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## **Virulence of *Aeromonas hydrophila* isolated from *Cyprinus carpio* reared in fresh water**

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**Abstract**--In India, fish bacteriology has a slow progress when compared to developed countries. The bacterial diseases reported so far are dropsy or hemorrhagic septicemia, fin rot, ulcer disease, columnaris disease, eye disease of *Catla*, skin disease of *Catfish*, streptococcal infection etc Among the Indian Major Carps are *Labeo calbasu*, *Cirrhinus mrigala*, *Cyprinus carpio*, *Catla catla* and *Labeo rohita*. *Cirrhinus mrigala* and *Cyprinus carpio* are the second fast growing and are widely consumed throughout India. *Cyprinus carpio* is susceptible to bacterial diseases in natural as well as cultured system and is infected with *Aeromonas hydrophila*. The survey revealed the occurrence in the region of Marathwada in most of the waterbodies surveyed. Two most virulent isolate among the isolated strains were tested for their ability to cause symptoms and subsequent death in the experimental fishes. The preliminary tests indicated that ulcers were associated with *Aeromonas hydrophila* AH-04 and AH-07 isolates. The LD<sub>50</sub> values were estimated as  $0.837 \times 10^5$ , and  $0.878 \times 10^5$  for isolates AH-04 and AH-07, respectively

**Keywords**--*Cyprinus carpio*, *Aeromonas hydrophila*, LD 50.

### **Introduction**

Diseases caused by bacterial are associated with substantial death in both wild and cultured fishes. The authentic part of these pathogenic microorganisms may vary from a prime pathogen to that of an opportunistic pathogen that imparts its host decline by initiating a disease process (Barde, 2021).

Infection in fishes brings about deleterious effects in their growth, resistance to stress and fecundity, and in addition, they are vulnerable to predation and sub optimal environmental factors. All these variables decimate the fish, indirectly impinging a heavy economic loss to the fish farmers, and it becomes imperative for them to control the infection by constant surveillance and development of

adequate control measures to prevent spreading of diseases and to promote healthy environment in all the farms.

It is common knowledge that cultivable fishes are susceptible to disease-causing pathogenic bacteria (Darak & Barde, 2014; Darak et al., 2021) and fungi (Barde et al., 2020, 2021). Bacterial diseases of fish have always caused night mares among fish farmers. Among bacterial diseases, furunculosis, ulcer disease, columnaris disease and several others have caused epizootics of such magnitude as to threaten the entire salmonid fisheries of the affected regions. Furunculosis of salmonid fish received more attention from pathologists, and has been a serious problem in hatcheries of many countries, like USA, Germany, Italy, Canada and UK. It is the first bacterial fish disease which has been worked out fully.

In India, fish bacteriology has a slow progress when compared to developed countries. The bacterial diseases reported so far are dropsy or hemorrhagic septicemia, fin rot, ulcer disease, columnaris disease, eye disease of Catla, skin disease of Catfish, streptococcal infection etc. (Darak and Barde, 2015). Therefore, it is necessary to throw emphasis on induced infections and to develop suitable control methods.

The Indian major carps are the most important food fishes and they are of very high commercial value. They are widely cultivated all over India, especially in central and South India and particularly in Tamil nadu. Private entrepreneurs have undertaken large scale fish farming by converting many a rice field into commercially viable fish farms. Among the Indian Major Carps are *Labeo calbasu*, *Cirrhinus mrigala*, *Cyprinus carpio*, *Catla catla* and *Labeo rohita*. *Cirrhinus mrigala* and *Cyprinus carpio* are the second fast growing and are widely consumed throughout India. *Cyprinus carpio* is susceptible to bacterial diseases in natural as well as cultured system. Hence, it was chosen as the experimental organism to find out the effects of pathogenic bacterial strain on the physiology and nutritional value of the fishes following infection by bacterial pathogen.

## **Material and Methodology**

### **Fish Samples**

Fish samples of fish species *Cyprinus carpio* species been collected typically from surrounding natural bodies of water and cultivated systems. After determining the source, some fish were also bought from the local markets for this investigation. Samples were taken for analysis after processing at the laboratory immediately. Immediately after catching with sterilized cotton swabs, samples of different body parts were obtained. Within 4 hours of selection the samples were cultivated. The fishes were selected and considering their easier availability, the health status of the fishes was given high priority.

### **Isolation and Maintenance of cultures**

The isolation of heterotrophic, aerobic and anaerobic bacterial communities in terms of cfu /ml were identified and enumerated by the standard methods described by Cheesbrough (1989) and Bergey's Manual for Systematic Bacteriology (1986). Cultures were preserved by aseptically transferring the bacterial cultures to freshly prepared sterile nutrient agar slants after every three weeks. The stock

cultures were stored at 4°C. For experimental works, subcultures were made from the stock cultures in suitable media before use. The cultures were also examined at regular intervals to test their pathogenicity.

