RESEARCH ARTICLE

Isolation and Characterization of Potential Bacterial Pathogens from *Cyprinus carpio* to find out the Impact of Water Pollution

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ABSTRACT: The most important fish pathogen is Aeromonas hydrophila (syn. A.liquifaciens, A.formicans), and this group is often referred to A.hydrophila complex. The prevalence of bacterial pathogen occurs in organically polluted waters. Predisposing risk factors include high temperature, overcrowding, organic pollution, and hypoxia. Motile aeromonads often invade skin wounds, commonly with water molds or ectoparasites. A.hydrophila is often associated with the protozoan Epistylis in causing widespread epidemic skin lesions known as red-sore disease. They are opportunistic pathogens of many immune compromised poikilotherms and homeotherms. A.hydrophila, Pseudomonas sp, Enterobacter sp., Serratia and Micrococci are some of the organisms highly abundant in the infected fishes. Among this, the maximum occurring A. hydrophila has been chosen for further microbiological and immunological studies. The direct bacterial count showed the maximum of 37.1 x 10^6 CFU/ml at 48th hour whereas by the total plate count the maximum bacterial count of only 21 x 10^6 CFU/ml was observed at 48th hour. The LD_{50} value was calculated as 3 x 10^6 cells for 16 gm average weight of experimental fishes. In the present study, potential bacterial pathogen like A.hydrophila was isolated and microbial characterizations were carried out to find out the impact of water pollution on the bacterial pathogenic potential.

KEYWORDS: *Aeromonas hydrophila, Cyprinus carpio,* water borne pathogen, fish diseases, LD_{50}

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1. INTRODUCTION

Motile aeromonad infection (MAI) is probably the most common bacterial disease of freshwater fish. MAI has been associated with several members of the genus *Aeromonas*, including *A.hydrophila, A.sobria, A.caviae, A.schuberri, and A.veronii.* Many other *Aeromonas* species had been recently taxonomically identified, but only few aeromonads had been strongly documented as true fish pathogens. *A. hydrophila* is a ubiquitous, free-living, Gram-negative bacterium prevalent in fresh and brackish water systems [1]. A variety of freshwater and brackish water fishes such as tilapia, carp, eel, milk fish, Indian freshwater bream, *Osteobrama belangeri*, and *Plecoglossus altevelis* [2-5] have been reported to be susceptible to the aeromonad infections.

Aeromonads cause wounds [6] and systematic infections [7] in fishes. Potential virulence factors include enterotoxin, haemolysins, endotoxins, cytotoxins and proteases [8,9]. The tissues of the fish act as a barrier to prevent the entry of the hydrophobic nature of toxins [10]. Secondary invasions with motile aeromonads also characterized a wide range of other diseases, such as epizootic ulcerative syndrome (EUS) of Asian rice-field fishes, furunculosis of salmonids [11], red-sore disease of largemouth bass (*Microplexus salmodes*) [12] and many

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parasitic conditions of tropical farmed fishes, which were all showed by acute haemorrhagic septicemia and ulceration [13]. In cyprinoids, the condition may be acute, with few signs, such as large chronic ulcers [14].

A.hydrophila when injected intramuscularly into healthy snakehead and catfish were found to induce dermo-muscular necrotic lesions. A dose of at least 10^6 cells of *A.hydrophila* was required to induce EUS-like lesions in snakehead and catfish [15]. *A. hydrophila* appears to be a component of the normal bacterioflora of the rearing pond water /lake sediment and golden snails in the areas sampled. It is probable that these were the natural sources for fish infection. *A. hydrophila* involvement in superficial infections of *Catla catla* was reported in India [16].

Lactic acid bacteria are commensal organisms in the human intestine and have been found to be inhibitory towards *A. hydrophila*. An inhibition zone of 5 mm was observed by agar spot test. Thus *Lactococcus lactis* provides inhibitory effect against *A. hydrophila* [17]. The main objective of the present study is to find out the impact of water pollution on the pathogenecity of common carp *Cyprinus carpio*. Various histological samples like ulcer or lesions noted in the skin of the fish and internal organ based samples include kidney, intestine, and liver are to be microbiologically analyzed. The microbial growth pattern of the predominant bacterial species *Aeromonas hydrophila* is to be studied in detail. The LD_{50} value of *A. hydrophila,* on *Cyprinus carpio* is planned to carry out to find the intensity of the microbial infection due to water pollution.

