

***Bacillus cereus* infection in stinging catfish, *Heteropneustes fossilis* (Siluriformes: Heteropneustidae) and their recovery by *Argemone mexicana* seed extract**

**Chandra G.\*; Bhattacharjee I.; Chatterjee S.**

Received: October 2012

Accepted: June 2015

**Abstract**

Mass mortality of stinging catfish, *Heteropneustes fossilis*, was observed in a fish farm of Kulgaria, Burdwan in West Bengal, India. The cumulative mortality rates reached up to 5% of the total fish in the farm per day. The clinical signs of the affected fish showed ulcers on the skin which gradually grew in size and progressed to form ulcerous dermatitis. Clinical signs of catfish experimentally infected with the isolate were similar to those observed in the affected fish of the fish farm. The isolate was identified as *Bacillus cereus* through biochemical tests and analysis of the isolate by 16S rDNA sequences (97%). With dip treatment (10 min/day) from day 5 to day 15 with *Argemone mexicana* 1 g/L chloroform: methanol seed extracts, the mortality rate decreased and the treated fish gradually recovered on day 15 and their ulcers completely healed on day 21. The hematological values attained following treatment were very close to that of the normal values ( $p>0.05$ ). This study is the first time report of *B. cereus* acting as a pathogen in fish and causing mortality in any fish farm.

**Keywords:** *Bacillus cereus*, *Heteropneustes fossilis*, Ulcer, Mass mortality, Hematology, *Argemone mexicana*

---

University of Burdwan, Golapbag, West Bengal, India, 713104

\*Corresponding author's email: goutamchandra63@yahoo.co.in

## Introduction

Stinging catfish, *Heteropneustes fossilis* (Siluriformes: Heteropneustidae) occur commonly in different aquatic bodies and can tolerate desiccation and low dissolved oxygen conditions (Talwar and Jhingran, 1991; Khanna and Singh, 2003). They are susceptible to several bacterial infections, mainly when reared in high density conditions. Disease outbreaks are responsible for elevated mortality rates and decrease in productivity efficiency, causing high economic losses to fish farmers (Grizez and Ollevier, 1995; Swain et al., 2002).

There has been occasional mention of *B. cereus* as a fish pathogen (Pychynski et al., 1981; Baya et al., 1992). It is reported to cause branchionecrosis in common carp (Pychynski et al., 1981) and striped bass (Baya et al., 1992). However, the supporting evidences are weak.

The problems in farms are usually tackled by preventing disease outbreaks or by treating the actual disease with drugs or chemicals. The use of antimicrobial agents has increased significantly in aquaculture practices (Alderman and Michel, 1992). Antibiotics used in both human as well as veterinary medicines have been tried experimentally to treat bacterial infections of fish. Problems including solubility, palatability, toxicity, cost, delivery and governmental restrictions have limited the available antibiotics to a select few, especially in food fish culture. Decreased efficacy and resistance of pathogens to antibiotics has necessitated the development of new alternatives (Smith et al., 1994).

Owing to easy availability, low cost, proven efficiency, and negligible side effects, medicinal plants have been extensively used in traditional medicine practices worldwide (Martin-Bettolo, 1980). Such plants are known to contain physiologically active principles that have been explored, incorporated, and subsequently exploited in traditional medicines for the treatment of various ailments including ulcers in humans and animals (Srinivasan et al., 2001; Chah et al., 2006). One such plant, *A. mexicana* L. (Papaveraceae), commonly known as prickly poppy, is used as a medicinal plant in several countries (Bhattacharjee et al., 2006).

In October 2009, mass mortality of stinging cat fish occurred in a catfish farm, at Kulgaria, a site near Burdwan, West Bengal, India. The aim of the present study was to identify the causative agent responsible for catfish losses in the farm and the cure of infection using medicinal plant extracts. To confirm the identity of the bacterium both phenotypic characteristics as well as 16S rDNA sequence analysis was done. It was also intended to verify the pathogenic capacity of the agent by the fulfillment of Koch's postulates, along with examination of changes in blood parameters.

