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Original Research Article

Pathogenicity of bacterial isolates to *Catla catla*

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A B S T R A C T

Catla catla fish were obtained from the Srivilliputtur fish farm. The infected fish samples were dissected from the infected muscles, gills and liver, the pathogenic strains were isolated. The isolated bacterial strains selective strains toxicity was studied against normal healthy fish. The highest microbial load $6.2 \pm 0.4\times10^7$ cfu g^{-1} was observed in muscle tissue of the dissected fish sample and the lowest load $4.1 \pm 0.7\times10^4$ cfu g^{-1} was found in intestine. The selected strains were administered to the healthy normal juvenile *Catla catla* for determination of LC_{50}. The LC_{50} value for the *Aeromonas hydrophila* was 5.4×10^6 CFU/ml, *Aeromonas salmonicida* was 2.51×10^6 CFU/ml *Vibrio* sp., 2.81×10^6 CFU/ml, *Escherichia coli* was 3.16×10^6 CFU/ml and *Staphylococcus aureus* was 3.16×10^6 respectively.

Keywords

Catla catla; toxicity; *Aeromonas salmonicida*; intestine.

Introduction

Fish are a heterogeneous group of animals comprising more than 21 700 species (Fänge, 1994). Fish live in a challenging environment facing so many problems. In the aquatic environment, fish are in constant interaction with a wide range of pathogenic and non-pathogenic microorganisms (Subramanian *et al.,* 2007). In the aquatic environment, fish are in constant interaction with a wide range of pathogenic and non pathogenic microorganisms (Subramanian *et al.,* 2007). Microbes play an important role in affecting fish health. Fish suffer from various types of diseases. All fishes carry pathogens and parasites as normal flora, and if the pathogenic load increases it lead to disease. Disease is prime agent affecting fish mortality, especially when fish are young (Sharma *et al.,* 2012). Pathogens which can cause fish disease comprise viral infections, bacterial infections, fungal infections, and mould infections (Axelrod, 1989). Fishes in farms are susceptible to several bacterial infections mainly when reared in high

density conditions. Disease outbreaks among fishes elevate the mortality rate and decrease the productivity leading to high economic loss to fish farmers (Sharma *et al.,* 2012). The prevalence of bacterial pathogens has been well documented in several cultured and wild fish water species (Moustafa *et al.,* 2010). Numerous candidates of antigen are now available to induce protective immune responses against opportunistic pathogens (Morin and Hopkins, 2002). The search for conserved protective antigens is an important element of this vaccination strategy because of the relatively large number of pathogens under consideration and the many serotypes, which might be clinically relevant (O'Riodan and Lee, 2004). Human infections caused by pathogens transmitted from fish or the aquatic environment are quite common depending on the season, patients contact with fish and related environment, dietary habits and immune system status of the exposed individual (Novotny *et al.,* 2004). Microbial investigation for characteristics of potential pathogenic microorganisms for fish will allow the application of adequate measures to prevent and control the main diseases limiting the production of fishes. *Aeromonas hydrophila* mainly causes motile aeromonad septicemia (MAS) and has also been reported to cause secondary infections associated with EUS outbreaks (Roberts, 1993). The disease caused by *A. hydrophila* has also been called 'Red-sore' disease (Huizinga *et al.,* 1979).

It does not usually cause problems in fish populations under normal conditions, but when fish are under environmental or physiological stress or infected by other pathogens (Fang *et al.,* 2000). Several studies have described a wide variation in the pathogenecity of *A. hydrophila* in different fish species (Sharma *et al.,* 2012). This is mainly due to the heterogenecity of strains and differences in the adhesive and enterotoxic mechanisms responsible for causing infection in fish (Fang *et al.,* 2004). Heuzenroeder *et al.,* (1999) showed that the mortality was dependent on the concentration of bacteria and the appearance of clinical signs in fish that eventually died of a major virulent factor, when its pathogenicity was studied using a suckling mice model infection. In the aquatic environment, fish are in constant interaction with wide range of pathogenic and non-pathogenic microorganisms (Subramanian *et al.,* 2007). Our emphasis in the present study is on the need for screening and isolation of bacterial and fungal from the infected fish sample and studied the LC_{50} values after the administration of the microorganism.

