Seroreactivity of somatic soluble proteins of *Vibrio alginolyticus*

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ABSTRACT

Serological characterization of *V. alginolyticus* was performed which might be quite useful in adopting immunodiagnostic approaches to detect vibriosis in shrimp. The sero-reactivity of *V. alginolyticus* soluble somatic protein antigen was assessed by agar gel precipitation test (AGPT), counter current immunoelectrophoresis and enzyme linked immunosorbent assay (ELISA) using hyperimmune serum against *V. alginolyticus*, raised in rabbit. SDS-PAGE analysis revealed 21 polypeptides in the range of 15 to 103 kDa in *V. alginolyticus* somatic soluble antigen preparation. No cross-reactivity of *V. alginolyticus* soluble protein antigen was noted with related pathogens, viz. *V. harveyi*, *V. fluvialis* and *Pseudomonas* sp. when counter current immunoelectrophoresis (CIE) was performed. From the present study, it was concluded that seroreactivity exists in *V. alginolyticus* somatic soluble proteins that might be exploited in diagnosis of vibriosis in shrimp.

Vibriosis is a major scourge of marine fish and shellfish worldwide (Egidius, 1987; Lightner, 1988; Austin and Austin, 1993). It can cause mortalities up to 100% in shrimps (Lightner, 1983). Causative agents of this disease are several *Vibrio* spp., *Vibrio alginolyticus* being one of the predominant species. Several identification techniques can be adopted for proper diagnosis of vibriosis having varying degree of practical utility. Out of those diagnostic methods, serological techniques may be preferred for rapid and cost-effective diagnosis of vibriosis in shrimp aquaculture. Seroreactivity of *V. vulnificus* sonicated antigens with specific antiserum was assessed by Nishibuchi and Seidler (1985) using agar gel precipitation test. ELISA and dot immunoassay (DIA) are sensitive, specific and rapid tests that have been used for animal disease diagnosis including those of shrimps (Waterstart et al., 1989; Castro et al., 1995). Mishra and Sekhar (1997) performed dot-ELISA for detection of *Vibrio* spp. in tiger shrimp *P. monodon*. They used different types of *V. parahaemolyticus* antigens in ELISA for testing their suitability as coating antigen. To characterize protein antigen mixture at the polypeptide level, SDS-PAGE can be used to resolve in smaller components. Like other
pathogenic antigens, several workers utilized SDS-PAGE to characterize vibrio antigens. Bogwald et al. (1991) performed SDS-PAGE and found the most important antigen on the surface of V. salmonicida were the rough form of LPS (< 14 kDa) and a 24 kDa protein. SDS-PAGE was performed to observe the comparison of different serotyping systems of V.anguillarum and V.ordali (Grisez and Ollever, 1995). Each of these serotype is characterized by a distinct polysaccharide banding pattern. SDS-PAGE was performed to observe the pathogenicity of V.harveyi to salmonids (Zhang and Austin, 2000). However, reports regarding serological reactivity of the protein antigens of V. alginolyticus is limited. In this context, the present work was undertaken to prepare and characterize somatic soluble protein antigens of V.alginolyticus by using different serological techniques that might be quite useful in serodiagnostic approaches.

Bacterial isolate (V. alginolyticus) was obtained from the laboratory of the Department of Fishery Pathology and Microbiology, Faculty of Fishery Sciences, WBUAFS. Biochemical tests were performed as per West and Colwell (1984) and identification of vibrio at species level was done as per Alisna and Blanch (1994).

For preparation of bulk culture, nutrient agar with 1% NaCl was used. V.alginolyticus strain from the pure culture was taken with sterile loop and transferred into the nutrient broth and kept for 24 hours in incubator at 37°C. On the next day, young broth culture was transferred to nutrient agar plate using sterile swabs and was incubated for 20-24 hours at 37°C. To harvest live bacteria, 5-10 ml sterile physiological saline was added to each plate and cells were removed with a sterile spatula. Heat killed antigen was prepared according to Mishra and Sekhar (1997) with some modifications. Briefly, live culture was taken in sterile tube and kept in a water bath for 30 min at 65°C. Then the tube was cooled and kept at -20°C. The somatic (sonicated) antigens were prepared by disintegration in an ultrasonicator (Anothos, Austria) for 30 cycles @ 60 sec / cycle with 30 sec rest each) using a titanium probe operated at 8-10 µm peak to peak at 0°C. Soluble sonicated extracts were centrifuged at 10,000 rpm for 30 min at 4°C. The pellet obtained (disrupted bacterial cells) was stained and observed under microscope to ascertain adequate disruption. Soluble supernatant was sterilized using 0.22 µm filters and the protein content was determined as per the method of Lowry et al. (1951).