MATERIALS AND METHODS

Sample Collection

In order to isolate the potential pathogenic bacteria, recently dead or moribund fish were collected from Melappalyam fish market, Palayamkottai, Tamil Nadu in sterile polyethylene bags and brought to the laboratory for further analysis.

Isolation of Pathogenic Bacteria

Samples were collected from the infected portions like ulcer, or lesions in the skin and from the internal organs such as kidney, intestine and liver of the diseased common carp for further enumeration and identification of pathogenic bacteria. Isolated colonies were stored in agar slants for further biochemical characterization. Cultures obtained were made free of contamination at frequent intervals by streak plating and sub culturing.

Biochemical Characterization

The standard morphological characterization criteria and biochemical tests were conducted to identify the important bacterial isolates based on Bergey's manual (1998) [18] (Table 1)

Bacterial Growth Studies

The growth pattern of selected isolates of bacteria *(A. hydrophila)* was studied in detail. Growth of the bacterial cells was measured by i) Direct count using Haemocytometer and ii) Total plate count method [19]. The number of cells was calculated after measuring the sample intensity or cell count at interval of 0,3,6,9,12,15,18,24,48 and 72 hours after inoculation of the cells in the fresh medium *i.e.,* each three hour interval of the incubation period cells were harvested by centrifugation. The pellet was serially diluted and total count was taken in Neubaur counting chamber. For viable count 0.1ml from the dilution was spreadplated on agar plates incubated and the colonies were counted. The count was plotted against time for estimating growth pattern.

Determination of LD_{50} Value of A. hydrophila on Cyprinus carpio

To find out the LD_{50} value of *A. hydrophila,* on *Cyprinus carpio,* eighteen hours old broth culture (at logarithmic phase) containing different loads of bacteria in physiological saline (0.85% NaCl; pH 7.2) were inoculated intraperitoneally. Six fishes were administered with a dose of 10^3 to 10^8 cells per 0.2ml. The LD_{50} value was calculated by Reed and Muench (1938) [20]. The fish were observed carefully for visible external systems and behavioral changes. Time taken to lose the balance and the individual death was noted. The fish were considered to be dead when there was no opercular movement or response for a gentle prodding. The number of fish died was noted and their individual length and weight were measured and noted. The mortality of the challenged fish was recorded and death due to *A.hydrophila* was confirmed by re-isolation of organism from the liver, spleen, body fluids and intestine.

RESULTS

Morphological, physiological and biochemical characterization of the isolates

The occurrence of *Aeromonas hydrophila* was noticed in the diseased fresh water fish *Cyprinus carpio* from markets. According to the colony morphology, four distinct colonies were picked up and the results are given in Table. 1.

Table.1 Biochemical characteristics of the pathogenic isolates from *Cyprinus carpio*

Biochemical tests	Aeromonas hydrophila	Pseudomonas sp.	Enterobacteriaceae	Serratia marcescens
Gram staining	-	-	-	+
Motility	+	+	D	+
Kovac's oxidase test	+	_	-	D
Oxidation fermentationtests	+	+	-	-
Catalase tests	+	-	D	+
Cytochrome oxidase	+	+	-	-
Huge & Leifson tests	F	N	F	F
Starch hydrolysis	D	D	-	D
Gelatin hydrolysis	D	D	-	+
NaCl tolerance (0%)	+	D	+	+
NaCl tolerance (5%)	-	D	+	D
NaCl tolerance (7%)	-	D	D	D
Methyl Red test	+	D	+	-
Voges Proskauer	+	D	-	D
Amino acid decarboxylase (Arginine)	+	D	D	-
(Lysine)	+	D	D	D
(Ornithine)	-	D	D	+
Urease test	_	D	D	D
Citrate utilization test	+	D	-	+
ONPG test	D	D	+	+
0/129 sensitivity test	-	D	D	D
Growth at 5^0C	-	D	D	D
Growth at 37^0C	+	+	+	+

Where, + more than 90% of identified genera show positive result;
- more than 9% of identified genera show negative result;
D -11-89% show positive result;
N- No reaction; F- Fermentation

The four chosen isolates were Gram negative except *Serratia* sp. They are all mesophilic, actively motile rods. Except *A.hydrophila,* all the other three isolates showed growth response at 5^0C. At 5% and 7% concentration of NaCl except *A.hydrophila* all other isolates showed positive growth response. In the Indole test *A. hydrophila* and *Serratia* sp showed positive results whereas *Pseudomonas* sp and *Enterobacter* sp showed negative result. Except *Serratia* sp, all were Methyl red positive; except *Enterobacter* all were VP (Voges Proskauer) positive and citrate positive.

