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RESEARCH ARTICLE

ISOLATION OF *Aeromonas hydrophila* FROM INFECTED ORNAMENTAL FISH HATCHERY DURING MASSIVE DISEASE OUTBREAK

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ABSTRACT

A wide range of pathogens is associated with ornamental fish diseases. Among the bacteria, *Aeromonas hydrophila* causes disease under stress conditions or in concert with infection by other pathogens in ornamental fish. The present study was undertaken to the *Aeromonas hydrophila* pathogenesis of goldfish (*Carrassius auratus*) and Koi (*Cyprinus carpio koi*), during massive fish disease outbreaks in various infected ornamental fish hatcheries in Kanyakumari District, Tamilnadu, India. Also the virulence factors such as protease, proteolytic activity and haemolytic activity was studied the virulence strains compared with the non-virulent *A. hydrophila* strains. The virulent *A. hydrophila* was challenged with the fingerlings of ornamental fish and the virulence was discussed.

INTRODUCTION

Fish disease is a major risk factor in commercial aquaculture with millions of dollars lost annually. *Aeromonas hydrophila* infection is the scourge of fresh and warm water fish farming worldwide and is considered as a significant economic problem, particularly in China and India over the past decade (Austin and Austin, 1987). *A. hydrophila*, gram negative facultative anaerobic short *Bacillus*, causes red fin disease, haemorrhagic septicemia, motile aeromonad septicemia and other infections in *C. auratus*.

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The Goldfish, *Carrasius auratus* has high susceptibility to motile aeromonads and are commonly valuable for experimental animals. Other pathologic conditions attributed to members of the motile aeromonad complex may include dermal ulceration, tail or fin rot, ocular ulcerations, erythrodermatitis, hemorrhagic septicemia, red sore disease, red rot disease, and scale protrusion disease. In the acute form of disease, a fatal septicemia may occur so rapidly that fish die before they have time to develop anything but a few gross signs of disease. When clinical signs of infection are present, affected fish may show exophthalmia, reddening of the skin, and an accumulation of fluid in the scale pockets. The

abdomen may become distended as a result of an edema and the scales may bristle out from the skin to give a "washboard" appearance. *A. hydrophila* produces a variety of biologically active extra cellular products (ECP), including enzymes and haemolysin compound (Wretlind *et al*., 1973) and they expressed the virulence factors including haemolysins, proteases, cholinesterases, enterotoxins, endotoxins, and adhesins all of which contribute to the overall development of the disease in fish . The present study, *A. hydrophila* was isolated from various tissues and body fluids of infected *Cyprinus carpio koi* and *Carrassius auratus* samples, based on morphological and biochemical confirmative tests. Their virulence factor were analyzed by various biochemical tests and challenged against healthy ornamental fishes.

MATERIALS AND METHODS

Source of Infected samples

Infected samples were collected from J. J. ornamental fish hatchery, Puthalum of Kanyakumari District, Tamilnadu, India. They were found with characteristic symptoms of dropsy in the abdomen, blisters, abscesses and hemorrhagic septicemia particularly in the gills, vent and abdomen. They were aseptically transported to the Laboratory and stored -20°C deep freezer until microbiological examinations were carried out.

Bacterial isolation and Phenotypic identification

The dominant colonies ware isolated from the hemorrhages as well as the body fluids of the infected fish. Triplicate samples were aseptically blended with Tryptic Soy Broth (TSB) (Hi media, India) and incubated at 37^0C overnight for enrichment. The culture was plated on Tryptic Soy Agar (TSA). The other colonies also identified by using nutrient broth and the culture were grown in nutrient agar. The selected isolates were identified by morphological, physiological and biochemical confirmations (Farmer and Hickman-Brenner, 1992) as well as based on the characteristics described in Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 1994). The pure cultures isolated from *C. karpio koi* were named as AHCK and *C. auratus* as named as AHCA further it was stored in nutrient agar slants at 4^0C for further experiments.

Biochemical characteristics of virulence factors

Proteolytic Assay

Casein dissolved at 1 % in 0.1mol/lt of Glycine sodium hydroxide buffer (p H 9.6). To that 1 ml of extra cellular protein to 1 ml of the above solution was added. It was incubated at 30ºC for 10 minutes. The reaction was stopped by adding 0.1 mol of 1 mol/lit of Trichloro acetic acid. Then it was centrifuged at 6000rpm for 10 minutes. Finally the supernatant was collected and the optical density was measured at 380nm.Tri chloro acetic acid was used as control.

Haemolytic Activity

Fish blood was collected and washed with PBS (pH 7.2) by centrifuged at 4000rpm for 5 minutes and a 2% standard erythrocyte was prepared. The experiment was done by mixing 0.5 ml of 2% standard erythrocyte with 4.5 ml of diluted extracts. It was incubated for 1 hour. Finally it was centrifuged at 4000rpm for 5 minutes and the supernatant was taken to measure the optical density at 540nm.

