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Survival Studies of Bacterial Pathogens and Their Immunization Effect on Infectious Cat Fish (Clarias Batrachus) in Glass Aquaria

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Abstract

Present study was carried out to examine the growth and survival of *Clarias batrachus* cultivated in glass aquaria. An experiment was conducted in four glass aquaria (size 90 × 30 cm) for a period of 21 days in January 2015. Six fishes of same size (age group) of *Clarias batrachus*, with mean initial length and weight of 6.5 ± 0.07cm and 5.8 ± 0.04 g respectively were assigned to each aquaria.The aim of this work is to determine the concentration of bacterial pathogens to be inoculated in *Clarias batrachus*, so as to induce bacterial infection but not death during a period of at least two days and, therefore, enable the development of treatment protocols. The tested concentrations were established through the Mac Farland scale and fish were subjected to bacterial *infection* concentrations (2.4 × 10 CFU/mL).The clinical examination was done 24 h after inoculation, and the clinical signs suggested bacterial infection in all fishes. In the lowest concentration, fishes demonstrated few clinical signs of disease, and in the highest concentration (4.5×10^{6} CFU/mL), all fishes died within 2 – 5 days of bacterial induction with acute infection. In the intermediate concentration, all fishes presented clinical signs and kept living at the beginning of the time of treatment. Therefore, 2.4×10^{6}CFU/mL concentration was defined as viable for the study of experimental infection in different bacterial pathogens.

Keywords:*Clarias batrachus,* Fish pathogens, LD_{50}

Introduction

Clarias batrachus is a catfish (Froese, Rainer, and Daniel Pauly, eds.2006). It is highly nourishing and valued as food. It is mostly used in laboratories for experimental purposes but also used as a food. The flesh has high nutritive value and its flesh is said to have wound healing effect and recuperative attributes. It is highly suitable for intensive culture due to its air-breathing habit. In central India it is commonly found in reservoirs of eastern Vidarbha region (M.S.). It is a faster growing fish than most of the other species of the genus. It is marketed live and fetches high prices in the market. Healthy fishes are prized for their table quality. However, this quality is influenced by several operational environmental factors.

Suggested that the type of micro-organisms that are found associated with particular fish depends on its habitat classified the bacterial pathogens associated with fish as indigenous and non-indigenous. The non-indigenous contaminate the fish or the habitat one way or the other and examples include *Escherichia coli, Clostridium botulinum, Shigella dynteriae, Staphylococcus aureus, V. cholera* and *Salmonella*.

The studies in the last decade (Kar*et al.,* 1993; 1994; 1995; 1996; 1997; 1998; 1999, 2000) showed that species like *Channa striatus, C.punctatus, Clarias batrachus and Anabas testudineus* have been severely affected by bacterial pathogens and the outbreak has been occurring during the period from November to March. Low temperatures appear to influence the severity of infectious lesions.

The causative agents of the severe acute infectious abdominal dropsy outbreak in Indian major carps. *Cirrhinus mrigala* was reported Shome et al (1996). However, the first observation on diseases in Indian major carps was found in descending order of susceptibility on *Catla catla*, *Cirrhinus mrigala* and *Labeo rohita* (Gopalakrishnan 1981). Other well recorded cases have been the severe epidemic due to the diseased condition of European carps (Snieszko 1954; Van Dujin 1956).

Sabur (2006) isolated and identified five species of *Aeromonas* bacteria in polyculture environment of five carp species namely *Labeo rohita, Cyprinus carpio, Cirrhina scirrhosus, Catla catla* and *Hypophthalmic hthysmolitrix*. Lately the bacteria *A. hydrophila* was isolated from Thai pangus *Pangasianodon hypophthalmus* (Siddik, 2009) and from climbing perch *Anabas testudineus* (Sayed, 2010). In the present work, experimental infection was done to know the pathogenicity of bacterial pathoens in *Clarias batrachus*. The virulence of the pathogen was estimated by experimental studies of the LD_{50} (median lethal dose) of *bacterial pathogens* in the glass aquaria.

