fixed as similar as in *rnp*B identification. The PCR conditions used were 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute, 72°C for 5 minutes and 30 cycles.

Results and Discussion

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 *rnp*B gene using genus specific primer. The *rnp*B gene is universally present in bacterial species and encodes the RNA subunit of endoribonuclease P, which is approximately 400 bp in length. This gene has been shown to be more suitable for phylogenetic discrimination of closely related taxa when compared with 16S rRNA sequences due to its higher rate of nucleotide variation (Tapp *et al*., 2003). In this study, *Aeromonas* sp. were identified by conventional and genotypic methods. To avoid misidentification, all the isolates were confirmed on the basis of *rnp*B gene amplification using a genus specific primer.Total DNA obtained from the isolates were quantified and 1 µg concentration of template DNA was subjected to *rnp*B gene amplification using genus specific primers. The expected amplicon size (400 bp) was observed in 200 isolates that were positive for *Aeromonas* (figure 1) in conventional tests.

Figure 1 Amplification of *rnp*B gene of *Aeromonas* sp.



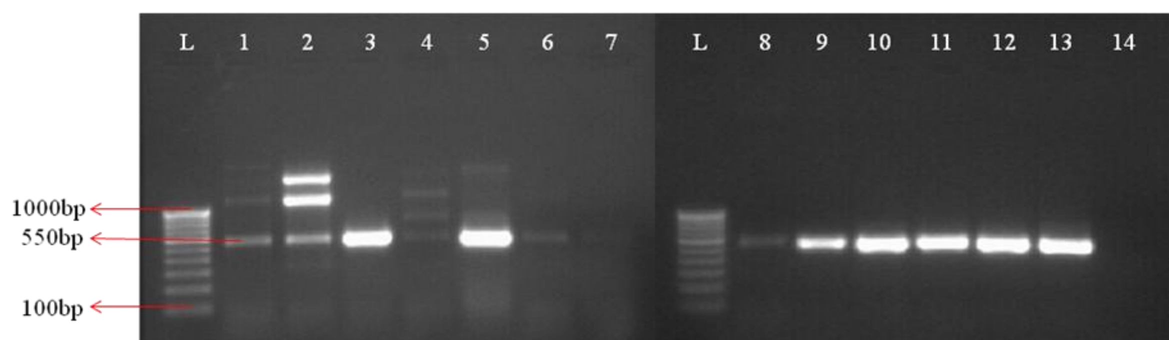
Lane L- Marker 100-1000bp, 400bp – amplicon

Distinct band in all the lanes except lane 15 indicates amplification of *rnp*B of *Aeromonas* isolates

Haemolysins are exotoxin protein produced by bacteria and the lytic activities of haemolysins on red blood cells are reported to be important for nutrient acquisition or for causing certain conditions such as anaemia (Griffiths *et al*., 1988). Haemolytic proteins are commonly isolated from pathogenic bacteria, and β-haemolysins are one of the important bacterial virulence factors (Erova *et al*., 2007). In a study conducted by Illanchezian *et al*. (2010) in Chennai, Tamil Nadu, it was found that 43.8% of *Aeromonas* isolated from 5 major fish markets, exhibited β-haemolysis. From this it is clear that the environmental aeromonads also possess pathogenic potential and may be a potential contaminant of water supplies, fishes and widespread in seafood.

In a study by Radu *et al.* (2003), observed that more than 90% of *Aeromonas* sp. isolated from fish market of Malaysia showed hemolytic activity. Chopra *et al*. (1991) and Wong *et al*. (1998) reported that, among the various virulence factors ascribed to explain the pathogenicity of *Aeromonas* sp, haemolytic activity seems to be related to enterotoxigenicity. Singh and Sanyal (1997) stated that the production of β-haemolysin could be correlated with enterotoxicity. Rahim *et al*. (2004) reported β-haemolytic activity by *A. hydrophila* around the wells on sheep blood agar plate. The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in Aeromonads and the property of haemolysis on blood agar is directly related to enteropathogenecity (Janda and Abbott, 2010). Ghenghesh *et al*., (2001) reported that 40% of *Aeromonas* sp. isolated from well water showed haemolytic activity. Thayumanavan *et al*. (2007) performed haemolytic activity for *A. hydrophila* isolates and reported that 84.9% of the isolates were β-haemolysin producers. According to Abbott *et al*. (2003), the increased activity of clinical isolates is due to horizontal transfer of haemolysin genes present in haemolytic *Aeromonas* to non-haemolytic species was recorded. Tsai and Chen,(1996) reported that about 87.5 and 100% of the strains of *A. hydrophila* isolated from fish and prawns of Taiwan exhibited haemolytic activity.The haemolytic activity of the isolates in the present study points to the potential pathogenic significance of *Aeromonas* sp. Since majority of the isolates in the present study showed haemolytic activity, this phenotype could be attributed to the activity of a product of *hyl* gene/ haemolysin gene. The presence of *hyl* gene which is responsible for haemolytic activity was also confirmed using PCR and 51.5% of the isolates were positive for *hyl* gene (550 bp) and among metagenomic samples 42% showed the presence of *hyl* gene. A possible explanation for the difference in *hyl* gene percentage and β-haemolytic activity might be due to the fact that gene is present but expression is low.

Figure 2 Amplification of *hyl* gene of *Aeromonas* sp.



Lane L- Marker 100-1000bp & 500-5000bp

550bp - amplicon

Distinct band in all lanes except lanes 14 indicates amplification of *hyl* of *Aeromonas* sp

Aeromonas sp. isolated from environmental and shellfish samples revealed that out of 38 isolates, 20 showed the presence of *hly*A gene (Yousr *et al*., 2007). Yogananth *et al*. (2009) also confirmed the haemolytic activity of *A. hydrophila* isolated from fish using the amplification of *hyl* gene. Escarpulli *et al*., (2003) in their work reported that 96% of *Aeromonas* sp. isolated from frozen fish from local markets of Mexico city carried haemolysin gene. The method described here may be a useful tool to assist the detection of *hyl* gene in *Aeromonas*, especially for food microbiologist. The presence of haemolysin gene in our isolates from marine fishes shows that they are virulent isolates and they are transferrable to humans during handling.

CONCLUSION

In this study about 44% of the isolates possessed the β-haemolytic activity.These findings show that the *Aeromonas* sp. isolated from marine fish gut are enterotoxigenic. This would pose serious public health concern. Thus the *Aeromonas* sp.is considered as emerging pathogen and identified as a high-risk carrier.

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ETHICAL STATEMENT

The authors declare that the manuscript has not been submitted to more than one journal for simultaneous consideration, has not been published previously, none of the data have been fabricated or manipulated**.**

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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