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Survival studies of bacterial pathogens and their Immunization effect on fish (*Channa marulias*) in glass aquaria

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Manuscript details:	ABSTRACT

ABSTRACT

Present study was carried out to examine for the growth and survival of *Channa marulias* 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 *Channa marulias*, 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 *Channa marulias*, 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 clinical exam 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 24 - 48 hrs 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 concentrations were defined as viable for the study of experimental infection in different bacterial pathogens.

Key words: *Channa marulias,* Bacterial pathogens*,* Wainganga river.

INTRODUCTION

Channa marulias is native to South Asia. In South India it is commonly found in reservoirs of eastern Vidarbha region. It is a faster growing fish than most of the other species of the genus. It is a carnivorous species. It is marketed live and fetches high prices in the market. Fishes are well known for their nutritional value. Healthy fishes are prized for their table quality. However, this quality is influenced by several operational environmental factors. Often, they are prone to microbial and parasitic infections. A well known economic loss to the fish industry was the major outbreak of bacterial infection in major carps. The causative agents of the severe acute infectious abdominal dropsy outbreak in Indian major craps.

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, 1961). Other well recorded cases have been the severe epidemic due to the diseased condition of Eropean carps (Snieszko 1994).

The studies in the last decade (Kar *et al.,* 1995) 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. Severe acute infectious abdominal dropsy outbreak in Indian major craps. *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 1961). Other well recorded cases have been the severe epidemic due to the diseased condition of European carps (Snieszko 1954). Sabur (2006) isolated and identified five species of *Aeromonas* bacteria in polyculture environment of five carp species namely *Labeo rohita, Cyprinu scarpio, Cirrhinas cirrhosus, Catla catla* and *Hypophthalmich thysmolitrix*. 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 *Channa marulias*. The virulence of the pathogen was estimated by experimental studies of the LD_{50} (median lethal dose) of *bacterial pathogens* in the glass aquaria.

MATERIAL AND METHODS

2.1 Study Area
This study was conducted on fish species collected for studies of bacterial pathogens and their immunization effect on hematological and biochemical indices in healthy and infected fish from Wainganga river In Gadchiroli district the river flows nearby Armori tehsil. The fish samples were collected from a freshwater during the period October 2015 to February 2016. The numbers of fishes caught were transported on the same day in a container filled with pond water to the

laboratory and the analysis was carried out. A total of 10 adult specimens of the species having mean length 20.14±0.40 cm, breadth 3.63±0.08 cm and weight 125.20 ± 4.18 g were utilized in the present investigation.

2.2 Laboratory Analysis
2.2.1 Fish samples
Forty fish samples were collected from Wainganga River between the periods of October 2015 to February 2016. Twenty samples of *Channa marulias* were collected aseptically and immediately from areas separately and transported in a thermal bag to the laboratory and processed within 3hrs of acquisition, and samples were kept in the refrigerator (4–8⁰C).

2.2.2 Sample preparation
Sample preparation was made using the method described by. About 10 g of the fish sample was cut from the head, middle and tail regions with a sterile knife. The cut samples were crushed into small pieces in a sterile mortar with about 10 ml sterile water. From the crushed sample, 1 ml aliquot volume was measured out and homogenized in a clean, dry sterile beaker containing 9 ml of distilled water giving a 1:10 dilution. This was done for the 40 fish samples (Myiazaki 1972) (Olufemi 1983) (Qureshi *et al.,* 2001) (Refai *et al.,* 1989).

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

2.3.1 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 and Selenite F broth which are dispensed in separate tubes. 10 fold serial dilution of the bacterial suspension inoculated in peptone water was prepared induplicate and viable aerobic bacterial counts were enumerated using 0.1ml and 1ml inoculums in standard plate count agar as described by (Slaby *et al.,*1981) and then incubated at 37ºCfor 48 hrs.

2.3.2 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 9 ml 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°C for 48 hrs as described by (Rodricks 1991). *Coliforms* organisms and gram negative enteric bacteria counts were determined using pour plate method with MacConkey agar, EMB Agar respectively (Chauhan 2013) (Chauhan *et al.,* 2014). Pseudomona isolation 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 (Bruno, 1980). The plates were incubated at 37°C for 24hrs. The observed colony growth were counted using Coulter™ Colony counter according to plate count method (Kvenberg 1991) (Laxmareddy, 2013). Identification of the organisms was done using the phenotypic and biochemical characteristics as described by and (Slaby *et al.,* 1981).

2.4 Estimate of mean colony forming unit per gram (CFUg-1)

The mean colony forming unit per gram (CFU g-1) denoted by (x) was calculated as Σ$f\chi$/Σf, where Σfx 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.