Materials and Methods

Study Area

This study was conducted on fish species collected from Wainganga River flowing through Gadchiroli and Chandrapur district, Vidharbha (M.S.) In Gadchiroli district the river flows nearby Armori tehsil and in Chandrapur district it is near Bramhapuri tehsil.

Sampling
The bacterial counts on the external surfaces, intestines and tissue were estimated as follows:

Skin Surfaces
Sample from different locations of the skin of 40 raw fish was taken by rubbing the sterilized cotton swab over the skin and then inoculated into 9ml of Nutrient broth, MacConkey broth which are dispensed in separate tubes. 10 fold serial dilution of the bacterial suspension inoculated in peptone water was prepared in duplicate and viable aerobic bacterial counts were enumerated using 0.1ml and 1ml inoculums in standard plate count agar as described by (Slaby, B.M., Martin, R.E., Ramsdell, G.E.1981)**,** and then incubated at 37^{o}C for 48 hrs.

Intestines, Gills & Tissues
1g of the fish sample was dissected out, blended and mixed properly in a mortar. It was aseptically transferred to a sample bottle containing 9mls of 0.1% sterile peptone water The bottle was closed and shaken thoroughly for 10 minutes and allowed to stand for 20 minutes, after which a 10 fold serial dilution was carried out in duplicates and viable aerobic bacterial counts were enumerated in standard plate count agar after incubation at 37^{o}C for 48 hrs as described by (Slaby, B.M., Martin, R.E., Ramsdell, G.E.1981).Coliform organisms and gram negative enteric bacteria counts were determined using pour plate method with MacConkey agar, EMB Agar respectively. Mueller-Hinton Agar for *Pseudomonas spp*. *Salmonella spp*. and *Shigella spp*., were enumerated using Salmonella Shigella Agar (SSA) and Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar for pathogenic *Vibrio spp*. The plates were incubated at 37^{o}C for 24hrs. The observed colony growth were counted using Coulter™ Colony counter according to plate count method. Identification of the organisms was done using the phenotypic and biochemical characteristics as described by (Cheesbrough, M. 1984).

Estimate of mean colony forming unit per gram (CFU g1)
The mean colony forming unit per gram (CFU g-1) denoted by (x) was calculated as $\Sigma f\chi/\Sigma f$, where $\Sigma fx$is the sum of the products of number of colonies and the colony forming unit per gram; while Σf is the summation of the number of colonies.

Collection and Stocking of Fingerlings
The experimental fingerlings of *Clarias batrachus* were collected from Wainganga River flowing through Gadchiroli and Chandrapur district, Vidharbha (M.S). For the feed trial experiment 04 glass aquaria (size 90 × 30 cm) were selected for a period of six months starting from January to Jun 2014. Two feed administrations (treatments) i.e. Feed A with 40%, Feed B with 39.40% (gross protein) were replicated twice. The experimental fish belongs to the same age group having mean length and weight of 6.5 ± 2.20 cm and were stocked at the rate of five fish/aquaria.

Median lethal dose (LD_{50}) experiment

An amount of 10 mg of fresh culture of the bacteria was carefully scraped and mixed with1 ml sterile physiological saline (PS) and desired dilutions were prepared by serial decimal dilution method. In a preliminary test the above stock dilution (10 mg in 1 ml) was calculated to contain around 10^6 CFU/ml. Four serial dilutions having an estimated concentration of 10^7, 10^6, 10^5 and 10^4 CFU/ml were used for the (LD_{50}) experiment. From each of the above 4 dilutions, 0.1 ml bacterial suspension was injected intramuscularly to each of previously stocked and acclimatized 10 fish making a group. Each group was then released in one aquarium properly labeled to understand the dose. The injected fish were observed up to 21 days. No feed was given to the experimental fish and water temperature was recorded twice daily. Immediately after death, each fish was transferred to laboratory, kidney was dissected out, touched with a sterilized loop and streaked onto TSA plates. The plates were incubated at 25°C for 48 hours for *A. hydrophila* colony appearance. From the mortality record, LD_{50} value was worked out according to the following formula:

