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Characterization and Quantification of Siderophores Produced by *Aeromonas hydrophila* Isolated from *Cyprinus carpio*

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Abstract: *Aeromonas hydrophila,* a gram negative bacterium was isolated from the lesions and ulcers of the diseased fresh water fish *Cyprinus carpio*. Haemolysin and siderophore production by the isolate was analysed. Haemolysin production was determined using blood agar. Spectrophotometric assay, $FeCl_3$ test and CAS agar test were performed to determine the siderophore producing ability of the bacterium. The bacterium isolated was found to induce haemolysis. The siderophore produced by *Aeromonas hydrophila* was invariably found to be of hydroxamate in nature. Maximum amount of siderophore production was recorded at 48 h of incubation, which declined thereafter.

Key words: *Aeromonas hydrophila*, siderophore, haemolysin, *Cyprinus carpio*

INTRODUCTION

Iron is an essential element for nearly all forms of life. It is the second most abundant metal after aluminium in the earth crust (Howard, 1999). However, its availability to the organisms is very limited due to the rapid oxidation of ferrous (Fe^{++}) to ferric (Fe^{+++}) state. Ferric ion is highly insoluble under physiological conditions and makes its acquisition by microorganisms (and indeed higher organism) a considerable challenge. In the biological fluids of the vertebrates, iron is found bound by transferrin, lactoferrin and in red blood cells (Neilands, 1995). In establishing an infection, pathogenic microorganism depends heavily on their ability to use the host complexed iron. To overcome the non-specific defense mechanisms of the host, to circumvent the solubility problem of iron and for its survival within the host, microorganism produce iron chelators called siderophores (Bullen and Griffiths, 1999; Messenger and Ratledge, 1985). Siderophores are low molecular weight (<1000D), avid ferric ion-binding compounds (iron chelating compounds) (Suryakala *et al*., 2004). They sequester ferric iron from environment under iron stress conditions. No system analogues to siderophores have been found for any other metal ion thus making iron unique in requiring such specific ligands. The siderophores are typically multidentate, oxygen-donor ligands that usually have hydroxamate, catecholate or carboxylate moieties (Baakza *et al*., 2004).

Aeromonas sp. have been recognized as opportunistic pathogens for fish and other poikilothermic and homiothermic animals (Alavandi and Ananthan, 2003; Rajeswari *et al*., 2005). The pathogen causes dreaded inflection in fresh water fishes not only in India but also in other countries (Tonguthai, 1985). Several virulence factors have been reported (i.e., proteases, enterotoxins, cytotoxins) as indicators of pathogencity in *Aeromonas hydrophila* (Santos *et al*., 1999). However, production of siderophores by *A. hydrophila* is not clearly determined.

The present study was designed to determine the haemolytic activity and haemolysin production of *A. hydrophila* isolated from the fresh water fish *Cyprinus carpio*. Further an attempt has been made to characterize and quantify the siderophore produced by the bacteria.

MATERIALS AND METHODS

Aeromonas hydrophila was isolated from the lesions and ulcers of diseased fresh water fish *Cyprinus carpio* by serial dilution and plating technique (Cappuccino and Sherman, 1999). The isolate was characterized based on morphological and biochemical tests as per Bergey's Manual of Systematic Bacteriology (Krieg and Hold, 1984).

Haemolytic activity of *A. hydrophila* isolated was tested on nutrient agar supplemented with 5% sheep erythrocytes with incubation at 37°C for 48 h. For haemolysin quantification, bacterium was cultured in TSB (Trypticase soy broth) at 37°C for 24 h. After incubation the culture was pelleted by centrifugation at 10,000×g for 10 min Supernatant (500 μL) was added to 1 mL of 1% suspension of sheep erythrocytes in phosphate- buffered saline and incubated at 37°C for 1 h and centrifuged at 5000×g for 5 min to remove unlysed erythrocytes. Lysis

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of erythrocytes was determined by measuring the absorbance of the supernatant at 545 nm. Blank was prepared by mixing the erythrocyte suspension with sterile TSB (treated similarly to the samples) and the absorbance corresponding to 100% of haemolysis was determined by adding a small amount of Sodium Dodecyl Sulfate (SDS) to blank tube.

For iron decontamination, in siderophore analyses the glasswares were rendered iron free by treating them with 6 M HCl and by rinsing with double distilled water.

For the determination of siderophore production, the *A. hydrophila* isolated was grown on modified Fiss Minimal Medium (5.03 g L^{-1} KH_2PO_4, 5.03 g L^{-1} L-asparagine, 5.0 g L^{-1} glucose, 40 mg L^{-1} $MgSO_4$ and 500 μg L^{-1} $ZnCl_2$, 139 μg L^{-1} $FeSO_4$ was added to the final medium containing 0.5 μM iron). After 48 h of incubation the cultures were centrifuged at 1000×g for 15 min and the supernatant was examined for siderophore production using $FeCl_3$ test (Jalal and Van der Helm, 1990) and CAS agar test (Schwyn and Neilands, 1987).

Hydroxamate, catecholate and carboxylate nature of siderophore was ascertained by examining absorption maxima (max) in Hitachi UV-Vis 160 A spectrophotometer. A peak between 420-450 nm and 495 nm on addition of 1 mL of 2% aqueous $FeCl_3$ to 1 mL of cell-free culture filtrate indicated the presence of ferric hydroxamates and ferric catecholate, respectively. A peak at 320, 250 and 210 nm indicated the presence of catecholate siderophore. A peak at 190-280 nm indicates the presence of copper-carboxylates. Chemical nature of siderophore produced was further confirmed by Tetrazolium salt test (Snow, 1954) for hydroxamate nature, Arnow (1937) for catecholate nature and for carboxylate nature which Vogel (1992) was performed.