Materials and Methods

Sample collection

The fish samples were collected in pre sterilized container from the fishing area in Srivilliputtur (931"0.012"N, 7737"59.880"E) Tamil Nadu, India. The collected fish samples were transported to the laboratory in an icebox for further analysis.

Enumeration of bacterial organisms

The infected fish samples were dissected and from the infected muscles, gills and liver, the pathogenic strains were isolated with help of sterile swab and spread over the nutrient agar plates. The plates were incubated at 37°C for 24-48 hrs, after incubation. The Total Heterotrophic Bacterial Population was enumerated and recorded.

Identification of Pathogens

The morphologically different microbial strains were identified in bacterial plates. The colonies were isolated and purified by restreak method. The isolated colonies were streaked on nutrient agar slants, incubated overnight at 37°C. The following tests were performed for identification of selected colonies isolated from the fish samples.

Determination of LD_{50}

From the isolated bacterial strains selective strains toxicity were studied against normal healthy fish *Catla catla*. The most common type of toxicity tests with aquatics events is the acute mortality test, which is usually conducted to obtain information about a medium lethal dose (LC_{50}).

LC_{50} is defined as the concentration of the test pathogen, which kills 50% of the test animals. LC_{50} value was calculated by variety of methods. In the present study, after 96 hours of the experiment, the LC_{50} values for five pathogenic strains were calculated as per formula described by Dhasarathan (2000).

$$\text{Proportionate Distance} = \frac{\text{\% of mortality at dilution next to above 50\%-50\%}}{\text{\% of mortality at dilution rate above 50\%- \% of mortality at dilution rate below 50\%}}$$

$$\text{Negative log } LC_{50} = \text{Negative log of dilution next to above 50\% of mortality} + (\text{Proportionate distance x dilution factor})$$

Result and Discussion

The isolation of microorganism is based on the infected fish species, its disease status, clinical signs and biochemical diagnosis. The results of the quantitative estimation of microbial count in muscle, gill, liver and intestine of diseased fish are given in the Table 1. The highest microbial load $6.3 \pm 0.4 \times 10^7$ cfu g^{-1} was observed in muscle tissue of the dissected fish sample. The lowest load $4.3 \pm 0.7 \times 10^4$ cfu g^{-1} was found in intestine. The percentage distributions of mycotic and bacterial isolates are shown in Table 2. One fungal species and nineteen bacterial species were isolated and identified. Among the 20 isolates, *A. invadans* was the only fungi and *A. hydrophila* was dominant in the bacterial isolates among *Pseudomonas* sp*, Vibrio* sp, *Acinetobacter* sp, *Enterobacter* sp, *Edwardsiella* sp, *Flavobacterium* sp, *Yersinia* sp, *Klebsiella* sp*, Haemophilus* sp, *Staphylococcus* sp, *Alcaligenes* sp and *V. parahaemolyticus* etc., isolated from muscle, gill liver and intestine. The identification of the fungi *A. invadans* was made on the basis of attachment to the surface, hyphae and sporangial morphology (Anon, 1994) and bacterial isolates were confirmed based on the morphological, physiological and biochemical characteristics of the isolates following Bergey's Manual of Classification (1998) and the results are recorded in the Table 3. *A. invadans* was found in the ulcerative tissue as macroscopic lesions in the muscles of the diseased *H. fossilis*.

The present study showed a high prevalence of motile aeromonad bacteria (35.8%) next to the *A. invadans* (41%) in all lesions (n= 167) and motile aeromonads were also recovered from internal organs of muscle, gills, liver and intestine in ulcerated fish indicating systemic invasion. The selected strains were administered to the healthy normal juvenile *Catla catla* for determination of LC_{50}. The mortality rates of *Catla catla* exposed to different concentration of bacterial strains are given in fig. 1.

Table.1 Enumeration of THBP fish sample muscle, gill, liver and intestine of diseased fish

S. No	Sample	Colony forming unit g-1 (CFU g-1)
1	Muscle	$6.3 \pm 0.4 \times 10^7$
2	Gill	$5.7 \pm 0.6 \times 10^6$
3	Liver	$7.2 \pm 0.9 \times 10^5$
4	Intestine	$4.3 \pm 0.7 \times 10^4$

(Values are mean ± Standard deviation).

