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Haemagglutinin and Chitinase Activities of Virulent Aeromonas Hydrophila Islolated from Cyprinus Carpio

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Abstract: In the present study the Hemeagglutination and Chitinolytic activity of extracellular products (ECP) secreted from Aeromonas hydrophila were examined. The present study showed infectivity experiments, incomplete creation of extracellular products and chitinase activity of the A. hydrophila. In haemagglutination assay in the present experiment, bacterial agglutination takes place in 1:1, 1:2, 1:4, 1:8,1:16,1:32 dilutions in the E.S-2 treated O blood groups and in 1:1,1:4 where as the ES -1and ES-3 failed to agglutinate. Generally, whole cells showed a wider range of enzymic activities than ECP. The results showed that extra cellular product chitin could be a promising source for pathogenic factor in microorganisms.

Key words: Extracellular Products, A. Hydrophila, C. Carpio, Pathogenic

1. INTRODUCTION

Possible virulence factors of *A. hydrophila*, which contribute to their pathogenicity, comprise the manufacture of extra cellular enterotoxins, hemolysin, cytotoxins and protease, the ability to stick on the cells, and the control of certain surface proteins (Howard and Buckley, 1985). Extracellular products (ECPs) include Siderophore for iron acquistion and array of exoenzymes and exotoxins (Santos *et al.*, 1987). These virulent determinants, most of whose the linkages of action remain to be determined, are taking an active part in making the bacteria to colonize, gain entry, establish, replicate and cause damage in host tissues and to avoid the host defense system and spread, finally killing the host (Yu *et al.*, 2004). Also, outer membrane plays an important role in infection and pathogenicity to the host (Tsolis, 2002). It is basically composed of protein, lipid and sugar, which could be accepted without difficulty as foreign substances by the host's immunological defense systems.

The ability of a pathogen to locate, to attach, and subsequently infect a susceptible host is a primary step in the maturity of the disease. The factors produced by motile aeromonads, which can facilitate contagion, are essential part of bacterial virulence. The moving aeromonads that have been isolated from an injured organ on diseased fish have been shown to have a lot of chemotactic reaction to skin mucus than isolates that were attain as free-living organisms from

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pond water (Cipriano, 2001). Chitin is a polymer of b-1,4-linked N-acetyl-D-glucosamine and is the second most abundant biopolymer on earth. Few enzymeswere given rise in the presence of individual forms of chitin, whereas others were unique to a particular chitin. In addition to the regulation by chitin and N-acetyl- D-glucosamine mono- and oligomers, chitinase activity appears to be highly regulated by other factors as well. The present study concentrates the chitinase virulence factors from the pathogenic *A. hydrophila* isolated from infected crustaceans.

2. MATERIALS AND METHODS

2.1 Sample collection, Isolation and Identification

The disease affected *C. carpio* samples were collected from infected farm for the isolation of *A. hydrophila*. The collected shrimp samples were held in icebox and carried to the laboratory and stored at -20^{0}C until the further work. The infected samples were washed 3 times with 100 ml of sterile sea water on sterile filters. It was homogenized in a sterile glass homogenizer with sterile water and the samples were serially diluted up to 10 fold. One hundred micro liters of these samples were plated on TCBS agar medium. All the plates were incubated between 28 and 30°C. After 2 to 7 days, colonies were observed and selected based on their morphological appearance and further purified by pure culture technique. Morphological, physiological and biochemical confirmations were identified from the selected isolates (Farmer and Hickman-Brenner, 1992) as well as based on the characteristics described in Bergey's Manual of Systematic Bacteriology.

2.2 Chitinase activity of A. hydrophila.

For measuring the enzyme activity, enzyme mixture was prepared using enzyme sample 100 µl , 500 µl colloidal chitin, 400µl of 125 mM Sodium acetate buffer (pH 5) and it was mixed well followed by shaking incubation at 37ºC with 200 rpm for 1 hour. After incubation, the enzyme mixtures were boiled at boiling water bath for 3 minutes followed by centrifugation at 10,000 rpm for 5minutes. The supernatant of each sample was collected andfrom each supernatant sample, 250µl was taken and mixed with 250 µl of colour reagent and was boiled at boiling water bath for 10minutes sample with high enzyme activity cleaned the colour reagent, and the absorbance was reader 420 nm in spectrophotometer (Harman *et al*., 1993).

1.3. Haemagglutination assay

This assay was performed in U – shaped micro well plates. Two fold serial dilution of serum samples were made in TBS. An equal volume of 1.5% RBC was added to each dilutionof serum sample. The plates were incubated at 25 ^{0}c for 30 min. Haemagglutination titer was recorded as the reciprocal of last dilution, resulting in agglutination after 30 minutes incubation. Negative controls comprised mixed equal volume of RBC & TBS.