## Materials and methods

### *Natural infection*

A disease outbreak occurred in a fish farm (average cumulative mortality on the farm was 5% per day) located at Kulgaria, Burdwan, West Bengal, India due to infection in response to stocking density of 4-5 kg m<sup>-2</sup>. Seventy five male and female

affected stinging catfish (*H. fossilis*) of market-size ( $150\pm2$ g) showing ulcers were collected. These fish were transported to the laboratory following standard protocol. The fish were maintained in 1.5 ton capacity fiberglass reinforced plastic (FPR) tanks supplied with dechlorinated tap water in a flow through system (dissolved oxygen  $6.0\text{-}6.7\text{ mgL}^{-1}$ , nitrite  $0.5\text{-}0.6\text{ mg L}^{-1}$ , temperature  $25\pm1^\circ\text{C}$ , and pH 6.8-7.1). The fish were subjected to clinical and postmortem examinations (Austin and Austin, 1993).

#### *Isolation and Identification of bacterial strain*

Bacteria were aseptically isolated from the liver and kidney of *H. fossilis*. For bacteriological analyses, liver and kidney tissue from 15 affected fish were inoculated on tryptic soya agar (TSA). After incubation at  $25^\circ\text{C}$  for 24h, a dominant colony on TSA agar plates was re-streaked onto TSA to obtain a pure isolate (Strain *S*). Morphological characteristics for preliminary identification and biochemical tests were performed according to *Bergey's Manual of Systematic Bacteriology* (Williams *et al.*, 1986). The DNA templates were amplified by a polymerase chain reaction (PCR) thermocycler. A total volume of  $100\text{ }\mu\text{l}$  PCR reaction mixture containing PCR buffer,  $0.2\text{ mM}$  deoxynucleoside triphosphate (dNTP),  $1.0\text{ mM}$  MgCl<sub>2</sub>, 20 pmole of bacterial primer 8f (5'-AGA GTT TGA TCC TGGCTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') was used for the study. This mixture was used to denature  $0.1\text{ }\mu\text{l}$  of fresh cells at  $95^\circ\text{C}$  for 4 minutes. Then, 1 unit of Taq

DNA polymerase was added to the reaction. This was followed by 30 cycles of denaturation at  $94^\circ\text{C}$  for 30s, annealing at  $48^\circ\text{C}$  for 30s and further extension at  $72^\circ\text{C}$  for 1 min. The final cycle was extended at  $72^\circ\text{C}$  for 2 min.

The reaction product was purified using PCR purification kits. The analyses of the sequences were performed using a genetic analyzer. The sequence obtained was analyzed to find the closest homolog of the microbes using a combination of NCBI (National Centre for Biotechnology Information) GenBank and RDP (Ribosomal Data Base Project). The distance matrix was also based on nucleotide sequence homology (using Kimura-2 parameter). Finally, phylogenetic trees were constructed using Mega 3.1 software by the neighbor joining method with Boostarp analysis to obtain information on the molecular phylogeny.

#### *Sensitivity test*

Antibiotic susceptibility of the isolate was assessed by the disc diffusion method (Bauer *et al.*, 1966) using Müller-Hinton agar (BD). High-potency bio-discs were procured from Himedia, Mumbai, India. The concentrations ( $\mu\text{g disc}^{-1}$ ) of different antibiotics used in the test were chloramphenicol (30), oxytetracycline (30), ampicillin (10), norfloxacin (10), ciprofloxacin (5), oxolinic acid (2), kanamycin (30) and erythromycin (15). The diameters of the inhibition zone were measured following 24 h of incubation at  $25^\circ\text{C}$ .

#### *Experimental infection*

A total number of 420 (210 male and 210 female) *H. fossilis* with an average body weight of  $149 \pm 2$  g were obtained from the said fish farm. The fish apparently healthy, free from any visible skin lesions and with normal behavioral reflexes were transported to the laboratory and maintained in conditions described above. The fish were allowed to acclimatize for 2 weeks prior to experimental infection with isolated fish pathogen.