Table.2 Distributions of mycotic and bacterial isolates in fish sample

S. No.	Microorganisms	% of colonies in fish sample				
		Muscle	Gill	Liver	Intestine	Total
1	*Aphanomyces invadans*	15	13.5	6.5	6	41
2	*Aeromonas hydrophila*	12.5	9.2	7.8	5.5	35.8
3	*Pseudomonas sp*	2	1	-	0.1	3.1
4	*Vibrio sp*	0.5	0.6	0.5	1.2	2.8
5	*Acinetobacter sp*	0.9	0.5	0.1	0.6	2.1
6	*Enterobacteria sp*	0.6	0.4	0.4	-	1.4
7	*Edwershilla sp*	0,7	0.2	0.4	0.4	1.7
8	*Flavobacteria sp*	0.4	0.1	0.3	0.1	0.9
9	*Yersinia sp*	0.2	-	0.3	-	0.5
10	*Klebsiella sp*	0.1	0.1	0.1	-	0.3
11	*Hemophilius sp*	0.2	0.1	-	0.2	0.5
12	*Staphylococcus sp*	0.3	0.1	0.2	0.1	0.7
13	*Alcaligenes sp*	0.5	0.5	0.3	0.4	1.7
14	*V.parahaemolyticus*	1.2	0.9	0.3	0.4	2.8
15	*A.salmonicida*	0.9	0.5	0.6	-	2..2
16	*Salmonella sp*	0.7	0.6	0.4	0.1	1.8.
17	*Escherchia coli*	0.6	0.1	0.1	0.4	1.2
18	*Micrococcus*	0.2	-	-	0.3	0.5
19	*Aquaspirillium*	0.6	0.3	0.2	0.2	1.3
20	*V.harveyi*	0.4	0.4	0.5	0.4	1.7
21	Others	0.5	0.3	0.5	0.4	0.3

Table.3 Biochemical characteristics of the pathogenic strain isolated from fish.

S. No	Biochemical tests	*A. hydrophila*	*Pseudomonas sp*	*Enterobacteria sp*
1	Grams, staining	-	-	-
2	Motility	+	+	D
3	Kovac's oxidase test	+	-	-
4	Oxidation fermentation tests	+	+	-
5	Catalase tests	+	+	D
6	Cytochrome tests	+	+	-
7	Huge & Leifson tests	F	N	F
8	Starch hydrolysis	D	D	-
9	Gelatin hydrolysis	D	D	-
10	Nacl tolerance (0%)	+	D	-
	Nacl tolerance (5%)	-	D	D
	Nacl tolerance (7%)	-	D	D
11	Indole	+	-	-
12	Methyl Red test	+	D	+
13	Voges Proskeur	+	D	-
14	Citrate utilization test	+	D	-
15	Amino acid decarboxlase (Arginine)	+	D	D
	(Lysine)	+	D	D
	(Ornithine)	-	D	D
16	Urease test	-	D	D
17	ONPG test	D	D	+
18	0/29 sensitivity test	-	D	D
19	Growth at 5^0C	-	D	D
20	Growth at 37^0C	+	+	+
21	H^2S	+	-	D

(+) **Positive; (-) Negative; (D)**

Table.4 LC_{50} value of *Staphylococcus aureus*

Concentration of *A. hydrophila (cfu/ml)*	Initial number of fish	Dead	Survival	Dead ratio (%)	Survival ratio (%)	Mortality	Cumulative mortality (%)
10^8	10	10	0	32	0	32/32	100.0
10^7	10	8	2	22	2	22/24	91.7
10^6	10	7	3	14	5	14/19	73.7
10^5	10	5	5	7	10	7/17	41.2
10^4	10	2	8	2	18	2/20	10.0

LC_{50} = 5.24 x 10^6 cfu/ml

Table.5 LC_{50} value of *Aeromonas hydrophila*

Concentration of *A. salmonicida (cfu/ml)*	Initial number	Dead	Survival	Dead ratio (%)	Survival ratio (%)	Mortality	Cumulative mortality (%)
10^8	10	10	0	29	0	29/29	100.0
10^7	10	6	4	19	4	19/23	82.6
10^6	10	5	5	13	9	13/22	59.1
10^5	10	5	5	8	14	8/22	36.4
10^4	10	3	7	3	21	3/24	12.5

