Typing of *Aeromonas hydrophila* of fish and human diarrhoeal origin by outer membrane proteins and lipopolysaccharides

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Abstract A study was undertaken to discriminate the strains of *Aeromonas hydrophila* isolated from fish and diarrhoeal samples by SDS-PAGE analysis of outer membrane proteins (OMPs) and lipopolysaccharides (LPSs). Common bands at 47 kDa positions for OMPs and at 31–38 kDa for LPSs were observed. No strain of *A. hydrophila* from clinical or fish samples was found identical in either OMPs or LPSs profile.

Key words · *Aeromonas hydrophila* · fish · diarrhoea · outer membrane proteins · lipopolysaccharides.

Introduction

Aeromonas hydrophila is an opportunistic as well as primary bacterial pathogen of a variety of aquatic and terrestrial animals including humans¹. The clinical manifestation ranges from gastroenteritis to soft tissue infection including septicaemia, urinary tract infections, ocular infections, peritonitis and meningitis². A strong association between gastroenteritis and *Aeromonas* has been shown in children and immunocompromised patients and frequently from patients of acute diarrhoea³.

These strains have also been isolated from a wide variety of sources including water, soil and human. *A. hydrophila* of the clinical samples were reported to be different from the isolates of water and fish⁴. This species is also a specific agent of disease in warm and freshwater fish⁵ and capable of producing hemorrhagic septicaemia, similar to those caused by other Gram-negative bacteria. A number of fish species including European eels⁶ have been reported to be infected by these organisms. *Aeromonas* strains have been serotyped on the basis of somatic antigen lipopolysaccharide⁷. There are several reports supporting the cell surface virulence factors of *A. hydrophila*.

Lipopolysaccharide (LPS) and carbohydrate reactive outer membrane proteins (OMP) have been implicated as *Aeromonas* intestinal colonization factors which lead to cause infections and act as prominent antigens. They play an important role in pathogenicity of many bacterial pathogens⁸. It activates the cellular immunity and induces a cytokine mediated systemic inflammatory response in the host⁹. On the contrary, a high variability in profiles of outer membrane protein (OMP) and lipopolysaccharide (LPS) has been described in *A. hydrophila* recovered from fresh and marine water fish.

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The main virulence factors of *Aeromonas* spp. can be associated with the liberation of cell surface factors, cell associated endotoxin⁷ and other biologically active extracellular products. Haemolysin, cytotoxin, enterotoxin, cytolytic enterotoxin (aerolysin) and other cell wall toxic components such as protease, lipopolysaccharides and outer membrane protein play important role in toxigenicity¹⁰.

The study on virulence characters, profiles of proteins and lipopolysaccharides can provide useful information with regard to the pathogenicity of *A. hydrophila*. The profiles can explain diversity among the strains of *A. hydrophila* based on their source. Although the role of *Aeromonas* in clinical infections has been reported extensively in various parts of the world, the incidence of infections with respect to Indian subcontinent has not been given much attention unlike *Vibrio* and other members of *Enterobacteriaceae*. The objective of this study was to determine the diversity of *A. hydrophila* strains among the fish and diarrhoeal isolates, based on the OMP and LPS profiles.

Materials and Methods

Bacterial strains: The haemolysin positive strains of *A. hydrophila*, which were isolated from fish and diarrhoeal sources (data not shown) were used. Three strains from fish (*Ah* F1, *Ah* F2 and *Ah* F3) and three strains from diarrhoeal (*Ah* D1, *Ah* D2 and *Ah* D3) sources were subjected to study of outer membrane proteins and lipopolysaccharides.