Mean lethal doses ( $LD_{50}$ ) for the isolated bacterium (Strain *S*) were estimated in *H. fossilis* according to Behrens and Karber, (1953); Yu et al. (2009). Five groups (Group I -V) each consisting of 40 fish, were used for experimental infection. The isolates (Strain *S*) were grown overnight on TSA medium at  $25^{\circ}\text{C}$ , and cell suspensions were prepared in sterile saline (0.85%). Fish were injected intra-peritoneally with a serial 10-fold dilution of the isolated strain *S* ( $0.1\text{ mL fish}^{-1}$ ) for each dilution. The final concentrations of the bacterium injected to groups I, II, III and IV of fish were  $10^3$ ,  $10^4$ ,  $10^5$  and  $10^6$  viable cells  $\text{mL}^{-1}$  respectively. A control fish group (Group V) was injected with 0.1 mL of 0.85% saline. Fish were not fed throughout the experimental period. Macroscopic alterations in the fish were recorded and dead fish were analyzed for the isolated pathogens.

#### *Experimental infection for further study*

After acclimation, the experimental fish were fed with commercial non-medicated fish pellet at a rate of 20% body weight once daily. The fish were divided into two groups; control ( $n=20$ ) and experimental ( $n=200$ ) of either sex. Each fish of experimental group was injected

intraperitoneally, using 21 gauge needles, with 0.1 mL of 24 hour broth culture containing approximately  $3.6 \times 10^5$  CFU  $\text{fish}^{-1}$  ( $LD_{50}$  value) of isolated strain *S*. Control fish received 0.1 mL of sterile broth. The surviving experimental fish, on day 5 of infection, were further divided into two subgroups (*sg*), each with 50 fish. One *sg* was with infected fish without any dip treatment (untreated). The second *sg* comprised of infected and dip treated fish (treated). Fish of the two *sg* were kept in two separate tanks. All groups and *sg* were maintained at  $25 \pm 3^{\circ}\text{C}$ , 14L: 10D in the laboratory conditions. Clinical changes were noted following days 5, 10 and 15 of infection. The tanks were checked daily for mortality to remove any dead fish. Reisolation of bacterium was attempted.

#### *Dip treatment*

For dip treatment, 200 mL of chloroform: methanol (1:1, v/v) was mixed with 50 g powdered *A. mexicana* seeds. The mixture was kept in a tightly sealed vessel and incubated at room temperature using a shaker incubator (temperature range 5- $50^{\circ}\text{C}$ ) for 24 h. The extract was filtered through double-layered cheese cloth and centrifuged at  $2000 \times g$  for 15 min at  $20^{\circ}\text{C}$ . The extracted liquid was subjected to rotary evaporation in order to remove the methanol. The residue was scrapped out of the glass beaker and dissolved in water (1 g/L). Infected fish of the treated *sg* were collected from experimental tanks with minimum stress using a hand net and immediately introduced into the treatment tank (75 L capacity), containing freshly prepared extract, for 10 min/day from day 5 to day 15. After treatment, the fish were

transferred back to their respective experimental tanks.

#### *Collection of blood*

Feed was withheld from fish 24h before collection of blood samples. From randomly picked fish ( $n=6$  from each sg) at 5, 10 and 15 day interval, after anaesthetizing with benzocaine solution (50 mg/L), blood was collected from the caudal vein with a 1 ml plastic syringe ringed with heparin and stored at 4°C and used on the same day. Blood samples were also collected without heparin, allowed to clot, centrifuged at 7000 x g and sera were collected and refrigerated. Pooled blood and sera was collected from six individuals randomly from each sg of infected experimental fish, both "treated" and "untreated" groups on days 5, 10 and 15, depending upon volume, for estimation of biochemical parameters. The red blood cell count (RBC;  $10^6 \text{ mm}^{-3}$ ) was determined in a 1:20 dilution of the blood sample in Hayem's solution and the white blood cell count (WBC;  $10^4 \text{ mm}^{-3}$ ) from a 1:200 dilution of the blood sample in Turke's solution using a Neubauer haemocytometer. The average of triplicate microhaematocrit values were used to determine the RBC volume at 10,000 x g for 5 min (Hct; %) (Larsen and Snieszko, 1961). 20  $\mu\text{L}$  of the blood sample was drawn from a heparinized capillary tube and mixed with 5.0 mL of cyanhaemoglobin reagent (Hycel). Blood hemoglobin (g/dL) content was determined following cyanomethemoglobin method (Van Kampen and Zijlstra, 1961). Serum was removed with a disposable transfer pipette. Total protein, albumin, globulin, albumin:

globulin, cholesterol, triglyceride, urea, Serum Glutamate Pyruvate Transaminase (SGPT) and Serum Glutamate Oxaloacetate Transaminase (SGOT) were determined by enzymatic methods with an automated analyzer.