A standard curve for siderophore was prepared using desferroxamine mesylate (DFOM; for hydroxamate siderophores). The amount of siderophore produced by the *A. hydrophila* at different time duration was extrapolated from the standard curve using CAS assay solution and reading the absorbance of the supernatant at 400 nm and denoted as μg mL (Trivier *et al*., 1995).

RESULTS AND DISCUSSION

The morphological, biochemical and physiological characteristics of *A. hydrophila* isolated from the fish *C. carpio* is presented in Table 1. Earlier studies consistently showed the presence of *A. hydrophila* as a single pathogen and dominant isolate from the body ulcers and lesions of the ulcerated fishes (Llobrera and Gautan, 1978).

A. hydrophila produce a clear zone around the streak of inoculum in blood agar indicating β haemolysis. The production of haemolytic toxins by *A. hydrophila* provides a strong evidence for its pathogenic potential. The extracellular haemolytic activity indicated 100% of haemolysis within 60 min of incubation. The results obtained in the present study revealed that the erythrocytes were highly sensitive to the action of haemolysin produced. Wong *et al*. (1998) demonstrated the presence of haemolytic genes in *A. hydrophila* strain. Murugappan and Gunasundari (Murugappan and Gunasundari, 1997) reported a reduction in the haemoglobin content and RBC count of the fish *C. carpio* infected with bacteria.

Production of siderophore by *A. hydrophila* was primarily confirmed by positive $FeCl_3$ test. Appearance of reddish brown colour on addition of 0.5 mL of 2%

Table 1: Morphological, physiological and biochemical characteristics of *Aeromonas hydrophila*

Character/Test	Reaction
Morphology	Straight rods
Motility	-
Growth in nutrient broth	Facultative anaerobic
Gram reaction	Negative
Colony character	Smooth edge, cream coloured colony
Catalase test	+
Citrate Utilization	+
Indole production	+
Voges-Proskauer test	+
Methyl red test	+
Starch hydrolysis	-
Oxidase test	+
Gas from glucose	+
Nitrate reduction	+
H_2S production	-
Protease production	+
Growth at different temperature	
10°C	-
20°C	+
30°C	+
37°C	+
Growth at different NaCl Conc.	
1%	+
3%	+
5%	+
7.5%	-

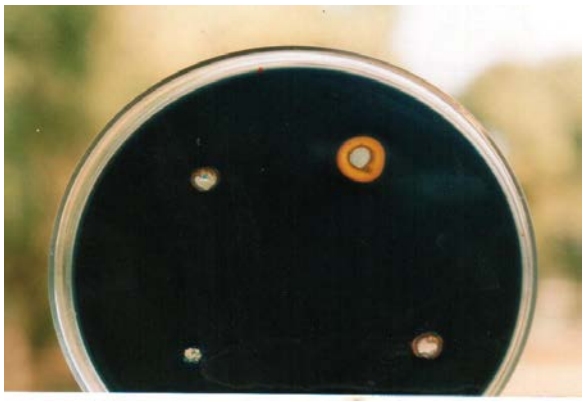

Fig. 1: Chrome azurol sulphonate assay

Table 2: Quantity of hydroxamate siderophore produced by *A. hydrophila* at different time intervals (average mean of three replicates)

Time (h)	CAS assay (OD at 400 nm)	Siderophore ($\mu g\ mL^{-1}$)
12	0.077	18.01
24	0.135	31.82
36	0.224	52.41
48	0.230	53.81
60	0.170	40.00
72	0.110	34.97

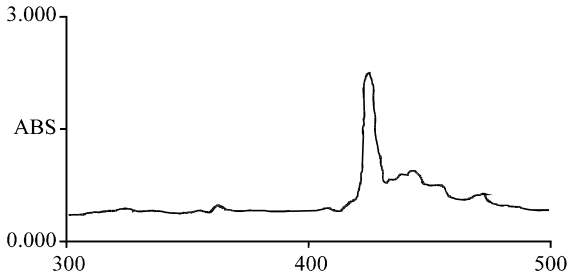

Fig. 2: UV spectra

aqueous $FeCl_3$ solution to 0.5 mL of cell free supernatant primarily indicates the production of siderophores. Formation of orange halo around the colonies in CAS agar plate further confirms siderophore production (Fig. 1). Positive results for Neilands spectrophotometric assay and tetrazolium salt test indicated hydroxamate nature of the siderophore produced. Neilands spectrophotemetric assay showed a maximum absorption at 430 nm indicating hydroxamate nature of siderophores (Fig. 2). Results for the detection of catecholate and carboxylate nature of siderophore were negative which confirms that the isolate produced only one type of siderophore. Mayer and Abdullah (1978) have reported mixed type of siderophores (hydroxamate and catecholate) produced by *Pseudomonas*.

In the present study, siderophore production by *A. hydrophila* increased gradually upto 48 h of incubation, which declined thereafter (Table 2). These results are in confirmatory with that of Gupta *et al.* (2001). Difference in quantity of siderophores produced with incubation period is a logical happening. Sharma and Johri (2002) suggested quantitative variation in siderophore production with space, time, environment and organisms concerned. Yeole *et al.* (2001) reported the influence of nutrients on siderophore production.

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