Median lethal dose (LD50) experiment

An amount of 10 mg of fresh culture of the bacteria was carefully scraped and mixed with1 ml 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 (LD50) 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. 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, LD50 value was worked out according to the following formula:

$$\text{Proportionate distance (PD)} = \frac{50\% \text{ mortality - mortality at dilution next below 50\%}}{\text{Mortality at dilution next above 50\% - mortality at dilution next below 50\%}}$$

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 1.06 x 10^6 and 21.54 x 10^6cfu/ml as shown in Table No.1. Out of the 40 fish samples analyzed, for theskin had the highest number of bacteria with 21.54 x 10^6cfu/ml in *C. marulias*.

Table 1. revealed the isolation of *Pseudomonas sp.* with the skin having the highest number count to be 18.78 x 10^6 cfu/ml. The *S. dysenteriae* isolated had the lowest count to be 1.06 x 10^6cfu/ml from the skin of *C. marulias* as compared with other parts of fish. The intestine is the most colonized part of *E. coli* examined areas in the fish with count to be 12.50 x 10^6 cfu/ml, while the lowest count was examined areas in the fish with count to be *C. marulias* (1.06x 10^6cfu/ml). The gills likewise showed possible colonization but in the lowest count as compared to other parts. Isolation of *Vibrio sp.* on the intestine of fishes. *Coliforms* isolation showed the highest count in *C. marulias* for skin (21.54 x 10^6cfu/ml).

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

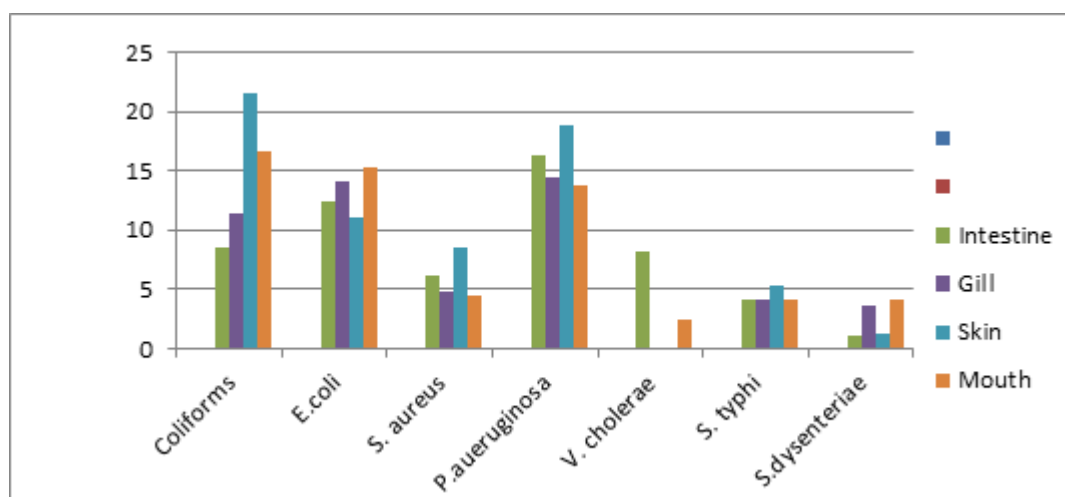| Fish	Parts	*Coliforms*	*E. coli*	*S. aureus*	*P. aueruginosa*	*V.cholerae*	*S.typhi*	*S.dysenteriae*
		(cfu/ml)	(cfu/ml)	(cfu/ml)	(cfu/ml)	(cfu/ml)	(cfu/ml)	(cfu/ml)
		10^6	10^6	10^6	10^6	10^6	10^6	10^6
Channa	Intestine	8.50	12.5	6.10	16.22	8.19	4.17	1.06
marulias	Gill	11.46	14.04	4.82	14.49	2.84	4.18	3.64
	Skin	21.54	11.08	8.46	18.78	3.24	5.24	1.26
	Mouth	16.64	15.26	4.48	13.84	2.48	4.16	4.14

Figure 1.Bacterial count in various parts of fish *Channa morulias*

Table 2: LD$_{50}$ pathogenicity test for fish *Channa marulius*.

Fish	Bacteria	Dose CFU/ml	No. of Fishes	No. of Fishes Died	Mortality	Post Infection Day of Mortality
Channa marulius	*S. aureus*	4.5 x 10^6	5	4	80%	4-6 days
		4.5 x 10^5	5	2	40%	4-6 days
	Escherichia coli	4.5 x 10^6	5	3	60%	2-4 day
		4.5 x 10^5	5	2	40%	2-4 day
	Streptococcus pneumoniae	4.5 x 10^6	5	3	60%	2-6 days
		4.5 x 10^5	5	2	40%	2-6 days
	Pseudomonas aeruginosa	4.5 x 10^6	5	5	100%	2-4 days
		4.5 x 10^5	5	3	60%	2-4 days
	V. cholera	4.5 x 10^6	5	4	80%	1-3 days
		4.5 x 10^5	5	2	40%	1-3 days
	S. typhi	4.5 x 10^6	5	5	100%	2-6 days
		4.5 x 10^5	5	4	80%	2-6 days
	S. dysenteriae	4.5 x 10^6	5	4	80%	2-4 day
		4.5 x 10^5	5	2	40%	2-4 day

The mouth and gills were also heavily populated by *E. coli* with the highest exhibited in the gills of *C. marulias*. *Staphylococcus aureus* had a low isolation rate in all samples analyzed 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 dysenteriae, S. aureus, Coliforms, S. typhi and* indicated in the Table 1.

