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Effect of pH and salinity on pathogenicity of *Flavobacterium columnare* and *Myxobacterium* sp. in Indian cat fish, *Clarias batrachus* (Linn.) and *Heteropneustes fossilis* (Bloch.)

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Abstract

Flavobacterium columnare (FC) and *Myxobacterium* sp. recorded persistently associated in fish hatchery and culture system of Himalayan and Sub - Himalayan regions were found to be pathogenic. The pH and salinity played a significant role on the pathogenicity of these potent pathogens in case of *Clarias batrachus* and *Heteropneustes fossilis*. LD_{50} value of FC was $10^{4.5}$ CFU in both the fishes and those of *Myxobacterium* sp it was 10^6 CFU ml^{-1} $fish^{-1}$. Fish challenged with *F. columnare* and *Myxobacterium* sp. (@ 0.2 ml $fish^{-1}$) individually consisting 10^{5-6}cfu ml^{-1} exhibited explicit symptoms of columnaris disease and marked with ulceration and saddle back lesion on the dorsal side of body. Maximum reisolation of inoculated bacteria was recorded at pH 7.0 and 7.5 and at 0.0-0.5 (*F. columnare*) and 0.0-1.0% (*Myxobacterium* sp.) salinity. Foregoing results elucidated that *F. columnare* was more sensitive to salinity in comparison to *Myxobacterium* sp. and their pathogenicity significantly ($p<0.05$) depends on the salinity and pH that might be one of the physical factors to control their proliferation.

Key words

Catfish culture, Columnaris disease, *F. columnare, Myxobacterium*

Introduction

Ecological conditions *viz.* water temperature, pH, alkalinity stocking density etc of aquatic environment play a significant role on the colonization of bacterial flora and incidence of diseases in culturable and wild species of fish (Snieszko, 2008 and Prasad, 2009). Alterations in these parameters may increase or decrease the proliferation rate of bacteria and accordingly influencing their virulency in fish and culture system. *Flavobacterium columnare* is aetiological agent of columnaris disease and has recorded mostly during the peak summer and rainy seasons and water temperature has been found to be an effective factor for the mortality of fish infected with this pathogen (Prasad and Verma, 2003). The growth of this bacterium was completely inhibited at 10% of NaCl solution (Bernardet, 1989) and used for the control of columnaris disease (Altinok and Grizzle, 2001). The relationship of environmental factors *viz.* tem, DO, alkalinity and management practices with the occurrence of *F. columnare* infection has been examined (Heidi *et al.,* 2009). Notwithstanding, very scanty information is available on pathogenicity of these bacteria which varies greatly and depends on the prevailing circumstances especially pH and temperature (Christopher *et al.,* 2008). *F. columnare* @ 2.5×10^5 cells ml^{-1} have conferred 100% mortality within 25 min. in chinook salmon, *Oncorhynchus tshawytscha* (Fujihara *et al.,*1971). Conversely, 10^{10} cells ml^{-1} (an extremely high dose) of *F. psychrophilum* led to the development of clinical signs of Rainbow Trout Fry Syndrome (RTFS) after 30 to 60 min at 10°C in rainbow trout, *O. mykiss* (Decostere *et al.*, 2000 and Michel and Garcia, 2003). It signifies that there exists a great relationship in the pathogenicity of pathogens and prevailing environmental conditions.

To correlate the role of physical factors *viz.* pH and salinity on the occurrence of flavobacterial infection in catfish culture in India very little progress has not been made. Though, persistent occurrence of columnaris disease has been recorded in fish hatchery and culture system of Himalayan and sub-Himalayan region ensuing mass mortality in fry and fingerlings marked with ulceration and saddle back lesion on the dorsal side of body including head region (Prasad, 2009). In last few decades, emphasis has been given on catfish culture and our Indian catfishes (*C. batrachus, H. fossilis*) are more promising for catfish farming due to their hardy nature and high phosphorous content (Venkatesh *et al.,* 1986). Hence, adaptation of modern methods for catfish culture would be of great significance. The stresses that confer pathogenicity in *F. columnare* and *Myxobacterium* sp. and their impact on cat fish in India have not been evaluated.