Proportionate distance (PD) =50% mortality - mortality at dilution next below 50%

Mortality at dilution next above 50% - mortality at dilution next below 50%

Dilution factor (DF) = Negative Log of lower dilutions

(Next above 50% mortality) …… (i)

PD × DF

…… (ii)

Log LD50 titer = (i) + (ii)

LD50 titer = 10[(i) + (ii)]

RESULTS AND DISCUSSION

In this study, for all the fish samples ranged between 6.60 x 10^6 and 25.60 x 10^6cfu/ml as shown in table 1. Out of the 40 fish samples analysed, for the skin had the highest number of bacteria with 22.89 x10^6cfu/ml in *C. batrachus*. The gills had the lowest isolation with 3.46 x 10^6cfu/ml in *C. batrachus*. The Coliform was found in *C. batrachus.*Table 1 revealed the isolation of *Pseudomonas spp*. with the skin having the highest number in *C. batrachus* (22.89x 10^6cfu/ml).The *Vibrio spp*. isolated had the lowest count of 1.34x 10^6cfu/ml from the skin of *C. batrachus* fish samples. The intestine is the most colonized part of the examined areas in the fish with *C. batrachus* having the highest count of 18.24 x 10^6cfu/ml. The gills likewise showed possible colonization but in the lowest count as compared to other parts. No isolation of *Vibrio spp*. on the gills and skin of fishes. *E. coli* isolation showed the highest count in *C. batrachus* for skin (14.44 x 10^6cfu/ml). The intestine and skin were also heavily populated by *E. coli* with the highest exhibited in the skin of *C. batrachus*. *Staphylococcus aureus* had a low isolation rate in all samples analysed as generally compared with other isolated organisms that had the lowest counts. The human bacterial pathogens that were isolated and identified include *Escherichia coli, Pseudomonas aueriginosa, Shigella* and *Salmonella typhi* as indicated in the table 1.

Table 1: count of bacteria present at different parts of examined sample fish

Fish	Parts	*Coliforms*	*E.coli*	*S. aureus*	*P. aueruginosa*	*V. cholerae*	*S. typhi*	*S.dysenteriae*
		(cfu/ml)	(cfu/ml)	(cfu/ml)	(cfu/ml)	(cfu/ml)	(cfu/ml)	(cfu/ml)
		10^6	10^6	10^6	10^6	10^6	10^6	10^6
C. batrachus	Intestine	12.86	12.56	5.2	18.24	1.34	4.48	4.32
	Gill	14.25	10.5	7.16	17.47	-	3.46	14.25
	Skin	18.3	14.44	20.1	22.89	-	3.18	2.13
	Mouth	11.36	7.84	16.47	16.43	1.48	2.1	0.95

Pathogenicity of bacterial pathogens

During the experimental period of pathogenicity test the average water temperature was 28°C. In aquaria resulted in 100% mortality at a dose of 4.5×10^6 CFU/fish and 60-80% mortality at a dose of 4.5×10^5 CFU/fish of the experimental fishes? No fish died from the control group of the experimental fish. Results of pathogenicity tests are shown in Table 2. *C. batrachus* was proved to be sensitive to bacterial spp.as shown by their mortality upto 100%, at a dose of 4.5×10^6 CFU/fish and 80%, at a dose of 4.5×10^5CFU/fish. Post infection days of mortality were observed to be from 2 to 5 days and 4 to 6 days respectively.