Five blood smears were prepared for each fish, from fresh blood, air-dried, stained with Leishman-Giemsa's stain and fixed in methanol. In each sample, three visual fields were identified at 1000 X for differential leukocyte counts (Houston, 1990). The whole experiment was repeated three times.

#### *Statistical analysis*

Available data were justified using Student's *t*-test (Zar, 1999).

## **Results**

*Clinical signs of naturally infected fishes*  
Naturally infected *H. fossilis* revealed skin ulceration, and abdominal distention. Gill samples of some of the affected fish revealed the presence of *Trichodina* sp., although this did not seem to be the major cause of the overall signs.

Internally, the affected fish showed abdominal dropsy, liver congestion and vent protrusion. The spleen and kidney were enlarged. Gram-stained imprints of the liver and kidney revealed numerous small rod shaped gram-positive bacterium. All the fish died within 10 -12 days.

#### *Bacterial characterization and identification*

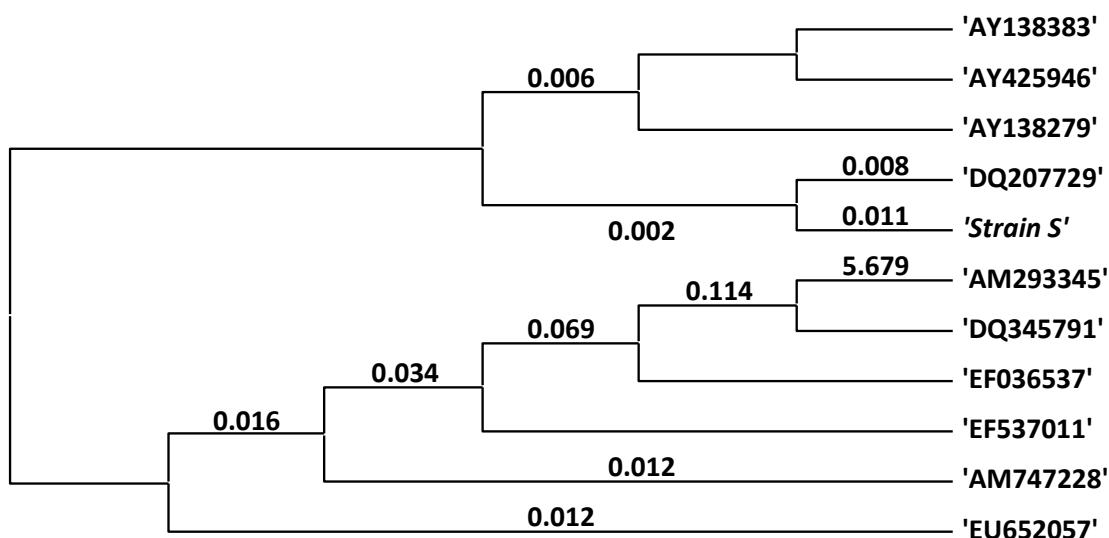
Morphological features coupled with biochemical profile revealed that the pathogenic isolate was a rod shaped

endospore (heat tolerance up to 45°C) forming, gram- positive bacterium. It grew between 20-45°C with an optimum growth temperature of 25°C. The isolate was found to grow within a pH range of 5-10 with optimum growth at pH 7. Biochemical tests revealed that they were negative to indole, and positive to VP, citrate utilization, caesin and gelatin hydrolysis, starch hydrolysis, oxidase and catalase testTABLEs. In order to gain more taxonomic information on the strain