Therefore, keeping stress factors in mind this experiment was conducted to evaluate the role of pH and salinity on the pathogenicity of two bacteria (*F. columnare* and *Myxobacterium* sp.) so as to alleviate their infections in catfish culture.

Materials and Methods

Catfishes, *C. batrachus* (n = 175) and *H. fossilis* (n = 175) with average length of 23 (±4) cm and weight 90 (±5) g sourced from Nanak sagar reservoir, Udham Singh Nagar, Uttarakhand, India were checked thoroughly for injury and disease conditions and only healthy fishes were used in this study. Fishes acclimatized to the food and laboratory conditions for 10 days in 500 l. plastic pools (3 x 2.5 fit) filled with non-chlorinated water were divided into different groups and experiment was conducted in triplicate comprising 10 fishes in each replicate (*C. batrachus*, n=5 and *H. fossilis*, n=5) using glass aquaria of 100 l. During the experiment fishes were fed with basal diet (rice bran 25, wheat bran 25, mustard oil cake 22, fish meal 26 and mineral mixture 2%) once in a day and the residuals were removed after 48 hr by siphoning. Physico-chemical parameters like pH, dissolved oxygen and hardness of aquaria water were recorded.

Determination of LD_{50}: To check lethal doge (LD_{50}) of bacteria, healthy *C. batrachus* and *H. fossilis* were inoculated intramuscularly @ 0.2 ml $fish^{-1}$ with bacterial suspension (0.3% BHI broth) consisting 2×10^3 - 10^8 and 2×10^5 - 10^7 cfu ml^{-1} of live *F. columnare* and *Myxobacterium* sp., respectively and value was enumerated as per Reed and Muench (1938). The controlled one received 0.2 ml of 0.3% BHI solution only. Challenged fishes were kept in aquaria filled with non-chlorinated tap water in which a temperature of 25 (±5) and 20 (±2)°C, respectively was maintained by using thermostat (Sobo, China). Mortalities in challenged fishes were recorded after 24 hr of bacterial inoculations. To full fill the Koch' postulate, samples were obtained from liver and kidney of freshly dead fish and streaked on selective medium (Flavobacterium isolation agar, FIA, Hi-Media, Mumbai) to check the presence and absence of targeted bacterium.

Effect of pH: The laboratory acclimatized fishes were sterilized by putting in glass tray kept inside the laminar airflow for 20 - 30 min at 25W UV-light for inactivating the external microbial populations. Thereafter, they were kept in pH adjusted (by adding few drops of 1M HCl or 1M NaOH solution in dH_2O) at 6.5 to 9.0 aquaria water fed with pH adjusted broth culture of *F. columnare* (@ $2x10^3$ cfu ml^{-1}) and *Myxobacterium* sp. (@ $2x10^5$ cfu ml^{-1}). The aquaria water maintained at pH 7 served as control. The experiment was conducted for 30 d at 25 (±5)°C for *F. columnare* and 20 (±2)°C for *Myxobacterium* sp. Mortality in fishes was recorded and inocula obtained from different organs of died fishes for the reisolation of targeted bacterium were streaked on FIA plate.

Effect of salinity: Different salinity of 0.5, 1.0, 1.5 and 2.0% (prepared by using NaCl, Merk in dH_2O) was used for experiment following Altinok and Grizzle (2001). The aquaria water maintained at salinity 0.0 acted as control. Briefly, sterilized (through UV radiation) fishes were kept in cleaned aquaria filled with water maintaining a salinity of 0.0 - 2.0% and fed with the broth culture of *F. columnare* (@$2x10^3$ cfu ml^{-1}) and *Myxobacterium* sp. (@ $2x10^5$ cfu ml^{-1}). The experiment was conducted for 30 d at pH 7.0 and temperature 25 (±5) & 20 (±2)°C for *F. columnare* and for *Myxobacterium* sp., respectively. Mortality in fishes was recorded and inocula obtained from different organs of died fishes to reisolate the targeted bacterium were streaked on FIA plate.

