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Clinical and pathological investigations in ulcer disease of *Cyrinus carpio* caused by *Aeromonas hydrophila*

Ravi D. Barde

Department of Zoology, SGB College, Purna, Maharashtra, India
Corresponding author email: ravibarde4u@gmail.com

Abstract---The Indian carps are the most important food fishes and they have a considerable economic impact on the country's economy. In several parts of the country, they are widely cultivated. The Indian Major Carps include the *Catla catla*, *Labeo calbasu*, *Cirrhinus mrigala*, and *Labeo rohita*, to name a few species. The fish *Cyprinus carpio*, both in the wild and in captivity, is vulnerable to *Aeromonas hydrophila* infections. Investigations carried out on *Cyrinus carpio* to better understand the implications of a pathogenic *Aeromonas hydrophila* isolates impacts on fish health revealed that the pathogen affects severely. The fish species that were injected with the bacterial suspension developed lesions of a similar nature, and dead fishes with ulcers on their bodies were found in all of the tanks. Even after 15 days of incubation and observation, no difference was observed in the fishes in the control group of fishes. Despite this, not all of the fish who got ulcers perished. Healing has been shown in some fish that had small ulcers, according to the research. *Cyprinus carpio* injected with a mixed bacterial suspension of AH-04 and AH-07 recorded a death rate was 65.0 percent. The pure bacterial suspensions AH-04 and AH-07 caused 35 percent and 40 percent death, respectively, in the test fishes.

Keywords---*cyprinus carpio*, *aeromonas hydrophila*, ulcer formation, mortality.

Introduction

Intensive culture of fish involves high rate of stocking and the abnormal density (crowding) of fishes, leading to susceptibility to communicable diseases, and consequent total destruction. There is a wide spread belief that an average hatchery is a hot bed of disease, where ailing fish are a rule rather than an exception (Barde, 2021). The primary factors responsible for fish diseases are

microbial, parasitic, environmental, nutritional and constitutional. Among the environmental factors may be mentioned low dissolved oxygen, super saturation of gases, temperature, eutrophication, malappropriate water chemistry, pollution, presence of intermediate hosts, toxigenic algae and lack of natural food in sufficient quantity and quality etc.

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. Fish bacteriology is not as advanced in India as it is in other nations. These include dropsy or hemorrhagic septicemia, fin or ulcer disease, and columnaris or Catla's eye disease, as well as streptococcal infection, which is the most common bacterial illness in fish (Darak and Barde, 2015). A focus on caused infections and the development of appropriate control strategies is essential.

The Indian carps are the most important food fishes and they have a significant economic value. Throughout the country, they are commonly grown. Catla catla, Labeo calbasu, Cirrhinus mrigala, and Labeo rohita are a few of the Indian Major Carps. Both wild and cultivated *Cyprinus carpio* are susceptible to *Aeromonas hydrophila* infections. To better understand the impact of a pathogenic *Aeromonas hydrophila* strain's effects on fish health, investigations were carried out to understand the implication of the infection on *Cyprinus carpio*.

Material and Methodology

Fish samples

Samples of *Cyprinus carpio* species were collected from natural water sources and farmed systems in the area. A few fish were also purchased from the local market after confirming the origins. After processing at the laboratory, samples were promptly collected for examination. In a matter of minutes, samples of various body areas may be collected after being caught with sterile cotton swabs. It took just four hours for the samples to be grown. The health of the fishes was given the highest importance because of their easier availability.

Isolation and maintenance of cultures

Bergey's Manual for Systematic Bacteriology (1986) describes the conventional procedures for identifying and counting the cfu /ml concentrations of heterotrophic, aerobic, and anaerobic bacteria. Freshly produced nutrient agar slants were used every three weeks to keep the cultures alive and viable. They were kept at 4 °C. Subcultures were made from the stock cultures before they were used in experiments. The pathogenicity of the cultures was also tested on a regular basis.

Effect of bacterial suspension

Within 24 hours of inoculation, more than 90% of the total number of fishes injected with the mixed bacterial suspension of the four bacteria AH-04 and AH-07 showed symptoms. All these inoculated fishes exhibited visible external symptoms of the disease. The external clinical symptoms differed little among the two species of fish.

Result

Aeromonas hydrophila isolation from different locations in the Marathwada area

Aeromonas hydrophila isolates were found in eight separate Marathwada district locations, for a total of 18 isolates. Nanded had four isolates apiece, Parbhani, Hingoli, Latur, Beed, Osmanabad, Jalna, and Aurangabad each had two isolates, and so on. These microorganisms were isolated based on the way they grew on Nutrient agar slants.