Preparation and extraction of outer membrane protein (OMP): The outer membrane proteins of *A. hydrophila* strains were prepared from overnight cultures in brain heart infusion (BHI) broth. Outer membranes were obtained by incubating the membrane suspension with Sarkosyl Tris HCl buffer (3% Sodium Lauryl Sarkocinate; 20mM Tris HCl; pH-8) for 20 min at room temperature. The cells were sonicated in phosphate buffered saline (PBS) and incubated at 80°C for 2 h in a water bath. After incubation, the contents were centrifuged at 16,000 rpm for 20 min. The pellets were dried and dissolved in minimum volume of PBS (1X) buffer¹¹. The protein was estimated by the method of Lowry *et al.*¹² The suspension was mixed with electrophoresis samples buffer (Tris HCl – 1.25 mL; glycerol – 1 mL; -mercaptoethanol – 0.5 mL; SDS (10 %) – 2 mL; bromophenol blue – 0.1 %) and boiled at 60°C for 1 min¹¹. The sample was loaded on to 1.5 mm, 12.5 % sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS - PAGE) gels.

Preparation and extraction of Lipopolysaccharide (LPS): The 24 h old culture on BHI broth was centrifuged at 12000 rpm for 15 min and the pellet was suspended in

50 mL of PBS. It was mixed with double strength electrophoresis sample buffer at 1:1 ratio and boiled for 10 min¹¹. The samples were digested with 30 µL of Proteinase K, electrophoresis sample buffer (0.25%), boiled at 60°C for 15 min and loaded on to 1.5 mm 12.5 % SDS-PAGE gel¹¹. Twenty milliliter of LPS suspension was applied in each slot. The separated LPSs were visualized by silver staining according to the method of Tsai & Frasch¹³.

Results and Discussion

The OMP and LPS profiles of *Ah* F1, *Ah* F2, *Ah* F3, *Ah* D1, *Ah* D2 and *Ah* D3 isolated from fish (lane 1 – 3) and diarrhoeal samples (lane 4 – 6) are shown in Fig. 1 and Fig. 2 respectively. The OMP profiles on 12.5 % SDS-PAGE showed the maximum molecular weight of 79 kDa in the fish strains of *A. hydrophila*. The OMP molecular banding pattern existed between 33 to 56 kDa in fish and diarrhoeal strains. Maji *et al.*¹⁴ (2006) reported 57 kDa and 23 kDa polypeptides of the OMP of *A. hydrophila*, possessing high immunoreactivity. Esteve *et al.*¹¹ found that all the Spanish isolates had two major bands with 45 and 50 kDa, while the Japanese and other isolates had different bands with varying molecular weight. Identical profiles OMP have been described in strains of *A. hydrophila* and *A. salmonicida*¹⁵. They showed similar features as our isolates. Based on the nature of S-layer or external / internal protein complexes, different bands may be exhibited in the acrylamide gel. The presence of species specific OMP indicated that these immunogenic polypeptides could be exploited to develop antibody-based diagnosis and the identification of *A. hydrophila*¹⁵. Esteve *et al.*¹¹ observed that the OMP bands ranged between 36 to 45 kDa in fish isolates of *A. hydrophila* and Kokka *et al.*¹⁵ reported that OMP of *A. hydrophila* O: 11 strains were found to contain a prominent band at the position of 52 to 54 kDa. The observation of 40 to 54 kDa OMPs are largely conserved among different mesophilic strains. Our results revealed that diarrhoeal and fish isolates of *Aeromonas* strains had variability in the OMP profile. Similar results were reported by Kuijper *et al.*¹⁷. Dooley & Trust¹⁸ reported three major antigenic outer membrane proteins such as 48, 50 and 52 kDa. The 52 kDa protein appeared to be the most abundant one. Lakshmanaperumalsamy *et al.*¹⁹ also reported that *A. hydrophila* strains shared a common band with 52 kDa of protein. The presence of species specific OMP indicated that these immunogenic polypeptides could be exploited to develop antibody-based diagnosis and the identification of *A. hydrophila*¹⁵.