Amino acid decarboxylase of arginine was found to be positive for all three isolates except *Serratia*. Lysine decarboxylase was positive for all isolates, and ornithine decarboxylase was positive for three isolates except *A.hydrophila.* All the four isolates showed ONPG positive. Except *Enterobacter* sp, all other three isolates showed positive for starch and gelatin hydrolysis. Based on the morphological, physiological and biochemical characteristics of the four isolates and on comparison with Bergey's manual (1998) [18], the identification of the bacterial isolates was confirmed. (Table.1). The four isolates were confirmed as *A.hydrophila, Pseudomonas* sp., *Enterobacter* sp. and *Serratia* sp.

Percentage composition of bacterial genera from diseased fish Cyprinus carpio

From the isolated samples from liver, kidney, intestine and body fluid, *A.hydrophila* was found to be higher in body fluid (46%) and liver (44%). In the intestine, both *A.hydrophila* and *Enterobacter* sp. were found almost in equal load (29%). But in the kidney sample, *Pseudomonas* sp. was found to be highest in maximum load (33%). Figure 1 to 4 shows that *A.hydrophila* count was found to be maximum except the kidney sample which was recorded second largest microbial count. The other microorganisms observed in the diseased histological samples of the fish *Cyprinus carpio* include, *Pseudomonas* sp, *Enterobacter* sp., *Serratia, Micrococci, etc.* The maximum microbial count of *A. hydrophila* was recorded during the studies. It has been chosen for further microbiological and immunological studies.

Bacterial growth studies

The viability of *A.hydrophila* isolates was counter-checked by growth studies at different time intervals. The total bacterial cells were obtained using the haemocytometer (direct count) and nutrient agar plates (total plate count). The direct bacterial count showed the maximum of 37.1 x 10^{6} CFU/ml at 48^{th} hour whereas by the total plate count the maximum bacterial count of only 21 x 10^{6} CFU/ml was observed at 48^{th} hour. The growth patterns of *A.hydrophila* by the direct count and total plate count methods are presented in Figure 5. The peak growth was observed during 48^{th} hour of culture at room temperature. Figure 6 shows the reisolate count *A.hydrophila* from the different organs and body fluids of the fish *Cyprinus carpio.*

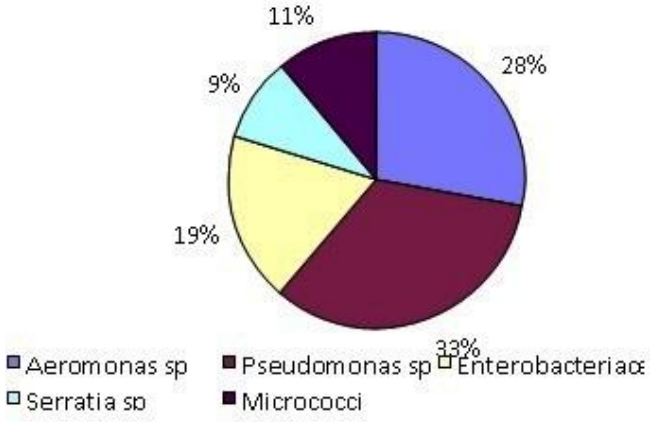

Figure 1: Percentage composition of bacterial genera observed in kidney of diseased fish

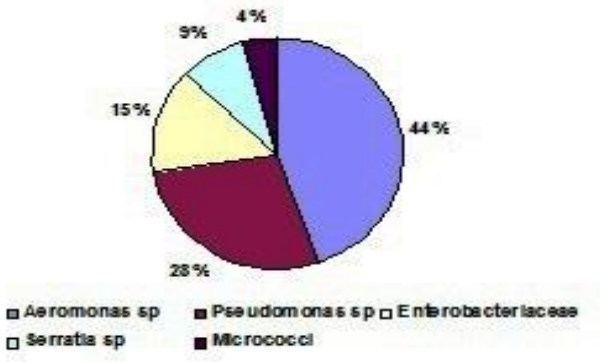

Figure 2: Percentage composition of bacterial genera observed in liver of diseased fish