Haemoagglutination

The slide test for Haemoagglutination of erythrocytes was performed as described by Powell and Finkelstein. Bacteria were harvested from overnight growth on Trypticase soy agar; about 109 cells were emulsified in a drop of 0.85% saline on a chilled glass microscopic slide. Blood was collected in sodium citrate (3.8%); the erythrocytes were washed three times and suspended drop (0.05 ml) of human erythrocytes suspended in saline was added to the bacterial emulsion. The slide was gently rotated to observe macroscopic Haemoagglutination.

Challenge against ornamental fish fingerlings

Healthy gold fish (*Carassius auratus)* weighing approximately 5± 1 g were purchased from a commercial hatchery. They were acclimatized and

kept in quarantine tanks for the period of 5 days to assess their disease-free health status. After acclimatizing, ten triplicate (10 X 3 = 30) experimental groups were stocked in 100 l capacity, flow-through aquaria with a water flow rate of 1 l/min. The fishes were challenged with a lethal dose of virulent *A. hydrophila* ($1X10^7$) injected with intramuscularly, and observed cumulative mortality and other for pathological signs for 7 days.

Data analysis

Data obtained from virulence factors as well as cumulative mortality were analysed using one way ANOVA ($P < 0.05$ as significant level). Means were also compared using SNK test.

Bacterial Isolates from diseased ornamental fishes

Major dominant micro biota such as Aeromonas sp, Vibrio sp, Bacillus sp and *E. coli* were isolated from the different samples such as gills, skin, gut, and body fluid of infected ornamental fishes *C. carpio koi* and *C. auratus* . Among the dominant micro biota Aeromonas sp was isolated the higher percentage (80 %). The other micro biotas are Vibrio sp (12 %), Bacillus sp (5 %) and *E. coli* (3 %) respectively (Fig 1).

Characterization of Virulence factors

The proteolytic as well as the haaemolytic activity of different strains were given in the Table 4.

Table 1. Virulence factors of *A.hydrophila* isolated from infected ornamental fishes in compared with MTCC and virulent strains

Virulent factors		*A. hydrophila* strains			
		MTCC	AHCK	AHCA	AHV*
Proteolytic activity		0.546 ± 0.01^a	0.612 ± 0.05^a	0.699 ± 0.001^b	0.765 ± 0.003^b
		0.324 ± 0.015^a	0.531 ± 0.00^b	0.578 ± 0.015^b	0.617 ± 0.01^b
Haemolytic activity		-	-	-	-
		+	+	+	+
		+	+	+	+
		+	+	+	+
	-Ve control	-	+	+	+
	+Ve contro	-	+	-	+
Haemoagglutination	1:2	-	-	-	+
	1:4				
	1:8				
	1:16				
	1:32				

*- *A. hydrophila* virulent strains already isolated from infected carp
Means with the same superscripts (a-b) do not differ from each other ($P < 0.05$)

RESULTS

Gross Clinical Sings of Naturally Infected Ornamental Fishes

The disease Signs of the koi carp, *C. carpio koi* were observed such as hemorrhagic skin, ulcerations, loss of scales and tail rot. Infected gold fish, *C. auratus* were found loss of scales, dropsy in the abdomen etc.

Among the different strain isolates, the lower activity was observed in the MTCC strains due to no virulence and it was significantly ($P<0.5$) increased to isolates of AHCK and AHCA. The higher activity was observed in the AHV strains of isolated from the infected carps already as the reference strains. *In vitro* haemagglutinin activity against human "O" group erythrocytes revealed that, *A. hydrophila* isolates (AHCA) agglutinate even in 1: 16 dilutions and AHCA agglutinate only in 1: 8 dilution. The MTCC had less reactivity of 1: 4 dilution only due to no virulence (Table 1).

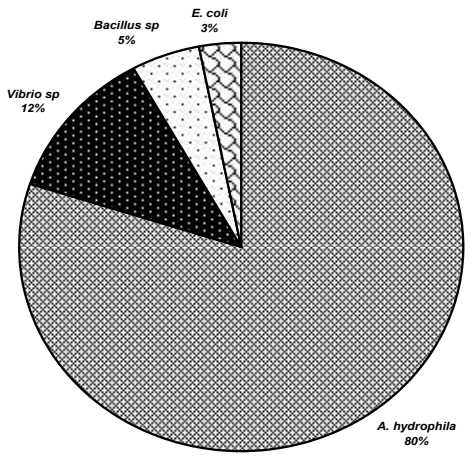

Fig. 1. Dominant microbiota isolated from the infected ornamental fishes such as *C. carpio koi* and *C. auratus*