The electrophoretic profiles of the selected strains of *A. hydrophila* showed LPSs with O polysaccharide side chains

Fig. 1 SDS–PAGE profiles of the outer membrane proteins of *A. hydrophila*, Lane 1 - *Ah* F1, Lane 2 - *Ah* F2, Lane 3 - *Ah* F3, Lane 4 - *Ah* D1, Lane 5 - *Ah* D2, Lane 6 - *Ah* D3, Lane M – Marker [PMWB-Protein marker (3–205 kDa)].

Fig. 2 SDS –PAGE profiles of the lipopolysaccharides of *A. hydrophila*, Lane 1 - *Ah* F1, Lane 2 - *Ah* F2, Lane 3 - *Ah* F3, Lane 4 - *Ah* D1, Lane 5 - *Ah* D2, Lane 6 - *Ah* D3.

of heterogeneous length (ladder like LPS). LPS banding patterns might be different depending on the serotypes of the strains. All the fish and clinical isolates exhibited similar banding pattern ranging between 31 to 38 kDa.

Antigenic LPSs patterns displaying O – polysaccharide of homogenous chain length were characterized by three or four bands¹¹. The fish isolates exhibited several bands and their molecular weights ranged from 13 to 59.5 kDa. The diarrhoeal isolates exhibited the LPSs bands ranging from 32 to 89 kDa. Previous research showed that the LPSs antigenicity ranged between 20 to 65 kDa¹⁵. Kokka *et al.*¹⁶ showed that homogeneous length O- polysaccharide side chains were characterized primarily by two or three bands and silver stain analysis of LPS profiles mainly consisted of core antigens. Our data relating to the banding position of the fish and diarrhoeal isolates showed similar results. *A. jandaei* produced diffused fast migrating bands, which may carry a lipid A core oligosaccharide fractions⁸. Our clinical isolates also produced diffusible bands (lane 1 and 4) with a molecular weight of 38 kDa. *A. hydrophila* from human leg wound, *A. sobria* and *A. caviae* from faecal samples showed bands ranging between 14 to 45 kDa¹⁵. In our study, the molecular weight of LPSs was higher than that of the above mentioned report, and it was in the range of 31 to 38 kDa. The LPS production was more at 37°C with high osmolarity and it has high virulence¹⁰. So it is easily expressed at human body temperature when the osmolarity increased in the ileum lumen. The high molecular weight bands of LPS might have played a role in the better performance of biofilm oral vaccine by eliciting a protective immune response²⁰.

The recent taxonomic changes with in the genus have assisted in establishing a correlation between the relative virulence of aeromonads to humans²¹. The species – specific proteins and lipopolysaccharides defined in this study, may be helpful in the detection and diagnosis of diarrhoeagenic aeromonads directly from stool samples in infected human. The comparison of outer membrane proteins and LPSs of both the fish and diarrhoeal isolates revealed the presence of more or less similar molecular weight LPSs and OMPs. It is also clear that food and environment may serve as the media for transmission of virulent *A. hydrophila* infection in human²².

On the other hand, this bacterium expresses a number of heat-stable exotoxins and exoenzymes, which may withstand heat treatment. Liberation of exotoxins is reported to be dependent on the presence of the O-antigen LPSs. Additionally, LPSs of *A. hydrophila* have pathogenic effects on animals. This is based on the strain – specific mechanism where the O-antigen LPS are lacking in rough strains but not in smooth strains. *A. hydrophila* strains with 47 to 54 kDa OMPs and 14 to 45 kDa LPSs are reported to be highly toxic¹⁵. *A. hydrophila* strains isolated from fish and diarrhoeal samples showed 33 to 56 kDa OMPs and 31 to 38 kDa LPSs. Asao et al.²³ first purified the haemolysin and it showed molecular weight of 49 to 52 kDa. The collective results may offer explanations and heterogeneity of OMP and LPS of *A. hydrophila* among the fish and diarrhoeal sources. The above property may express to their virulence, disease spectrum and pathogenic properties in to host. They may also express more virulent phenotypes as a result of gene activation through bacterial communication (quorum sensing) or gene transfer among human and environmental strains of *A. hydrophila*

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