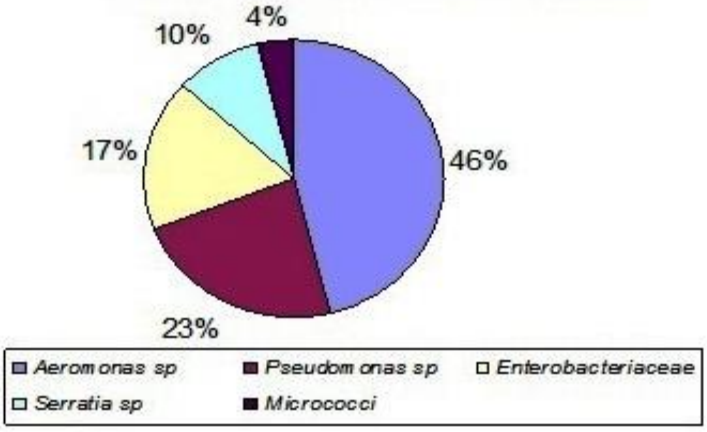

Figure 3: Percentage composition of bacterial genera observed in body fluid of diseased fish

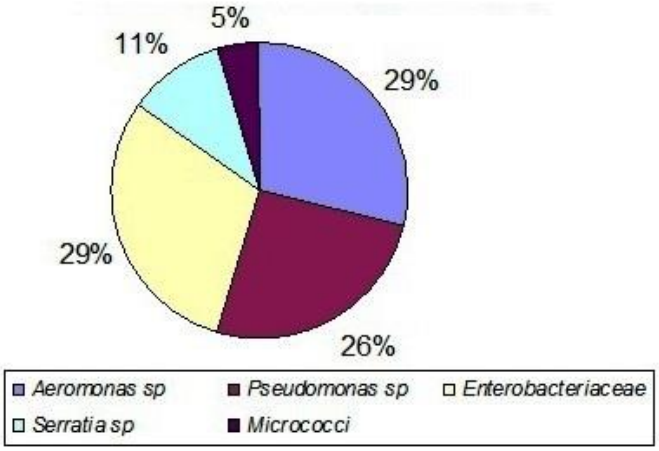

Figure 4: Percentage composition of bacterial genera observed in the intestine of diseased fish

