Diagnostic potentiality of fractionated antigen of \textit{Aeromonas hydrophila} isolated from gold fish, \textit{Carassius auratus} (Linn.)

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ABSTRACT
With the aim of isolating purified antigens of extra cellular products (ECP) of \textit{A. hydrophila} that might be exploited for early sero-diagnosis, bacteria was isolated from ulcerative lesions of gold fish. Polypeptide profile of ECP of \textit{A. hydrophila} showed 12 bands having molecular weight in the range of 22 – 55 kDa in SDS-PAGE analysis. Fractionation (By DEAE-cellulose) and serological characterization of ECP were subsequently performed. One of the fractionated antigen (D1) possessing mainly of 45 kDa polypeptide revealed sero-reactivity with \textit{A. hydrophila} antiserum having no cross-reactive component(s) with related bacterial antigens, viz. \textit{Vibrio harveyi}, \textit{V. alginolyticus} and \textit{Pseudomonas fluorescence}. Hence, 45 kDa polypeptide of ECP of \textit{A. hydrophila} showing potentiality of sero-diagnosis should be given due attention.

Introduction
Among different cultured ornamental fishes, gold fish constitute the maximum share of profit but are infected by ulcerative lesions frequently, causing often high mortality in winter season. \textit{Aeromonas hydrophila} is associated with this dreadful bacterial disease. Though motile \textit{Aeromonas} species are wide-spread inhabitants of aquatic environments and are reported to be constituents of normal gut flora of fish (Neilson, 1978; Trust and Sparrow, 1974), strains of \textit{Aeromonas hydrophila}, most important opportunistic pathogens of fresh water aquarium fishes, are commonly associated with ulceration. \textit{Aeromonas hydrophila} produces a variety of extracellular products (ECP) including toxins, haemolysins and proteases (Allan and Stevenson, 1981; Ljungh and Wadstrom, 1982; Dooley and Trust, 1988) that constitute the virulence factors of this bacterium for ornamental fishes. Development of serodiagnostic kit for early detection of this disease is vital for the aquaculture industry. It may be mentioned here that there is paucity of published information on fractionation, characterization and serological reactivity of ECP antigen of \textit{Aeromonas hydrophila} isolated from ulcerative lesion of gold fish. In-fact, knowledge of antigenic components of \textit{Aeromonas hydrophila} is quite needed while taking serodiagnostic approaches. In this context, the aim of the present work was to perform immunobiochemical
Material and methods

Bacteria: In this study, 12 bacterial isolates were generated from ulcerative lesions of moribund gold fishes. All the strains were isolated using Tryptose Soya Agar (TSA), Selective Starch Ampicillin Agar (SAA) and Pseudomonas Agar (PA) (Himedia, Bombay). All the isolates were identified on the basis of morphological, physiological and biochemical tests following basically the classification of Popoff and Veron (1976) and the identifications were confirmed using BIOLOG following Dierckens et al. (1998).

Antigen: The extracellular products (ECP) from a representative strain of Aeromonas hydrophila were obtained using cellophane technique (Liu, 1957) and the protein content in the ECP was estimated as per the method of Lowry et al. (1951).

Fractionation of crude antigen: ECP was then fractionated using DEAE – cellulose as column bed-material. The column was equilibrated with tris-HCl (pH 8) buffer and the bound proteins were eluted using continuous elution buffer (0.025 M tris – HCl, pH 8 containing 3M Urea) prepared from different molalities of sodium chloride ranging from 0.15 M to 0.3 M (Joardar and Ram, 1999). The elution was collected in test tubes containing 3 ml each and optical density of each fraction was measured at 280 nm.

SDS-PAGE: Protein samples (both crude and fractionated ECP) were denatured and run on SDS – PAGE gel (12.5%) according to the procedure of Laemmli (1970). The polypeptides were stained by coomassie blue R 250 and the molecular masses were determined by comparison with medium molecular weight marker.

Antiserum: Antiserum was raised against crude ECP of Aeromonas hydrophila following Mishra and Sekhar (1997) with some modifications. Briefly, one healthy New Zealand White (NZW) male rabbit (weighing 900 gm) was injected deep intramuscularly with 5 doses of ECP antigens mixed with equal volume of adjuvant (Sigma, USA) at 10 days intervals with increased subsequent doses ranging from 600 µg to 1200 µg per injection. First dose was given with Freund's Complete Adjuvants (FCA) and subsequent four doses with Freund's Incomplete Adjuvants (FIA).

Seroreactivity: To determine the immunogenicity of fractionated antigen of ECP, agar gel precipitation test (AGPT) was performed using this fractionated antigen and antiserum to crude ECP.