(*Strain S*), the 16S rDNA was partially sequenced and the isolates were presumptively identified as *Bacillus cereus* (97%). The nearest homologous species was found to be *B. cereus* strain CCM 2010 (GenBank Accession Number: DQ207729). The aligned sequence data have been presented in Fig. 1. The phylogenetic relations of the bacterial strain with other closely related species have been presented in the dendrogram (Fig. 2).

```
TGGAGAGTTGATCGTGGCTCAGGATGAACGCTGGCGGCGTCATAATACATGCAAGTCGAGCGA
ATGGATTAAAGAGCTTGCCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGTAACCTGC
CCATAAGACTGGGATAACTCCGGAAACCGGGGCTAATACCGGATAACATTTGAACGTGCATGGT
TCGAAATTGAAAGGCAGCTCGGCTGTCACTTATGGATGGACCCCGCGCATAGCTAGTTGGTG
AGGTAAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCCGTGAAGTGAAGGCTTCGGGCGTAAAAGTCTGTTAGGGAAAGAA
CAAGTGCTATTGAAATAAGCTGGCACCTGACGGTACCTAACAGAAAGCCACGGCTAACTACGT
GCCAGCCGCCCGGTAATACGTAGGTGGCAAGCGTTACCGGAATTATTGGGCGTACAGCGCGC
CAGGTGGTTCTTAAGTCTGATGTGAAAGCCCACGGCTAACCGTGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGG
AGGAACACCAGTGGCGAAGGCAGCTTCTGGTCTGTAAGTGAACACTGAGGCGCAGACGTGGG
AGCAAACAGGATTAGATACCTGGTAGTCCACGCCGAAACGATGAGTGTAAAGTGTAGAGGGT
TTCCGCCCTTACTGCTGAAGTTAACGCATTAAGCACTCCGCTGGGAGTACGGCCGAAGGCTG
AAACTCAAAGGAATTGACGGGGGCCGCACAAGCGGTGGAGCATGTGGTTAATTGAAAGCAACG
CGAAGAACCTTACCAAGGTCTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCTCGGGAGC
AGAGTGACAGGTGGTAGGTGACATGGTGTGTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCAC
GAGCGCAACCCCTGATCTTAGTTGCCATCTTAGTTGGCACTCTAAGGTGACTGCCGTGACAA
ACCGGAGGAAGGTGGGATGACGTCAAATCATGCCCCCTATGACCTGGCTACACACGTGCT
ACAATGGACGGTACAAAGAGGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACGTTCTCAG
TTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGC
CCGGTGAATACGTTCCGGCCTTGTACACACCGCCCGTACACCCACGAGAGTTGAAACACCCG
AAGTCGGTGGGTAACCTTTGGAGGCCAGCCCTAAGGTGGGACAGATGATTGGGTGAAGTC
GTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGAACACCTCCTT
```

**Figure 1:** Aligned sequence data (1547 bp) of the isolated bacterium *Bacillus cereus* (“Strain S”).



**Figure 2:** Dendrogram showing phylogenetic relations of the bacterial strain “*Strain S*” with other closely related bacteria.

#### Sensitivity test

Antibiotic susceptibility tests indicated that the isolates were sensitive to chloramphenicol (17mm), norfloxacin (16mm), ciprofloxacin (20mm), erythromycin (23mm), and oxolinic acid (19mm) but resistant to ampicillin (2mm), oxytetracycline (3mm) and kanamycin (3mm).

#### Experimental infection

##### Experimental infection to determine LD<sub>50</sub> value

Injection with a bacterial dose of 10<sup>3</sup> CFU fish<sup>-1</sup> (Group I) led to neither visible signs nor any mortality on day 5. In case of injection with an infectious dose of 10<sup>4</sup> CFU fish<sup>-1</sup> (Group II), the mortality rate was 30% on day 5 post infection (p.i.). Mortality rate reached 48.5% on day 5 p.i. when infectious doses of 10<sup>5</sup> CFU fish<sup>-1</sup> (Group III) were used. All fish died within 3 days p.i. when injected with 10<sup>6</sup> CFU fish<sup>-1</sup> (Group IV). Group V (control) did not show any abnormality or clinical signs or mortality during the experiment. Based

on these findings, a dose of 3.6 × 10<sup>5</sup> CFU fish<sup>-1</sup>, which was very close to LD<sub>50</sub> value, was deemed appropriate for further infection trials.