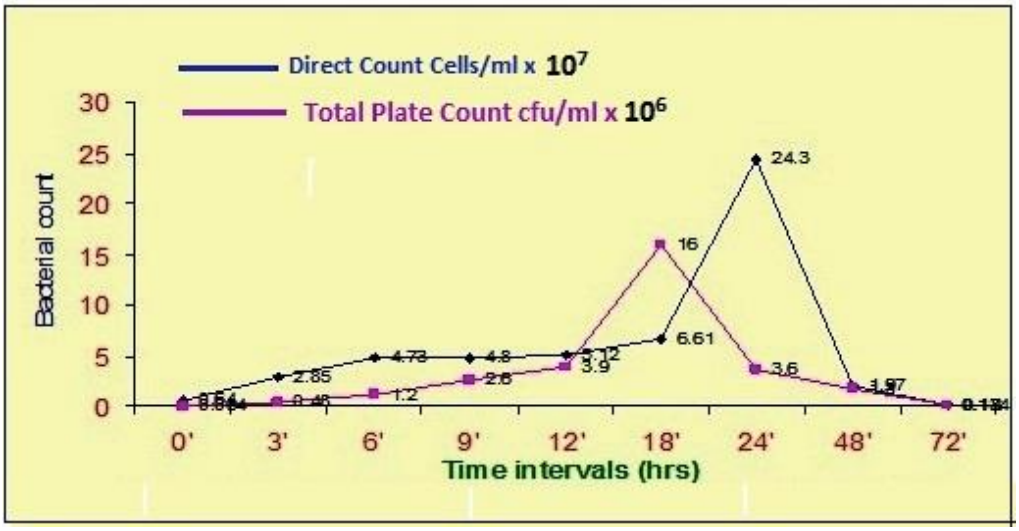

Figure 5: Growth of *Aeromonas hydrophila* isolates at different time intervals

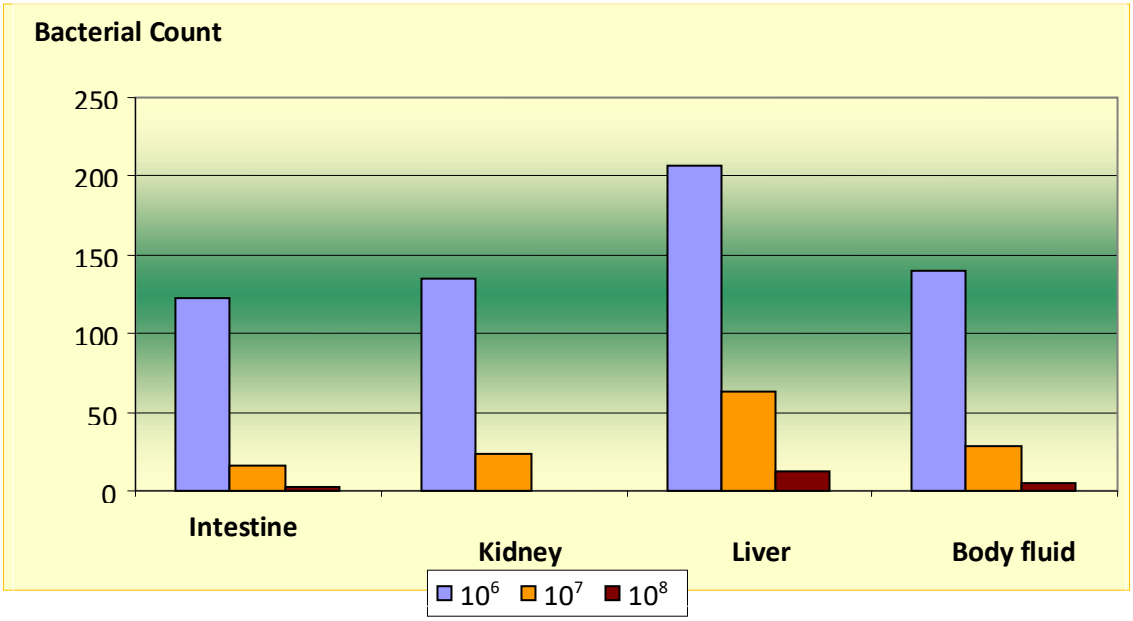

Figure 6: Reisolation of *Aeromonas hydrophila* from different organs and body fluids

Determination of LD_{50} Live cells of A.hydrophila

The lethal dose (LD_{50}) value of *A.hydrophila* live cells for *Cyprinus carpio* was calculated from the probit chart and the percentage mortality for different cell densities is given in Table 2. The LD_{50} value was calculated as 3 x 10^6 cells for 16 gm average weight of experimental fishes.

DISCUSSION

Most of the bacterial fish diseases reported from many Asian countries are contributing major share for the aquaculture production [21]. Bacteria are considered as serious pathogens, which cause diseases and subsequent economic losses in aquaculture. Among the bacterial diseases, the fishes were predominantly affected by Gram-negative bacteria such as *Aeromonas, Pseudomonas* and *Vibrio* and among the three *Aeromonas sp.* are the main pathogenic organism for the fresh water fishes [22]. Carps are the group of fishes which were used as a research model organism considered as a main source of protein rich food. Species from *Aeromonas* and *Pseudomonas* are widely isolated bacterial strains from the carps and its culture environment [23]. *A. hydrophila* is a causative agent for many diseases for the aquatic organisms and it is major organism investigated by many researchers [24]. Three Indian major carps include *Catla catla, Labeo rohita, Cirrhinus mrigala* and exotic species like *Cyprinus carpio*, Hypophthalmichthys molitrix and *Ctenopharyngodon idella* are some of the carps tested with *Aeromonas* species. This infection may cause diseases in human beings also through fishes in the food chain [25-28].

Isolation of pathogenic bacteria from diseased Cyprinus carpio

Based on the morphological, biochemical and physiological characteristics, the isolates were compared with literature reports. The isolated colonies from the diseased *Cyprinus carpio* were identified as *A.hydrophila, Pseudomonas* sp., *Enterobacter* sp and *Serratia* sp. Among the bacterial isolates *A.hydrophila* was found to be predominant. Lio Po et al., (1992) [16] recovered bacterial isolates from the fish sample, culture water, soil and the golden snail and reported 89% of the total isolates were the *A.hydrophila,* 6% of *Enterobacter* sp and 5% of *Pseudomonas* sp. Munn and Trust (1984) [20] also reported *Aeromonas salmonicida* as the primary causative agent of furunculosis affecting mostly the *Salmonoids* in both fresh water and sea water. Samples from water, fishes, food stuffs and environment are tested for the presence of motile *Aeromonas* species. Stress may be occurred to the fishes when cultured in polluted water which is affected by the pathogens of *Aeromonas* species [29,30] and

this can be considered to be a potential pathogens affecting carps [21].