Pathogenicity in ulcer disease in *Cyprinus carpio* by two *Aeromonas* isolates

The skin of the fish were infected with two strains of *Aeromonas* (AH-04 and AH-07). Studies on fish afflicted with these harmful bacteria were justified given that the germs had been isolated from ulcer tissue of the fish. These bacteria's pathogenicity and virulence were researched in great depth. A study of the bacteria AH-04 and AH-07's pathogenicity AH-04 and AH-07 were shown to be related with ulcers in preliminary experiments. We believed it was a good idea to find out how much inoculum healthy fish needed to get sick before moving forward with the experiment. The LD50 values of these bacteria have not been documented in any fish. Therefore, studies were done to determine the LD50 values of these bacteria, and a comparison research was conducted on the susceptibility of different fish species to these harmful bacteria.

Susceptibility of species of fishes to AH-04 and AH-07

Different fish species susceptibility to the same bacterial pathogens are known to vary significantly. Since, the fish are commercially important species of freshwater fish, an attempt has been made to research the comparative pathogenicity of AH-04 and AH-07 in *Cyprinus carpio*. Saline suspension of the fish bacterial pathogenic isolates AH-04 and AH-07 was injected intramuscularly (0.5 ml I 100 gm body weight) into healthy fishes *Cyprinus carpio* and *Cirrhinus mrigala* in pure and mixed culture conditions. The dose of 1×10^5 cfu was used for inoculation. All the bacterial suspension were injected into 20 fish of each species and sterile saline (0.85 per cent NaCl) was injected into the control group. Under similar conditions, the experiment was replicated 3 times. Thus, bacterial suspension was injected into the fish species and all the fishes were kept 15 days under observation.

Observation of external pathological symptoms

Both the fish species inoculated with bacterial suspension developed similar type of ulcers and dead fishes with ulcers on their body in all aquariums. No change was recorded even after 15 days of incubation and observation in the control set of fishes. Nevertheless, not all fish which developed ulcers died. Healing has been found in some fish with mild ulcers was recorded.

Fishes treated with mixed bacterial suspension

The region around the injection site initially turned reddish. Gradually, swelling was observed and around the small red spot, a discoloured region of 3 to 4 mm diameter of the skin was observed. No notable change in the swimming behavior was observed. By this point the skin was almost intact and this form of lesion was called superficial ulcer. The scales were almost intact in fish with scales, and only the mucous layer was disturbed. By this point, not one of the fishes died.



Figure 1. Ulcer on body of *Cyprinus carpio*

The ulcers formed on the surface of the skin of fishes were observed between 20 to 30 hours after inoculation. Thereafter, the ulcers increased in size - 8 mm to 14 mm. In *C. carpio* the surface layer of the skin was degenerated. In *Cirrhinus mrigala*, the scales fell off and degeneration of the skin was observed. The movement of the fishes became sluggish with irregular opercular movement. This stage of ulcer was termed as moderate ulcer. Some fishes died at this stage (Figure 1). The ulcers became deep and necrotic in some of the surviving fishes after 48 to 72 hours. The underlying muscle layer was degenerated and the skeletal musculature was exposed. The fish mainly remained motionless on the floor of the aquarium. Some of the fishes floated near the surface making 45 to 90° angle of their body to the surface of the water. This type of ulcer was termed as severe ulcer. All fishes at this stage ultimately died with deep open sores on their body surface. Healing of the surviving fishes with moderate ulcers was noticed after 7 or 8 days of injection and it took about 12 days for complete healing.

Fishes treated with pure bacterial suspension

Approximately 80% of the fishes exhibited reddish swelling at the site of the injections within 24 to 48 hours of inoculation when injected with pure bacterial suspension AH-04 and AH-07. The skin was degenerated and the muscle layer was revealed slowly after 48 hours. The skin become fragile and the scales were

slumped at the location of the ulcer. The resultant ulcer was small with a diameter between 8 and 14 mm. The fish began to die in the injection site with mild sores after 72 hours. After 7 to 8 days of inoculation the remaining fish were healed, and the complete healing took between 12 and 14 days.

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 1).