LC_{50} = 2.51 x 10^6 cfu/ml

Table.6 LC_{50} value of *Aeromonas salmonicida*

Concentration of *Vibrio sp. (cfu/ml)*	Initial number	Dead	Survival	Dead ratio (%)	Survival ratio (%)	Mortality	Cumulative mortality (%)
10^8	10	10	0	39	0	39/39	100.0
10^7	10	9	1	29	1	29/30	96.7
10^6	10	9	1	20	2	20/22	90.9
10^5	10	8	2	11	4	11/15	73.3
10^4	10	3	7	3	11	3/14	21.4

LC_{50} = 2.81 x 10^5 cfu/ml

Table.7 LC_{50} value of *Escherichia coli*

Concentration of *E. coli* (cfu/ml)	Initial number	Dead	Survival	Dead ratio (%)	Survival ratio (%)	Mortality	Cumulative mortality (%)
10^8	10	10	0	30	0	30/30	100.0
10^7	10	8	2	20	2	20/22	90.9
10^6	10	5	5	12	7	12/19	63.2
10^5	10	5	5	7	12	7/19	36.8
10^4	10	2	8	2	20	2/22	9.9

LC_{50} = 3.16 x 10^6 cfu/ml

Table 8. LC_{50} value of *Vibrio* species

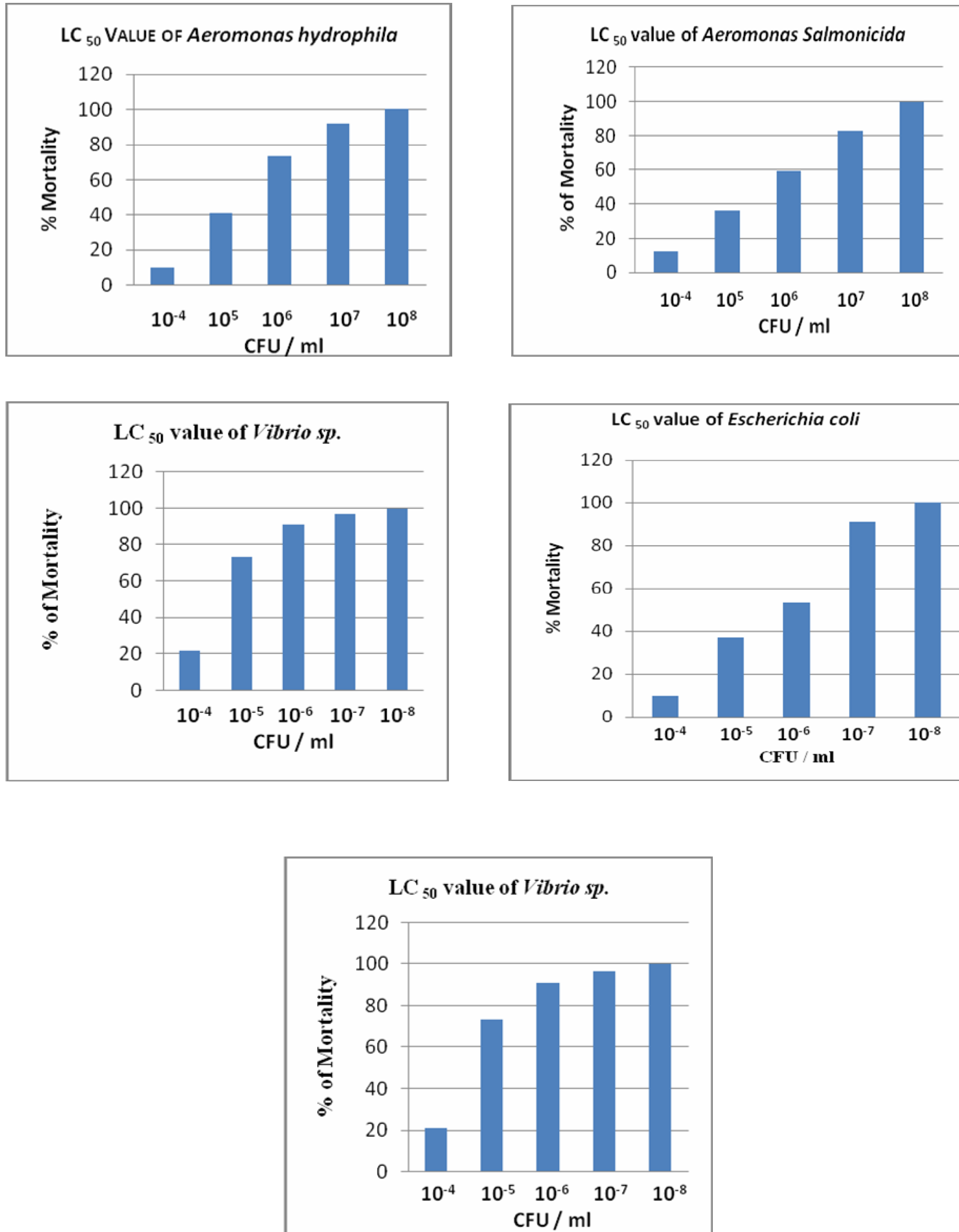
Concentration of (*cfu/ml*)	Initial number	Dead	Survival	Dead ratio (%)	Survival ratio (%)	Mortality	Cumulative mortality (%)
10^8	10	10	0	30	0	30/30	100.0
10^7	10	8	2	20	2	21/22	95.5
10^6	10	5	5	12	7	14/19	73.7
10^5	10	5	5	7	12	8/19	42.1
10^4	10	2	8	2	20	3/22	13.6