The present investigation is similar to the observations of Areerat et al. [31], Roberts et al. [13] and Liu et.al., [32] who have identified acute infections and abdominal dropsy as the most common syndrome of *Aeromonas* infected *C.carpio*. Amin et al., [2] reported that *A.hydrophila* is frequently associated with diseases in carps, eels, milk fish, channel catfish, tilapia and etroplus. *A.hydrophila* is a highly affecting species which increases mortality rate in a variety of fishes [33,34] and causes enormous economic loss [35].

Previous studies of the bacterial microflora of some fresh water fishes in tropical water showed that *Aeromonas* species were the most predominant microorganisms isolated from the skin and gills of the fish [36,37]. The same organism has also been isolated from healthy and moribund fishes [38]. Maya et.al, [39] concluded that the interaction of microorganisms with aquatic biota is unique and diverse. When fish are under stress due to the surrounding aquatic environment, saprophytic microorganisms on the skin, gills and in the alimentary tract will turn into pathogen. *A.hydrophila* has been consistently associated with EUS in fish and the pathogenicity of this bacterium for EUS susceptible fish had been reported [40-42]. *A. hydrophila* induced severe dermo-muscular necrotic lesions in both catfish and snakehead.

Determination of LD_{50}

In order to find out the suitable root of administration for development of effective management strategies, the intraperitoneal injection was chosen for the inoculation of *A.hydrophila* at $3x10^6$ cells. Similar type of scientific approach was also made by Irionto and Austin (2002) [43], who have reported the minimal lethal dose of rainbow trout as 10^5 cells per fish.

Khalil and Mansour [44] and Daskalov [25] reported that *A.hydrophila* was found to produce haemolytic and proteolytic exotoxin that are lethal to tilapia and the LD_{50} value was 2.1×10^4 cells/fish. The lethal effect was attributed to the stable unknown virulent factors that were responsible for 20% mortality. Other pathogenic isolates of fish including *Pseudomonas aeruginosa* and *A.hydrophila* were also tested for their pathogenicity [45]. He also observed that the fish isolates of *P. aeruginosa* had a lethal dose of $1.5x10^5$ cells/fish of (*C.carpio*) and $4.2x10^5$ cells/fish for (*Oreochromis mossambicus*). The fish pathogen *A.hydrophila* had lethal doses of $2.1x10^6$, $6.8x10^5$ and $3.2x10^6$ cells/ fish respectively for *C.carpio, Labeo rohita,* and *O.mossambicus.*.

Lio-Po et al., [46] and Rashid et al., [47] reported that *A. hydrophila* a minimum dose of 10^6 cfu/ml injected intramuscularly was required to induce dermal lesions in walking catfish. Similarly, Supriyadi [48] showed that *A. hydrophila* was the most virulent pathogen to walking catfish, and was slightly virulent to giant gourami *Osphronemus gourami,* and avirulent to common carp *C.carpio* at the dose of 10^5 cells/fish. Most of the bacterial fish diseases were reported from temperate regions. Bacteria are considered as serious pathogens, which cause diseases and subsequent economic losses in aquaculture. Among the bacterial diseases, the fishes were predominantly affected by Gram-negative bacteria such as *Aeromonas, Pseudomonas* and *Vibrio* and among the three *Aeromonas sp.* are the main pathogenic organism for the fresh water fishes [22, 49].

CONCLUSION

Water pollution causes acute loss of aquatic production by its excessive load of pathogenic bacteria like *Aeromonas hydrophila.* Identification of the water-borne diseases to the protein rich fishes which are considered to be main source of food caters protein supplement to the population in and around the water bodies. In the present study, various pathological bacteria like *Aeromonas hydrophila*, *Pseudomonas* species, *Serratia marcescens and enterobacteriace* species were isolated. Among the bacterial strains *Aeromonas hydrophila* was found to be the predominant species selected for the growth studies. LD_{50}value was calculated to find the potential virulence of the *Aeromonas hydrophila* on *Cyprinus carpio*, common carp. The present observation showed better understanding of the pathogenic bacteria recorded in the polluted water and created a better option to produce quality aquaculture production for fulfilling the protein need of the society.

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