##### Experimental infection for further study and clinical signs of experimentally infected fish

All dead fish showed typical external and internal clinical signs comparable to those found in naturally infected fish in the farm. Affected fish showed hemorrhagic spots, which progressed to form ulcerous dermatitis. Initially, the area around the probing site turned reddish which swelled gradually. Mucous layer was affected. Swimming behavior was disturbed. The ulcers were observed to grow in size (25–35 mm in diameter), skin further depigmented, and underlying muscles eroded. The infected fish refused food. Fish became sluggish, followed by agony and finally all the infected fishes “untreated” died between days 10–12. The internal manifestations included ascites of varying intensities and predominant

oedema on the skin and various internal organs. Liver congestion and vent protrusion was also noted. The spleen and kidney were enlarged.

#### Dip treatment

Dip treated fish gradually recovered on day 15 and their ulcer cured and completely healed on day 21. On day 15

their ulcer size reduced and the treated fish became energetic. Their intake of food and their movement was restored.

#### Hematological indices and differential leukocyte count

The changes in primary hematological parameters are indicated in Table 1.

**Table 1: Selected blood parameters (mean±S.E.; n=3) in *Heteropneustes fossilis* infected with *Bacillus cereus* at chosen days during the experimental period receiving daily dip treatment with *Argemone mexicana* seed extract.**