### Determination of LDW values of the pathogenic bacteria

The two pathogenic bacteria, *Aeromonas spp.* (AH04 and AH07) were grown in Brain Heart Infusion Broth at 30°C for 48 hours. These cultures were used to make five 10-fold dilutions of calculated by the spread plate method. 0.05 ml of each of these diluted cell suspensions were injected intraperitoneally to 10 healthy fishes. The doses received by the fishes ranged from  $1 \times 10^3$  to  $1 \times 10^7$  cfu. Each of the 10 control fishes received 0.05 ml of sterile 0.85% NaCl solution. Following injection, the fishes were observed for a 15 days period to record the appearance of disease symptoms and mortality. The dead fishes were immediately sacrificed and parts of liver and kidney were incubated in nutrient broth for re-isolation of the bacteria. The lethal dose 50% end, (LD50) was calculated from the relationship between the probits of percentage mortalities and the logs of the dilution series of bacterial suspension.

### Result

#### Isolation and maintenance of *Aeromonas hydrophila* from various location of Marathwada region

A Total of eighteen (18) isolates of *Aeromonas hydrophila* were isolated from eight different location of Marathwada districts. 04 isolates each were isolated from Nanded district, 02 isolates each were isolated from Parbhani, Hingoli, Latur, Beed, Osmanabad, Jalna and Aurangabad districts each (Table 1). These isolates were identified and maintained on their characteristics of growth on Nutrient agar slants.

Table 1  
Isolate codes of *Aeromonas hydrophila* with their location of isolation

Sr. No	District	Natural culture system	Isolate code of <i>Aeromonas hydrophila</i>	Artificial culture system	Isolate code of <i>Aeromonas hydrophila</i>	Total
1	Nanded	Godavari (Vishnupuri)	Ah-01	Kandhar	Ah-03	04
		Asna (Devapur)	Ah-02	Petwadaj	Ah-04	
2	Hingoli	Kayadu (Balapur)	Ah-05	Bhategaon	Ah-06	02
3	Parbhani	Masoli (Gangakhed)	Ah-07	Yeldari	Ah-08	02
4	Aurangabad	Nagzhari (Tembapuri)	Ah-09	Paithan	Ah-10	02
5	Jalna	Galhati (Ambad)	Ah-11	Ghanewadi	Ah-12	02

6	Latur	Manjra (Latur)	Ah-13	Sakol, Udgir	Ah-14	02
7	Beed	Sindphana (patoda)	Ah-15	Mehakari, Ashti	Ah-16	02
8	Osmanabad	Sina (Paranda)	Ah-17	Khasapur	Ah-18	02
			09		09	18

### **Pathogenicity Studies of two Aeromonas (AH-04 and AH-07) isolates in fish ulcer disease using *Cyprinus carpio***

Two isolates Aeromonas (AH-04 and AH-07) were purified from the skin ulcers of the fish concerned. Since these bacteria were isolated from the fish's ulcer tissue, it was considered logical to study the clinical symptoms, histopathology and hematology of fish infected with these pathogenic bacteria. The study of the pathogenicity and virulent characteristics of these bacteria were studied in detail.

### **Pathogenicity studies of the bacteria AH-04 and AH-07**

The preliminary tests indicated that ulcers were associated with AH-04 and AH-07. It was thought proper to determine the amount of inoculum required to cause symptoms in healthy fishes. There are little records of these bacteria's LD50 values in any fish. Therefore, experiments were conducted to establish the LD50 values of these bacteria, and a comparative study on the sensitivity of these fish species to these pathogenic bacteria was carried out.

### **Determination of the LD50 value**

Bacteria isolates AH-04 and AH-07 cell suspensions were injected into healthy fish in serially diluted doses ranging from  $1 \times 10^2$  to  $1 \times 10^7$  cfu in 0.05 ml of inoculum. Materials and Methods gives details of procedures followed. Fish mortality rates increased with all bacterial suspensions with increasingly concentrated. The bacterial isolates AH-04 and AH-07 could be purified from the dead fish lesion of liver and kidney. At the highest concentration ( $1 \times 10^7$  cfu), 85% of the fish inoculated with AH-04 died and AH-07 died at the lowest concentration ( $1 \times 10^3$  cfu). The LD50 values were estimated as  $0.837 \times 10^5$ , and  $0.878 \times 10^5$  for isolates AH-04 and AH-07, respectively (Table 2). The inoculums concentration  $1 \times 10^2$  did not induced death or symptoms in fishes.