2. RESULTS

3.1 Morphological and biochemical confirmation of pathogenic

A. hydrophila The pathogenic *A. hydrophila* were isolated from *C. carpio* Annai aquarium located Azhigiyamandapam, Tamilnadu Pradesh. The infected *C. carpio* were reddish colouration due to inflamation. They were confirmed by morphological and biochemical tests and the colony morphology were given the Table 1.

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3.2 Screening for Chitinolytic activity

The isolated bacterial strains were screened for ½ LB medium supplemented with colloidal chitin which forms a zone of enzyme activity. The highest zone activities were determined ES-2 strain and also it indicates virulence of the microorganism. The results were recorded in Fig 1a to 1b.

3.4 Hemeagglutination assay

Haemagglutinin assay was performed by human blood groups against *A. hydrophila* pathogen by Microwell titre plate assay technique. The agglutinin takes place 1:1, 1:2, 1:4 and 1:8 dilutions in ES- 2. There was no agglutination in 1:16 and 1:32 dilutions of *A. hydrophila* (Table 2.).

4. DISCUSSION

In the present study, the morphological and the biochemical characters of the virulent bacterial isolates were confirmed as *A. hydrophila* from infected ornamental fishes, as identified by Bergy *et al.* (1984). Nordmann and Poirel (2002) reported that Aeromonas sp. are Gram-negative, rod shaped, mainly motile, facultative anaerobic, oxidase positive and glucose fermenting bacteria. Chopra *et al.* (2000) reported that, Aeromonas sp. produce hemolysin that are cytotoxic and cause lysis of erythrocyte and play significant act in pathogenesis. Culture filtrates of two *A. hydrophila* strains isolated from patients with diarrhea and assumed to be causative agents of the infections were shown to contain enterotoxic, cytotoxic, and hemolytic activities. Selected pathogenic *A. hydrophila* strains secrete extracellular products those lethal for rainbow trout and showed proteolytic, hemolytic, and cytotoxic activities (Santos *et al*., 1968).

Multiple virulence-associated biological activities, including enterotoxic, cytotoxic, cytolytic, and proteolytic activities, have been identified in culture supernatant fluids of clinical and environmental isolates of *A. hydrophila*. The role of protease enzyme is to supplynutrients by separating host proteins into small molecules that can be entered into the bacterial cell (Cicmanec and Holder, 1979). Chitins can vary by the arrangement of *N*- acetylglucosamine strands, degree of deacetylation, and role of cross-linked structural components, such as proteins and glucans (Gooday, 1990). The end products of chitin hydrolysis, e.g., *N*-acetylglucosamine, glucosamine, and chitobiose, are known to induce chitinase synthesis in *A.hydrophila*.

In haemagglutination assay in the present experiment, bacterial agglutination takes place in 1:1, 1:2, 1:4, 1:8,1:16,1:32 dilutions in the E.S-2 treated O blood groups and in 1:1,1:4 where as the ES -1and ES-3 failed to agglutinate. This may be due to the activation of antigen in blood. If an individual's serum contains specific anti bacterial antibodies, then antibodies will bind to the bacteria and interfere with haemagglutination by the bacteria.

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Table 1. Biochemical confirmation of the pathogenic A.hydrophila

S. No	**Biochemical Tests**	**Isolated strains**		
		ES -1	**ES -2**	**ES -3**
1.	Gram staining	-Ve	-Ve	-Ve
2.	Motility	motile	motile	motile
3.	Oxidase	+Ve	+Ve	+Ve
4.	Catalase	+Ve	+Ve	+Ve
5.	Indole	+Ve	+Ve	+Ve
6.	Methyl red	+Ve	+Ve	+Ve

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7.	Voges proskauer	+Ve	+Ve	+Ve
8.	Citrate	+Ve	+Ve	+Ve
9.	Starch	+Ve	+Ve	+Ve
10.	Glucose	+Ve	+Ve	+Ve
11.	Sucrose	+Ve	+Ve	-Ve
12.	Lactose	-Ve	-Ve	-Ve
14.	Mannitol	+Ve	+Ve	-Ve
15.	Maltose	+Ve	+Ve	+Ve

ES-1: Experimental strain 1, ES -2: Experimental strain strain 2, ES -3: Experimental strain 3

Table 2. Haemagglutinin assay performed by human 'O' blood group against *A.hydrophila*

Dilutions	**Agglutination**		
	ES -1	**ES -2**	**ES -3**
- ve control	-	-	-
+ve control	+	+	+
1:1	+	+	+
1:2	+	+	+
1:4	+	+	+
1:8	+	+	+
1:16	-	+	-
1:32	-	+	-

- : No agglutination takes place; + : Agglutination takes place

Figure 1. Chitinase activity of pathogenic E.S -2: *A.hydrophila* strain 2