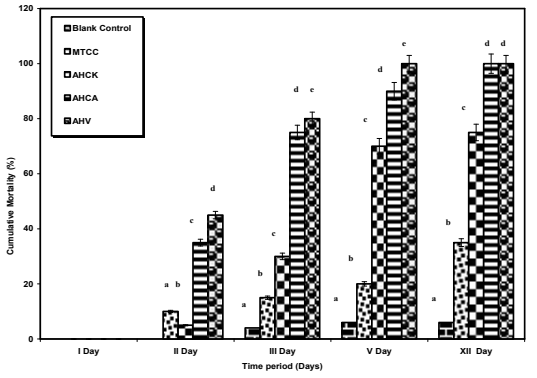

Fig. 2. Cumulative Mortality (%) of gold fish *C. auratus* challenged with different strains of *A. hydrophila* challenge

Determination of virulence by challenge test

The percentage of cumulative mortality of *C. auratus* after 7 days of *A. hydrophila* was given in the Fig 2. The fish challenged with MTCC strain had low cumulative mortality of 35 % with in seven days due to less virulence. The other strains such as AHCK and AHCA are responsible for high virulence and the cumulative mortality of 75 and 100 % respectively with in seven days and statistically significant ($P<0.05$). The AHV responsible for higher virulence and all fishes succumbed to death with in five days.

DISCUSSION

The environmental stresses are an important factor contributing to outbreaks of disease due to *A. hydrophila* in ornamental and other cultured fresh water fin fishes. This is a common inhabitant of healthy fish and the aquatic system; it is also an established opportunistic pathogen infecting fish under physiological or environmental stress. The present study investigates that, the dominant micro biota as *A. hydrophila* among with other pathogens during the massive out break of bacterial infection in the ornamental fish hatchery. The virulence factors is responsible for hemorrhages, ulceration and loss of scales etc. De Figueredo and Plumb (1977) found that strains of motile aeromonads isolated from diseased fish were more virulent to channel catfish than were those isolated from pond water.

Also *A. hydrophila* was quickly able to produce lesions and establish a systemic infection, evidenced by isolation of the organism from the kidney (Llobrera and Gacutan, 1987). The protease plays a critical role in the early stages of the infective process, protecting the bacterial cells against complement-mediated killing or other serum bactericidal effects. Subsequently, proteolytic activity would act to provide nutrients for continued growth and proliferation. These functions could presumably be carried out by either the heat-stable metalloprotease studied here or by the additional serine protease produced by many strains of *A. hydrophila* (Allan *et al*.,1981). In the experimental ornamental fish hatchery, we observed that there is high mortality found in mostly earlier stages of gold fish with loss of scales and dropsy in the abdomen. But in *C. carpio koi* mostly the external symptoms are blood hemorrhages and tail rot. These external symptoms are due to the secretions of virulent protease and haemolysis etc.

Many studies have attempted to further delineate the virulence mechanisms of motile Aeromonads. Kou (1973) found that many of the virulent, avirulent, and attenuated aeromonads that he studied possessed hemorrhagic factors and lethal toxins. The virulent *A. hydrophila* had

quantitatively more toxic potential than did their avirulent one. The present study among the four comparisons, the isolates from infected fishes are high virulent than the MTCC strains. It may due to the reflection of virulence. The haemoagglutination assays revealed that the higher virulent strains were very effective at least 1: 16 dilutions against human erythrocytes. The virulent/ less virulent strains had effective only lower dilution such as 1:4.

The *A.hydrophila* injected intraperitoneally is pathogenic to *Clarias batrachus* fingerlings, causing 93% mortality in fish infected with 10^7 cfu/ml, with peak mortalities occurring on days 14 and 15. (Thune *et al*., 1982). The present challenge experiments against the different strains of *A. hydrophila* to the gold fish revealed that, there is very little mortality were observed in the MTCC strains challenge gold fish at the end of experiment. Surprisingly the virulent strains responsible for cent percent mortality till the end of *A. hydrophila* challenged experiment against the ornamental gold fish *C. auratus*. Carp that had been intramuscularly injected with *Aeromonas hydrophila* showed both hemolysis and anasarcous changes (Kanai and Takagi 1986). These changes were caused by toxic substances produced by the bacterium. The nephroses, hepatocyte degeneration and cardiac hemorrhage that were observed in the present study appear to have been caused by aeromonad toxins. The toxins appear to have been produced by bacteria that grew in edematous scale-sacs and ascitic fluid as well as skin ulcerative lesions. Further study need to characterize the other virulence factors and extra cellular products in molecular level. It will be useful to study the pathogenesis at molecular level.

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