Statistical analysis: Statistical analysis of data obtained was done using SPSS-7.0 version and applying one way ANOVA and suitable superscripts were attributed to mean values for statistical significance.

Results and Discussion

We could record 100% mortality in both the fish, *C. batrachus* and *H. fossilis* inoculated intramuscularly with 0.2 ml suspension of FC8 isolate of *F. columnare* (comprising 2×10^6 cfu ml^{-1}) kept at 25 ± 5°C, pH 6.94 - 7.61, DO 7.5 - 8.0 mg ml^{-1} and hardness 155-167 mg l^{-1} (as CaCO3) within 72-96 hr. On the contrary 70 and 50% mortality was noted at a concentration of 10^5 and 10^4 CFU ml^{-1} which was significantly ($p<0.05$) higher than other bacterial isolates. The LD_{50} concentration of this FC8 bacterium was calculated to $2\times10^{4.5}$ CFU ml^{-1} at 25 ± 2°C. FC10 isolate conferred 50 and 30% mortality at a concentration of 10^8 and 10^7CFU ml^{-1}. On the contrary, myxobacterial concentration of 10^7 and 10^6 led significant ($p<0.05$) mortality of 80 and 50 in both the species kept at 20±2°C. The LD_{50} concentration of this bacterium was enumerated to 2×10^6 cfu ml^{-1} at 20 ± 2°C. The maximum mean mortality values were 9.67±0.33 and 5.33±0.33 in case of FC8 and FC10 isolates, respectively at 96 hr (Table 1). The mean values of mortality on 24-28 d was significantly lower in comparison to other two groups and value of 20 days significantly ($p<0.05$) higher in comparison to other group. Result advocated that FC8 isolate of *F. columnare* belonged to high virulence because its 10^6cfu concentration conferred 100% mortality while 10^8 cfu of FC10 led to 50% mortality only and hence categorized as low virulent group. This is in consistent with

Table - 1: Mortality in *C. batrachus* and *H. fossilis* intramuscularly challenged with different doses of *F. columnare* and *Myxobacterium* sp.

Bacteria	cfu ml^{-1}	hrs D^{-1}	Fish mortality		Total fish mean mortality (CB&HF)	Average mortality %
			C. batrachus (CB)	*H. fossilis* (HF)		
F. columnare						
FC 8 isolate at 25±2°C	2X10^6	72-96h	5.00±0.00	4.67±0.33	9.67±0.33c	100
	2X10^5	7-9d	3.33±0.33	3.67±0.33	7.00±0.58bc	70
	2X10^4	15-18d	2.33±1.2	2.67±0.33	5.00±1.15b	50
	2X10^3	25-30d	1.00±0.58	1.00±0.58	2.00±1.15a	20
F. columnare						
FC10 isolate at 25±2°C	2X10^8	72-96h	2.67±0.33	2.67±0.33	5.33±0.33c	50
	2X10^7	22-23d	1.67±0.33	1.33±0.33	3.00±0.58b	30
	2X10^6	24-28d	0.67±0.33	0.67±0.33	1.33±0.33a	10
Myxobacterium sp.						
M6 at 20±2°C	2X10^7	4-5d	3.67±0.33	4.00±0.58	7.66±0.88c	80
	2X10^6	14-16d	2.33±0.33	2.33±0.33	4.66±0.33b	50
	2X10^5	22-26d	1.00±0.58	1.00±0.58	2.00±0.58a	20

Table - 2: Effect of pH on survival of *F. columnare* and *Myxobacterium* sp. in *C. batrachus* and *H. fossilis*