The present study was carried out to understand the pathogenicity of *A. hydrophila* and median lethal dose (LD_{50}) in experimentally infected *C. batrachus*. The average body weight of the experimental fish was 6.5 ± 2.20 cm. During the experimental period of pathogenicity test and LD_{50} the average water temperature was 28^0c. Akhlaghi and Vafaie (2002) isolated pathogenic bacterial pathogens from diseased frog-eyed fish (*Carassius*sp.) at 20°C. Mostafa (2007) calculated LD_{50} of *A.hydrophila* in *Heteropneustes fossilis*at 28°C. In the present pathogenicity test, the bacterial isolates were proved to be highly invasive. Pathogenicity of bacterial spp.was measured intramuscularly at 28°C with two different doses of 4.5×10^6 CFU/fish and 4.5×10^5CFU/fish and showed mortality to 100% and 60-80% within 2 to 5 days.

Median lethal dose (LD_{50})

Results of LD_{50} test are presented in Table 2. All the fish died with 4.5×10^6 CFU bacteria/fish within 2 - 5 days. With the dose of 4.5×10^5 CFU/fish, 3-4 fish died outof 5. Among them three fish died at the day of doses, one fish died at 2nd day, one fishdied at 4th day of doses. In the case of 4.5×10^4 CFU/fish, 2 fish died out of 5. Amongthem one fish died at 3rd day and another fish died at 6th day. In case of fishes, streaking and incubation from each dead fish gave rise to the appearance of pure colonies of bacterial pathogens.

Table 2: Result of Ld_{50} pathogenicity test of fish *Clarias batrachus*.

FISH	BACTERIA	DOSE CFU/ML	NO.OF FISHES	NO. OF FISHES DIED	MORTALITY	POST INFCTION DAY OF MORTALITY
Clarias batrachus	*E. coli*	4.5×10^6	5	5	100%	2-4 day
		4.5×10^5				
	P.vulgaris	4.5×10^6	5	3	60%	4-6 day
		4.5×10^5				
	Pseudomonas sp.	4.5×10^6	5	4	80%	2-4 days
		4.5×10^5				
	Klebsiella sp.	4.5×10^6	5	4	80%	2-5 days
		4.5×10^5				
	Staphylococcus sp.	4.5×10^6	5	3	60%	4-6 days
		4.5×10^5				
	Vibrio sp.	4.5×10^6	5	5	100%	1-3 days
		4.5×10^5				
	Aeromonas sp.	4.5×10^6	5	5	100%	1-3 days
		4.5×10^5				

The study proved that bacterial pathogens, though opportunistic, was a serious pathogen for human beings. It was also proved that the pathogenesis of the pathogen was very active at least in liver, kidney and intestine of the experimental fish, investigated. As a ubiquitous species, of bacteria are available in water, fish body, and other aquatic animals and even in their feed. From the above discussion it is clear that the pathogen might be an important disease causing agent of fishes in Chandrapur and Gadchiroli (M.S.) aquaculture. Generally pathogenic bacteria are found to cause disease in fishes associated with fungus, *Aphanomyces invadans* to produce EUS (Hasan, 2007). As a bacterial pathogen, it is causing severe losses of fish by decreasing fish production and ultimately hampering the national economy. It has been isolated from lesions of almost all infectious diseases. So, proper preventive as well as curative measures should be taken for the reduction of the disease conditions caused.

CONCLUSIONS

It was confirmed that pathogenic bacteria was found to be a serious pathogen for the aquaria as was also found for carps, catfishes, eels and snake heads. Pathogenesis of Experimental infection of fish *C. batrachus* with bacterial pathogens in the gills, skin, mouth and intestine was very active. Further researches are necessary to prepare antibody against this bacteria, to serotype all fish water bodies' isolates, to prepare

whole vaccines or biological control and purified vaccines and to try vaccination and biological control agents in susceptible fishes to save our fish the people against this pathogen.

The fish in this study harboured human disease causing organisms that cause diseases such as food poisoning, diarrhea, typhoid fever and Shigellosis. (Claucas, I.J., Ward, A.R.1996) Suggested that when present in food, pathogens such as *S.aureus, Salmonella, Shigella* and *Pseudomonas* are most likely to cause food-borne diseases. The high incidence of *Salmonella* in the fish from the river is a major health concern. In addition to salmonellae, the presence of diverse enteric bacteria in fish indicates the contamination representing a potential hazard to human health.

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