Table 2

Cumulative mortalities of *Cyprinus carpio* after 15 days of inoculation with serial 10 fold dilutions of AH- 04 and AH-07 (*Aeromonas hydrophila*)

Dose (c.f.u.)	number of fishes inoculated	Number of dead fishes	
		AH-04	AH-07
$1 \times 10^2$	20	0	0
$1 \times 10^3$	20	3	4
$1 \times 10^4$	20	6	6
$1 \times 10^5$	20	9	10
$1 \times 10^6$	20	11	11

1 X 10 <sup>7</sup>	20	15	16
LD50		0.837 X 10 <sup>5</sup>	0.878 X 10 <sup>5</sup>
c.f.u 0.05 ml of inoculum			
b Calculated number of bacteria required to kill 50% of injected fish. (Calculated from relationship between probits of percentage mortalities and the logs of the dilution series of bacterial suspension).			

### Comparative mortality rate of the two fish species

The mortality rate was 65.0% when inoculated with a mixed bacterial suspension of AH-04 and AH-07 in *Cyprinus carpio*. The pure bacterial suspensions, AH-04 induced 35 % and AH-07 induced 40 % mortality (Table 3).

Table 3

Percentage mortality and Nature of ulcer formation in *Cyprinus carpio* injected intramuscularly with saline suspensions of AH-04 and AH-07 in pure and mixed condition

Bacterial strain	Number of fishes inoculated	Number of fishes dead	Nature of ulcer	Percentage mortality
Control	20	0	-	-
AH-04	20	8	moderate	40.0%
AH-07	20	9	moderate	45.0%
Mixed	20	14	severe	70.0%

Control set of fishes were intramuscularly injected with sterile saline suspension

Cumulative mortalities of fishes with respect to number of days after inoculation is shown in Table 4 The table shows that in all cases there is a steep rise in the number of mortalities after one after inoculation of pathogenic bacteria AH-04. The maximum number of death of fishes occurred during 4 to 7 days hours after inoculation. After 7 days, the death rate fell steeply and no further death in the fishes were recorded. In *Cyprinus carpio* the death rate of fishes injected with AH-04 was slightly varied in fishes' species inoculated. The percentage mortality was higher in *Cyprinus carpio*.

Table 4

Relation of time and Mortalities of the *Cyprinus carpio* after injection with bacterial suspension of AH-04

Days after inoculation	Number of fishes inoculated	AH-04		AH-07	
		Number of fishes dead	% mortality	Number of fishes dead	% mortality
1	20	0	0	0	0
2	20	3	15	2	10
3	20	8	40	7	35
4	20	10	50	9	45
5	20	12	60	11	55

6	20	13	65	12	60
7	20	14	70	13	65
8	20	14	70	14	70
9	20	14	70	15	75
10	20	14	70	15	75
11	20	14	70	15	75
12	20	14	70	15	75

## Discussion

In the present work, the serially diluted dose of different bacterial suspensions produced consistent trends of mortality. Isolation and identification of injected bacterial strains from external lesion, liver, and kidney of freshly dead fishes clearly indicate that the cause of death was associated with these bacterial strains. Various workers have observed changes in the liver and kidney of fishes infected by pathogenic *Aeromonas* strains (Pal and Pradhan, 1995). Prasad et al., (1995) could reisolate the pathogenic strain of *Aeromonas hydrophila* from liver, kidney and spleen of fish experimentally infected by the bacteria.

The LD50 values of the two *Aeromonad* (AHS02 and AHS03) in fishes were found to be  $1 \times 10^5$  c.f.u., for all four isolates. Descriptions of the degree of virulence by previous worker (Santos et al., 1996) suggested that strains of *Aeromonas* sp. showing LD50 less than  $1 \times 10^6$  could be classified as virulent, those with LD50  $10^6$  to  $10^8$  as weakly virulent and those with LD50 more than  $10^8$  as nonvirulent. Accordingly AHS02 and AHS03 could be denoted as virulent. There was no significant difference between the virulence of these. The virulence was similar as suggested by its slightly higher LD50 value.

Sahu et al., (1996) found that experimentally infected *Aeromonas hydrophila* could produce lesion in the catfish, *Clarias batrachus* on 30th day and in Rohu (*Labeo rohita*) on the 10th day. However, the infection was limited to ulcer in the skin and muscle only. Histopathological changes in the internal organs were not as remarkable as it was in the skin and muscle. Besides *Aeromonas* sp. and *Pseudomonas* sp., various other types of bacteria were also found to be associated with epizootic ulcerative syndrome. Jhingran and Das (1990) observed that *Micrococcus* sp. isolated from lesions and haematopoietic tissues of epizootic ulcerative syndrome affected fishes could induce ulcers within 72 hours both through inoculation and when kept in association with the bacteria. Ali and Tamuli. (1991) found *E. coli* and *Pseudomonas aeruginosa* to be associated with epizootic ulcerative syndrome affected fishes.