Hyperimmunisation of rabbit for antiserum preparation against V.alginolyticus was done according to Mishra and Sekhar (1997). Briefly, one healthy NZW breed rabbits (male) weighing 800 gm was injected deep intramuscularly with 5 doses of heat killed V.alginolyticus antigen mixed with equal volume of Freund's complete adjuvant (Sigma, USA) and subsequently with Freund's incomplete adjuvant at 10 days interval. Inoculation in the form of booster was one with an increasing dose of 600 µg - 1200 µg of proteins. Serum was collected 6 days after the last injection and kept at -20°C until used.

Agar gel precipitation test (AGPT) of sonicated soluble antigen and hyperimmune serum was done using 0.9% agarose gel, made in normal saline solution. Antigen was placed in central well and antiserum (diluted) in peripheral wells. Different two fold
dilutions of antiserum (i.e. neat, 1:2, 1:4 and 1:8 ratio) were used. Enzyme linked immunosorbent assay (ELISA) was performed according to Mishra et al. (1997). Soluble V. alginolyticus protein antigens were analysed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as per Laemmli (1970). Counter current immunoelectrophoresis (CIE) of V. alginolyticus somatic antigen and antiserum was performed in 1% agarose gel, prepared in 0.05 M tris-boarate buffer, pH 8.6 for one hour at 3mA constant current.

Cross-reactivity of V. alginolyticus antigen with other antigens (mentioned below) was carried out using CIE. Here, four pairs of well were made. One well of every pair was filled with V. alginolyticus antiserum and the other well of second pair was filled with V. alginolyticus antigen. The remaining wells were filled with V. harveyi, V. fluvialis and Pseudomonas soluble somatic antigens (obtained from the Department’s laboratory).

To assess sero-reactivity of V. alginolyticus sonicated antigen, AGPT, CIE and ELISA were performed using V. alginolyticus antiserum, raised in rabbit. While performing AGPT, different dilutions of V. alginolyticus antiserum (i.e. neat, 1:2, 1:4, 1:8 ratio) was used. After 24 hours, clear precipitating bands were observed with all the dilutions of antiserum, indicating the titre of the antiserum was more than 8 (results not shown). In a similar study, immunodiffusion of sonicated V. vulnificus cells illustrated a single major precipitation line with antiserum prepared from whole cells of the same species (Nishibuchi and Seidler, 1985). In CIE, clear multiple precipitating bands were observed when V. alginolyticus soluble antigen and corresponding antiserum were used (Fig.1). Multiple band formation indicated that antigen preparation was a mixture of several proteins against which several polyclonal antibodies were present in the rabbit antiserum. The
reactivity of the antigens with different dilutions of rabbit antiserum as assessed in ELISA was expressed in O.D. values (at 492 nm) and shown in Fig. 2. In all the cases (i.e. 1:50, 1:100 and 1:200 dilutions of the serum), the values of hyperimmune serum were considerably higher than the normal rabbit serum, indicating specific seroreactivity of V. alginolyticus with its anti-serum. Earlier, specific seroreactivity of V. alginolyticus, V. anguillarum and V. parahaemolyticus was assessed by ELISA (Adams, 1991).

When V. alginolyticus soluble somatic antigen was resolved in 12.5% polyacrylamide gel, 21 polypeptide bands were observed upon Coomassie blue staining (Fig. 3). Out of 21, 7 were major bands and 14 were minor. The individual molecular weights of the polypeptides were 105, 103, 101, 98, 96, 84, 72, 70, 65, 68, 54, 50, 48, 40, 35, 30, 25, 4, 22, 18 and 16 kDa. SDS-PAGE was used by several workers to assess the antigenic profile of protein antigens of vibrio (Grisez and Ollevier, 1995; Montero and Austin, 1999). SDS-PAGE analysis of cell envelope proteins of V. anguillarum showed that regardless of the sero groups of source of isolation, all the strains possessed protein components with molecular masses ranging between 97 to 14 kDa (Santos et al., 1995). Analysis of cell envelope proteins showed that V. anguillarum related organisms possessed major protein components with molecular weight ranging form
approximately 33-45 kDa (Santos et al., 1997). Here, we report V. alginolyticus somatic soluble antigen possess 21 polypeptide components ranging from 105 to 16 kDa.

There was no band formation in CIE when V. alginolyticus antiserum and of V. harveyi, V. fluvialis and Pseudomonas antigens were used (Fig.4). V. alginolyticus antiserum showed band formation only with V. alginolyticus antigen, indicating no crossreactivity with related species. In short, from the present study it can be concluded that serological reactivity exists in V. alginolyticus soluble antigen as assessed in AGPT, ELISA and CIE that can be exploited in serodiagnosis of vibriosis in shrimp. Furthermore, V. alginolyticus somatic soluble antigen was found to be a mixture of 21 polypeptides in the range of 16 kDa. Hence, identification of immunodominant polypeptide antigens(s) by Western blot analysis should be the next step while preparing serodiagnostic kit for vibriosis.

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**References**