Cross-reactivity: To determine cross-reactivity of the fractionated antigen with crude ECP antigens of related bacteria viz. Vibrio alginolyticus, V. harveyi, Pseudomonas fluorescence (obtained from the Department of Fishery Pathology and Microbiology, WBUAFS) counter – current immunoelectrophoresis (CIE) was performed using the antigens and the antiserum, raised against crude ECP antigen of A. hydrophila.

Results and discussion

In this study, all the isolated strains were identified as Aeromonas hydrophila. The protein content of the crude ECP antigen of the representative isolate was obtained to be 1.71 mg/ml. It was quite higher than the ECP antigen (0.07 – 0.08 mg/ml) produced by Santos.
Polypeptide profile (Fig. 1) of extracellular products (ECP) of *A. hydrophila* by SDS–PAGE analysis showed 12 bands having molecular weight in the range of 22 to 55 kDa. Common bands in the range of 20–97 kDa were observed when SDS–PAGE was done with outer membrane proteins (OMP) of *A. hydrophila* (Rahman et al., 2001). In the *A. hydrophila* strains examined, major protein of molecular weight 30 kDa and proteins in the molecular weight range of 45 to 55 kDa were observed (Dooley and Trust, 1988).

To identify the principle component(s) of ECP antigen responsible for seroreactivity, fractionation of the crude antigen was performed using anion–exchange chromatography. One prominent peak (D1) containing positively charged cationic proteins followed by one prominent peak (D2) was observed using salt gradient in elution buffer, indicating presence of anionic proteins (Fig. 2). Protein in those peaks were pooled, sucrose concentrated, filter sterilized and kept at -20°C in aliquots.

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**Fig. 1:** Polypeptide profile of crude and fractionated antigens of *A. hydrophila* as assessed by SDS-PAGE.

Lane M – Molecular weight marker (medium range)
Lane C – Crude antigen
Lane F – Fractionated (D1) antigen

**Fig. 2:** DEAE-cellulose column chromatography of ECP antigen of *A. hydrophila*. Buffer used for elusion - Tris-HCl (0.025 M, pH 8) containing 3M urea with salt gradient of NaCl (0.15-0.3M).

**Fig. 3:** Sero-reactivity of fractionated antigen (D1) with hyper-immune serum as assessed by agar gel precipitation test.

Ag : Fractionated (D1) antigen
Ab : *A. hydrophila* (crude ECP) antiserum
Two fractionated antigens (viz. D\textsubscript{1} and D\textsubscript{2}) thus obtained were subsequently subjected to AGPT analysis to determine their seroreactivity. But only D\textsubscript{1} revealed seroreactivity showing clear precipitation in AGPT (Fig. 3).

A 45 kDa polypeptide was observed as major band when D\textsubscript{1} was analyzed by SDS-PAGE (Fig. 1) and this result corroborates with the observation of Perez et al. (2002) who fractionated ECP by molecular exclusion chromatography. An extracellular toxin, produced by A. hydrophila from cultured crucian carp with septicemia, was fractionated by column chromatography using DEAE-cellulose and Sephadex G-100. The fraction was a single polypeptide with a molecular weight of 52.5 kDa as determined by SDS–PAGE (Tu and Lin, 1992).

Seroreactivity of antigens prepared from Aeromonas hydrophila was shown by Leblanc et al. (1981). In the present study, immunogenecity of crude ECP antigen was observed while performing agar gel precipitation test (AGPT) (result not shown). Fractionated ECP antigen (D\textsubscript{1}) constituted mainly of 45 kDa polypeptide, showed reactivity (by forming precipitation) with the ECP antigen of A. hydrophila but no cross-reactivity (band formation) was observed with the ECP antigens of V. harveyi, V. alginolyticus and Pseudomonas fluorescence in CIE analysis (Fig. 4). Earlier, sero-specificity was shown with the antiserum raised against protease fractionated from ECP of A. hydrophila (Strain ATCC 7966) but this protease had no cross-reaction with protease of ECP from another strain of A. hydrophila (Strain NRC 505) (Leung and Stevenson, 1988).

From our present study, it was clear that one of the fractionated antigens (D\textsubscript{1}) of ECP of A. hydrophila having seroreactivity, with no cross-reactive components (epitopes) with related bacterial antigens might be exploited in diagnostic preparation(s).

Hence, 45 kDa polypeptide of ECP of Aeromonas hydrophila possessing potentiality of sero-diagnosis should be given due attention to exploit it as a tool for rapid early sero-diagnosis of ulcerative conditions in gold fish.
Acknowledgement

Authors are thankful to the Vice-Chancellor, WBUAFS, for providing necessary research facilities.

References