Ali and Tamuli (1991) isolated three types of bacteria from ulcers of four species of affected fishes and reinfection studies showed that *Aeromonas* sp. induced only mild infections, *Vibrio* sp. induced similar disease symptoms and *Micrococcus* sp. failed to induce any disease symptom. Mukherjee et al., (1995) reported constant isolation of chemoautotrophic nocardioform (CAN) bacteria from epizootic ulcerative syndrome affected fishes which bears close relationship to the human/rat and demonstrated its pathogenicity in mouse model.

Qureshi et al., (1995) isolated nine types of bacteria from epizootic ulcerative syndrome affected fishes out of which *Aeromonads* were found to be highly pathogenic while *Micrococccans* and *Cytophagans* were less pathogenic. These previous findings show that there is little similarity in the type of bacteria isolated from epizootic ulcerative syndrome positive fishes though many of these bacteria are virulent and are able to induce epizootic ulcerative syndrome like lesion when injected to healthy fishes. AHS02 and AHS03 also belong to different genera and species though they are almost equally virulent. A possible explanation for this discrepancy in bacterial types may be that epizootic ulcerative syndrome is not caused by any single bacterium. It seems to be a complex disease caused by mixed infection.

The range of incidences of ulcerative disease recorded from the different species of naturally infected fish shows that *Channa punctatus* is one of the most susceptible fishes. The incidence percentage ranges from 20% to 100%. The other two fish species are also highly susceptible in natural waters. The incidence percentage ranges from 10% to 20% in *Heteropneustes fossilis* and 10% to 55% in *Anabas testudineus* (Das and Das, 1993). The relative susceptibilities of various other fish species to several bacterial pathogens have been investigated by several workers (Soltani *et al.*, 1994). In natural infections, though most of the pond cultured fishes are susceptible, some like the Nile tilapia (*Oreochromis niloticus*) is found to be resistant to epizootic ulcerative syndrome (Ahmed and Rab, 1995). Thus, a comparative susceptibility data among all types of pond cultured fishes is necessary because this will provide vital clues in fish farm management. Choice of fish species other than which are highly susceptible to the disease will minimize the losses and segregating fishes which are more susceptible can lower the risk of disease outbreak.

Globally *Aeromonas* sp. is one of the most common bacteria associated with fish diseases. Although many strains are regarded as opportunistic pathogens, others are clearly primary pathogens in their own right (Trust, 1986). The fish diseases which involve *Aeromonas hydrophila* includes motile *Aeromonad* septicemia, red spot disease of European eel, *Anguilla anguilla* (Schaperclaus, 1934), red disease of Japanese eel, *A. japonica* (Hoshina, 1962) and red disease of carp, *Cyprinus carpio* (Egusa, 1978). Jo and Onishi (1980) isolated *A. hydrophila* from all diseased cultured ayu, *Plecoglossus alfivelis* characterized by exophthalmus and subcutaneous ulceration. Rahim et al., (1985) isolated *A. hydrophila* from the wounds of five species of fishes in Bangladesh. Okpokwasili and Okpokwasili (1994) found that *Pseudomonas* spp. and *A. hydrophila* isolated from brown patch disease of tilapia were more virulent to tilapia fingerlings when infected by a mixed culture than *A. hydrophila* or *Pseudomonas* spp. alone. Esteve *et al.*, (1993) isolated *Aeromonas hydrophila* and *Aeromonas jandaei* from diseased European eel (*Anguilla anguilla*) from an eel farm in Spain which caused ulcerous disease by intraperitoneal injection (LD50 dose:  $1 \times 10^5$  to  $1 \times 10^7$  c. f. u. / fish) and also by bath exposure to  $10^7$  to  $10^8$  c.f.u. / ml in healthy eels.

*Aeromonas liquifaciens*, another pathogenic strain of *Aeromonas* sp. was isolated from scale protrusion disease in carp by Kusuda and Takahashi (1970), which affected fish farms of Japan. Reinfection studies in carp and gold fish showed that percentage mortality of carp was higher than that of gold fish. *Aeromonas*

*punctata* has been regarded as the etiological agent of infectious dropsy in carps by Schaperclaus (1965) and he thought that primary infections resulting in disease may be induced by *Pseudomonas fluorescens*. Later he indicated that 3 different microorganism, *A. punctata*, *P. fluorescens* and a virus may be involved in this disease which manifest itself in several forms (Schaperclaus, 1969).

This tends to indicate that *Aeromonas* sp. are highly opportunistic pathogens which invade the fish once the skin barrier is breached. This however does not eliminate the fact that these bacteria are primary pathogens. In order to arrive at a conclusion on this aspect, a detailed study on the role of these bacteria in causing epizootic ulcerative syndrome is necessary.

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