Table 1
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

Discussion

A similar pattern of death was observed when various bacterial samples were serially diluted in the current study. Experiments on newly deceased fish have revealed that injected bacterial strains found in the exterior lesion, liver, and kidney were the direct cause of death. Toxic *Aeromonas* strains have caused liver and kidney damage in several fish studies (Okpokwasili and Okpokwasili, 1994). The pathogenic strain of *Aeromonas hydrophila* was able to be re-isolated from fish liver, kidney, and spleen by Prasad et al. (1995). Intramuscular injections of AH04 and AH07 were given to two economically important species *Cyprinus carpio* in pure and in mixed form. Mortality data showed that the mixed bacterial suspension was more virulent than the pure bacterial suspensions. Severe ulcers were induced at the injection site in fishes treated with a mixed suspension while moderate ulcers were induced in fishes injected with pure bacterial suspensions of AH04 and AH07.

Aeromonads were found to experimentally induce ulcerative symptoms like skin lesion when injected intramuscularly to healthy *Catla catla* and *Labeo rohita* using at least 1×10^5 c.f.u./ml (Leung et al., 1995). Karunasagar et al., (1995) isolated a number of *Aeromonas* strains from external ulcer and internal organs of epizootic ulcerative syndrome positive fish and found that these bacteria had very low LD50 values ranging from 10^3 to 10^5 in catfish fingerlings and 10^4 to 10^6 in mouse. Prasad et al., (1995) observed that *Cirrhinus mrigala* intraperitoneally injected by virulent *Aeromonas hydrophila* strain isolated from epizootic ulcerative syndrome affected *Mastocembelus arnatus* exhibited symptoms of epizootic ulcerative syndrome including lethargic behaviour and haemorrhages on

the general surface of the body. *C. mrigala* showed 100%, 90%, 60% and 30% mortality in 2, 3, 4 and 5 days after intraperitoneal injection with 0.1 ml of viable bacterial suspension containing 2.0×10^3 , 1.6×10^3 , 1.2×10^3 and 0.8×10^3 c.f.u. respectively.

Clarias batrachus and Rohu (*Labeo rohita*) were both experimentally infected with *Aeromonas hydrophila*, which caused lesion on the 30th and 10th days, respectively. Only an ulcer in the skin and muscle were infected (Sahu et al., (1996). Compared to the skin and muscle, histopathological alterations in the internal organs were not as glaring. Epizootic ulcerative syndrome has been linked to a number of additional bacteria besides *Aeromonas* sp. and *Pseudomonas* sp. Within 72 hours of inoculation, Jhingran and Das (1990) found that the micrococcus sp. isolated from the lesions and haematological tissues of epizootic ulcerative syndrome-infected fish could produce ulcers. *E. coli* and *Pseudomonas aeruginosa* were discovered to be related with epizootic ulcerative syndrome-infected fish by Kar et al., (1990).

A study on the pathogenicity of these bacteria in two economically important fishes in pure as well as mixed form was undertaken. When compared among the fish species, the obtained data indicated that the mortality rates inoculated by all the bacteria AH04 and AH07 and mixed culture were slightly lower although the differences were not statistically significant. The goldfish ulcer disease is caused by an atypical strain of *Aeromonas salmonicida* which is responsible for loss of both wild and cultured goldfish (Whittington et al., 1987). *Aeromonas salmonicida* has been reported to be isolated from head ulcer of eel, *Anguilla japonica* characterized by ulcerative lesion on the head (Ohtsuka et al., 1984) and carp erythrodermatitis characterized by cutaneous ulcerative lesion (Csaba et al., 1984).

Scale protrusion disease in carp was shown to be caused by *Aeromonas liquifaciens* (Kusuda and Takahashi, 1970), a pathogenic strain of *Aeromonas* sp. Researchers found that the death rate of carp was significantly greater than that of gold fish in reinfection trials. According to Schaperclaus (1965), *Pseudomonas fluorescens* may cause primary infections that result in illness in carps, and this has been supported by other studies. Later, he stated that three separate microorganisms, *A. punctata*, *P. fluorescens*, and a virus, may be implicated with this sickness, which manifests itself in multiple forms (Schaperclaus, 1969).

Several other workers (Harikrishnan & Balasundaram, 2005; Vivekanandhan et al., 2005; Darak & Barde, 2014; Barde, 2021.) also reported the association of mainly *Aeromonas* and occasionally *Pseudomonas* with epizootic ulcerative syndrome. Among *Aeromonads*, Karunasagar et al., (1989) and Singh et al., (2008) had recovered *Aeromonas hydrophila* and *Aeromonas sobria* more often than other bacteria. Rahim et al., (1985) on the other hand found *Aeromonas caviae* and two other fluorescent *Pseudomonads* to be involved in epizootic ulcerative syndrome. 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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