Bacteria	pH	Fish mortality		Total fish mean mortality (CB&HF)	Isolation of bacteria
		C. batrachus (CB)	*H. fossilis* (HF)		
F. columnare					
@ 2x 10^3 cfu ml^{-1} at 25±2°C	6.5	2.67±0.33	3.33±0.33	6.00±0.58c	0.0
	7.0	0.00±0.00	0.00±0.00	0.00±0.00a	10.0
	7.5	1.00±0.58	1.33±0.33	2.33±0.67b	8.0
	8.0	1.00±0.58	1.00±0.58	2.00±1.00b	0.0
	8.5	3.67±0.33	4.33±0.33	8.00±0.58d	0.0
	9.0	5.00±0.00	5.00±0.00	10.00±0.00e	0.0
Myxobacterium sp.					
@ 2x 10^5 cfu ml^{-1} at 20±2°C	6.5	3.33±0.33	3.33±0.33	6.67±0.67e	0.0
	7.0	0.00±0.00	0.00±0.00	0.00±0.00a	10.0
	7.5	0.67±0.67	0.67±0.58	1.33±0.88ab	9.0
	8.0	1.00±0.58	1.00±0.00	2.00±0.58b	3.0
	8.5	4.00±0.00	4.33±0.33	8.33±0.33cd	0.0
	9.0	4.67±0.33	5.00±0.00	9.67±0.33d	0.0

Table 3: Effect of salinity on survival of *F. columnare* and *Myxobacterium* sp. in *C. batrachus* and *H. fossilis*

Bacteria	Salinity (%)	Fish mortality		Total fish mean mortality (CB&HF)	Isolation of bacteria
		C. batrachus (CB)	*H. fossilis* (HF)		
F. columnare					
@ 2x 10^3 cfu ml^{-1} at 25±2°C	0.0	0.00±0.00	0.00±0.00	0.00±0.00a	10.0
	0.5	0.67±0.58	0.67±0.58	1.33±0.33b	9.0
	1.0	0.00±0.00	0.00±0.00	0.00±0.00a	0.0
	1.5	0.00±0.00	0.00±0.00	0.00±0.00a	0.0
	2.0	1.33±0.33	2.00±0.58	3.33±0.33c	0.0
Myxobacterium sp.					
@ 2x 10^5 cfu ml^{-1} at 20±2°C	0.0	0.00±0.00	0.00±0.00	0.00±0.00a	10.0
	0.5	1.00±0.58	1.33±0.67	2.33±1.20b	8.0
	1.0	0.33±0.33	0.33±0.33	0.67±0.33ab	5.0
	1.5	0.00±0.00	0.00±0.00	0.00±0.00a	0.0
	2.0	1.33±0.33	1.00±0.00	2.33±0.33b	0.0

Fig. 1a: Ulceration, erosion on the body surface and rottening of fins (arrow) of C. *batrachus* experimentally infected with *F. columnare* FC 8

Fig. 1b: Erosion of skin on the ventral side (arrow) of C. *batrachus* experimentally infected with *Myxobacterium* sp.

Bernadette *et al*. (1996) who reported LD_{50} doses of $<1.3\times10^3$ to 1.3×10^2 and 1.7×10^5 cfu ml^{-1} of *F. columnare* in barramundi (*Lates calcarifer*) and goldfish (*Carassius auratus*), respectively. Moreover, virulency of bacterial isolates especially *F. columnare* has not been universal as it varied from bacterial strain, fish species and prevailing ecological conditions and accordingly some isolates may produce symptoms at low concentration while others lead mortality at high value (Decostere *et al.*, 1998; Prasad, 2009).