LC_{50} = 3.16 x 10^6 cfu/ml

From the mortality rates the LC_{50} value was calculated and recorded in tables 4 to 8. The LC_{50} value for the *Aeromonas hydrophila* was 5.4×10^6 CFU/ml, *Aeromonas salmonicida* was 2.51×10^6 CFU/ml, *Vibrio* sp., 2.81×10^6 CFU/ml, *Escherichia coli* was 3.16×10^6 CFU/ml and *Staphylococcus aureus* was 3.16×10^6 respectively.

The mean of bacterial load was observed to be higher that fungal load. The mean bacterial load was found to be high in muscle load ($6.3 \pm 0.4\times10^7$) cfu g^{-1} followed by gills load ($5.7 \pm 0.6\times10^6$) cfu/ml, liver ($7.2 \pm 0.9\times10^5$) cfu/ml and intestine ($4.3 \pm 0.7\times10^4$cfu/ml). Similarly Al-Harbi *et al.,* (2003) stated higher bacterial load in gills followed by intestine of hybrid Tilapia. Totally 19 bacteria and one fungus were isolated from infected *Carassius auratus*. These results are supported by Katoch *et al.,* (2003) who has reported 25 bacterial and fungal species in fresh water carp at Himachal Pradesh, India. Similarly, a total of 17 bacterial and mycotic species were isolated and identified in *Channa striatus* in India with most of the isolates from muscle and gills (Dhanaraj *et al.,* 2008). The fungal species *A. invadans* were found in all the samples and lesion of infected individuals. The fungus *A. invadans* was identified by the attachment to the surface, hyphae and sporangial morphology. *Aphanomyces invadans*, *Aspergillus flavus*

Fig.1 LC_{50} value of different types of bacterial antigens against fish *Catla catla*



and *Aspergillus fumigates* were the main fungi isolated from the Nigerian freshwater fish culture (Opkonasili *et al.,* 1998). *A. hydrophila* was dominant in the bacterial isolates found in infected samples. The findings of the study are supported by Thampuran *et al.,* (1995) who have reported the dominance of *Aeromonas hydrophila* in the EUS affected *C. striatus*. Motile aeromonads have been associated with the surface of lesion in EUS fishes (Karunasagar and Karunasagar, 1996). The predominance of *Aeromonas hydrophila* in EUS affected fish has also been reported previously by Kumar *et al.,* (1989) in India. Baruah *et al.,* (2012) reported that several species of bacteria and fungi were found to be associated with EUS affected snakehead *C. striatus* and that 89% of the total isolates were *Aeromonas hydrophila*. Some of these *A. hydrophila* strains have been characterized as virulent (Torres *et al.,* 1990) or cytotoxic (Yadav *et al.,* 1992). SabinaYesmin *et al.,* (2004) have reported that *Aeromonas hydrophila* is one of the important pathogens of fish in freshwater and brackish water. In the present study, *Pseudomonas* sp., *Flavobacteriumsp, Alcaligenessp, Vibrio* sp., etc., were found in addition to *Aeromonas hydrophila* and *Aeromonas invadans*. Thampuran *et al.,* (1995), have also reported the presence of *Pseudomonas* sp., *Alcaligenes* sp., *Micrococcus* sp. and *E. coli*, in infected *C. striatus*.

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