Median lethal dose (LD$_{50}$) for *Channa marulius*
The pathogenisity tests are shown in Table No. 4.11 of *Channa marulius* was proved to be sensitive to bacterial species as shown by their mortality upto 100%, at a dose of 4.5 × 10^6 CFU/ml shown by *Pseudomonas aeruginosa* and *Salmonella typhi* compared to 4.5× 10^5 CFU/ml contain 60% and 80% respective bacteria. While, maximum mortality of *Staphylococcus aureus, Escherichia coli., Streptococcus pneumoniae, Vibrio cholerae* and *Shigella dysenteriae* shown that 80%, 60%,

80% and 80% respectively in 4.5×10^6 CFU/ml dilution factor compared to 4.5×10^5 CFU/ml contained 40%, 40%, 40% and 40% respected pathogens during 2-6 days of period.

Results of LD_{50} test are presented in Table No. 2 shown that all the fish died with 4.5×10^6 CFU /ml within 2 - 5 days, among them 2 fish died at the day of doses, 1 fish died at 2nd day, 1 fish died at 5th day and 1 fishes died at 6th day. With the dose of 4.5×10^5 CFU/ml, 4 fishes died out of 5. Among them 1 fish died at the day of doses, 1 fish died at 2nd day, 1 fish died at 4th day and 1 fishes 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.

DISCUSSION

A high population of bacteria in food indicates the general quality of the food and the degree of spoilage it might have undergone. The occurrence of total bacterial counts of many of the samples investigated having > 5 X 10^6 CFU/g raises concern about the hygienic status of the production and point of sale environment. The results from this study and according to published microbiological guidelines as cited by (Gilbert *et al.* 1996) suggest that the microbiological quality of the fish examined is unacceptable and pose a potential risk to public health. The diversity of potential pathogens from the samples of fish is of concern particularly at a time when many in our communities are immunologically compromised as a result of various illnesses. These opportunistic and pathogenic bacteria were also previously isolated by several other researchers from fish (Mhango *et al.,* 2010).

The fish in this study harbored human disease causing organisms that cause diseases such as food poisoning, diarrhea, typhoid fever and Shigellosis. (Claucas and Ward, 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.

The isolation of *Salmonella, Shigella, and E.coli* indicate faecal and environmental pollution. Coliforms such as *E.coli* are usually present where there has been faecal contamination from warm blooded animals (Chao *et al.,*2003). The organism *E.coli* is recognized as the

reliable indicator of faecal contamination in small numbers and in large number sit is an indicator of mishandling. In similar studies, *Escherichia coli, Pseudomonas aueriginosa, Shigella dysenteriae, Staphylococcus aureus and Salmonella typhi were* isolated from the gills, intestines. This was attributed to the heavy load of sewage disposal into the seas which could act as a suitable environment for the growth and survival of the human pathogens.

CONCLUSION

Seven human bacterial pathogens i.e. *Escherichia coli, Pseudomonas aueriginosa, Shigella dysenteriae, Staphylococcus aureus, Vibrio cholerae,* and *Salmonella typhi* were isolated from the two fish species *Channa marulias* collected from Wainganga river of Gadchiroli District.. The presence, in large populations of these bacterial pathogens indicates high levels of faecal contamination in the river. The presence of enteric bacteria may be attributed to faecal contamination due to improper sewage disposal and or water pollution. The fish act as a reservoir of human pathogens and the presence of highly pathogenic agents such as *Salmonella, Shigella* species and of opportunistic pathogens is a potential health risk/hazard to human beings and may cause diseases to susceptible individuals especially the immune-compromised consumers. Moreover the recoveries of various organisms, which are potentially pathogenic to humans, in the fish suggest that if they are improperly handled, undercooked or consumed raw may contribute to the spread of the pathogens in the community. Further examination of fish especially for the presence of pathogens, during handling, storage and up to the very point of consumption is needed for the protection and maintenance of community health by keeping food borne diseases to a minimum.

As the bacteria are species specific parasites, it was found from the present study that bacteria are highly pathogenic to fresh water ornamental fish *C. marulias* causing parasitism. There may be certain toxins present in given species of bacteria which cause pathogenesis in fish lead to change in hematological parameters and varying degree of destruction in the tissue which leads to mortality of fish.

Conflicts of interest: The authors stated that no conflicts of interest.

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