Fishes experimentally infected with FC8 isolate exhibited overt signs of columnaris and myxobacterial diseases similar to those of naturally infected fishes and marked with deep ulceration, saddle back lesions (Fig. 1a) erosion and necrotization of skin on the dorso-lateral sides of the body on the onset of 25-30 days with 20-30% mortality. While fish infected with *Myxobacterium* sp. showed symptoms of white lesions on the ventro-lateral (Fig. 1b) side and ulcerations near the caudal peduncle, dark pigmentation and saddle like lesions on the dorsal surface on the onset of 18-25 days with 15-20% mortality. Appearance of such symptoms in challenged fishes suggested that the bacterial isolates of present investigation are virulent in nature and responsible for the initiation of columnaris disease. This is in accordance with Hussain and Summerfelt (1991). Different strains of these bacterial species with diverse virulence ability have been described but these differences have not been correlated with the presence or absence of specific virulence factors (Dalsgaard, 1993). In the present study experiments have not been conducted on the attachment of *F. columnare* or *Myxobacterium* to the gills and its role in pathogenesis but Decostere (2002) have reported that the attachment of *F. columnare* to the gills play a significant role in its pathogenesis which is based on suitable ecological factors like pH, salinity, hardness etc. They might be playing important role in the adhesions of these bacteria and enabling them to withstand clearing mechanism operating on body surface (Christopher *et al.,* 2008; Heidi *et al.,* 2009).

When the fishes were kept at different pH it was found that there were no effects of pH up to 24 hr. All experimental fishes were showing normal behaviours and accepting the feed properly. There were little development of haemorrhages and reddening at the anal region after 24 hr onwards. Results of *in vivo* survival of test bacteria evaluated in terms of reisolation from challenged fish kept at various pH explicated that pH 7.0-7.5 would be highly suitable for *F. columnare* proliferation because none of the isolates grew at pH 8.0 or 9.0 (Table 2) as bacteria were not recovered from the challenged fish at this pH. Each bacterium was found to be pH specific i.e. some tolerated low pH while other tolerated high pH. Appearance of clinical signs were noticed on 30 days in *C. batrachus* and *H. fossilis* when a dose of 2×10^3 (cfu ml^{-1}) of *F. columnare* and 2×10^5 (cfu ml^{-1}) of *Myxobacterium* sp. was inoculated in them and pH was maintained at pH 7.0 to 7.5 in case of *F. columnare* and pH 7.0 in case of *Myxobacterium* sp. These findings suggested that *F. columnare* preferred pH of 7.0 to 7.5 while, *Myxobacterium* sp. tolerated pH of 7.0 to proliferate as it has also been evident from *In vitro* experiment and concur to those of Snieszko (2008) and Prasad (2009) who also reported the occurrence of *F. columnare* and *Myxobacterium* sp. infection in experimentally infected fishes at pH 7.0 to 7.4.

The experimental fishes subjected to different salinity exhibited no effects of salinity on fish health up to 12 hr as their performance was at par with controlled one. First mortality was recorded after 2 days in case of *C. batrachus* and *H. fossilis* and no bacteria were reisolated from the dead fishes. *F. columnare* recovered at 0-0.5% salinity but no such bacteria were obtained at 1.0-2.0% salinity in both the fishes. On the contrary, *Myxobacterium* sp. was recovered at 0.5-1.0% salinity (Table 3). Salinity tests suggested that experimentally infected fishes were not exhibiting the symptoms when kept in 1 to 2% salinity, however, 10 and 30% mortalities were recorded in challenged catfishes. Bacteria were not recovered from the fishes exposed to 1.5 to 2.0% salinity. It suggests that 1 to 2% salinity did not favour the growth of *F. columnare* and *Myxobacterium* sp. considering the above results, the limit of tolerance for both the bacteria ranged between 0.5 and 1.0%, which is close to the total salt concentration of physiological saline of fish tissue. The present result is comparable to those of

Altinok and Grizzle (2001) who reported very less adhesion of *F. columnare* to gill arches in 3% than 0.3% NaCl solutions.

In conclusion, this investigation provides strong evidence that virulence of *F. columnare* and *Myxobacterium* sp. depends on salinity and pH. Maintaining of pH 7.5 and below 7.0 may mitigate the severity of infection of these pathogens in aquaculture.

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