Parameters Days	Control 1	Status	Days		
			5	10	15
Hb (in dl <sup>-1</sup> )	12.00 ± 1.42	Untreated	11.05 ± 1.10 <sup>N.S.</sup>	7.05 ± 0.20*	6.39 ± 0.30*
		Treated	7.56 ± 1.10 <sup>N.S.</sup>	8.90 ± 1.03 <sup>N.S.</sup>	10.58 ± 0.10 <sup>N.S.</sup>
Hct (%)	34.04 ± 0.90	Untreated	23.96 ± 2.39 <sup>N.S.</sup>	22.00 ± 2.09 <sup>N.S.</sup>	16.25 ± 2.36**
		Treated	21.20 ± 0.10 <sup>N.S.</sup>	28.56 ± 0.88 <sup>N.S.</sup>	31.00 ± 0.70 <sup>N.S.</sup>
WBC (10 <sup>4</sup> mm <sup>-3</sup> )	2.50 ± 0.18	Untreated	3.18 ± 0.20*	3.40 ± 0.16**	3.65 ± 0.60**
		Treated	3.19 ± 0.70*	3.02 ± 0.10*	2.59 ± 0.12 <sup>N.S.</sup>
RBC ((10 <sup>6</sup> mm <sup>-3</sup> )	1.05 ± 0.05	Untreated	1.18 ± 0.30 <sup>N.S.</sup>	1.17 ± 0.40 <sup>N.S.</sup>	1.13 ± 0.39 <sup>N.S.</sup>
		Treated	1.11 ± 0.42 <sup>N.S.</sup>	1.06 ± 0.08 <sup>N.S.</sup>	1.05 ± 0.31 <sup>N.S.</sup>
LYM (%)	27.00 ± 0.60	Untreated	26.20 ± 1.50*	21.80 ± 0.73**	20.50 ± 0.05***
		Treated	24.96 ± 0.76**	22.70 ± 1.20*	27.50 ± 0.20 <sup>N.S.</sup>
MON (%)	6.00 ± 0.30	Untreated	6.98 ± 0.40 <sup>N.S.</sup>	7.67 ± 0.20*	8.31 ± 0.20**
		Treated	8.92 ± 0.20*	7.00 ± 0.16 <sup>N.S.</sup>	6.51 ± 0.31 <sup>N.S.</sup>
NEU (%)	66.00 ± 0.02	Untreated	65.00 ± 0.70 <sup>N.S.</sup>	60.30 ± 0.20***	58.00 ± 0.40***
		Treated	64.80 ± 0.40 <sup>N.S.</sup>	67.00 ± 1.70 <sup>N.S.</sup>	68.70 ± 0.80 <sup>N.S.</sup>
EOS (%)	1.00 ± 0.28	Untreated	1.82 ± 0.10 <sup>N.S.</sup>	1.17 ± 0.40 <sup>N.S.</sup>	1.17 ± 0.80 <sup>N.S.</sup>
		Treated	1.32 ± 0.20 <sup>N.S.</sup>	1.33 ± 0.90 <sup>N.S.</sup>	1.00 ± 0.20 <sup>N.S.</sup>
Blood Urea (in mg/dl)	20.90 ± 0.85	Untreated	16.10 ± 0.20**	14.80 ± 0.28***	12.70 ± 0.34***
		Treated	17.00 ± 0.37**	18.90 ± 0.25*	20.00 ± 0.37 <sup>N.S.</sup>
Serum Cholesterol (in mg/dl)	526.60 ± 0.55	Untreated	428.60 ± 0.97***	400.15±11.68***	384.60±12.62***
		Treated	433.50±13.75***	472.00 ± 4.64***	493.20 ± 0.70***
Serum Triglyceride (in mg/dl)	123.20 ± 1.12	Untreated	138.40 ± 0.30 <sup>N.S.</sup>	143.60± 5.01 <sup>N.S.</sup>	150.40± 5.60 <sup>N.S.</sup>
		Treated	136.70 ± 3.16 <sup>N.S.</sup>	135.40± 0.40 <sup>N.S.</sup>	130.20± 0.47 <sup>N.S.</sup>
Serum Protein (in gm/dl)	7.30 ± 0.15	Untreated	3.25 ± 0.03***	3.02 ± 0.04***	2.54 ± 0.12***
		Treated	3.60 ± 0.16***	4.95 ± 0.03**	6.30 ± 0.08 <sup>N.S.</sup>
Albumin (in gm/dl)	1.20 ± 0.01	Untreated	0.98 ± 0.01 <sup>N.S.</sup>	0.84±0.07*	0.78 ± 0.06**
		Treated	1.00 ± 0.03 <sup>N.S.</sup>	1.07±0.005 <sup>N.S.</sup>	1.10 ± 0.01 <sup>N.S.</sup>
Globulin (in gm/dl)	4.10 ± 0.01	Untreated	3.81± 0.02 <sup>N.S.</sup>	3.58± 0.22 <sup>N.S.</sup>	3.12 ± 0.05***
		Treated	3.62 ± 0.08**	3.76 ± 0.06 <sup>N.S.</sup>	3.98 ± 0.01 <sup>N.S.</sup>
Albumin : Globulin	0.29 ± 0.01	Untreated	0.25 ± 0.01 <sup>N.S.</sup>	0.21 ± 0.01*	0.16 ± 0.02**
		Treated	0.20 ± 0.01*	0.28±0.005 <sup>N.S.</sup>	0.27 ± 0.003 <sup>N.S.</sup>
SGPT (in IU/L)	49.40 ± 0.36	Untreated	52.40 ± 0.15 <sup>N.S.</sup>	55.80±2.1 <sup>N.S.</sup>	60.20 ± 1.67 <sup>N.S.</sup>
		Treated	52.20 ± 1.71 <sup>N.S.</sup>	51.10±0.25 <sup>N.S.</sup>	50.20 ± 0.20 <sup>N.S.</sup>
SGOT (in IU/L)	282.40 ± 0.28	Untreated	117.00±2.20 <sup>N.S.</sup>	116.50 ± 0.32 <sup>N.S.</sup>	115.20 ± 0.17 <sup>N.S.</sup>
		Treated	296.20 ± 1.00 <sup>N.S.</sup>	302.50±4.25 <sup>N.S.</sup>	311.70 ± 5.13 <sup>N.S.</sup>
Alkaline Phosphatase (in IU/L)	113.90 ± 0.50	Untreated	117.20 ± 0.36 <sup>N.S.</sup>	290.60±0.87 <sup>N.S.</sup>	288.20 ± 0.36 <sup>N.S.</sup>
		Treated	120.10±2.44 <sup>N.S.</sup>	126.20 ± 3.38 <sup>N.S.</sup>	

hemoglobin (hb), haematocrit (hct), white blood cell (WBC), red blood cell (RBC), lymphocyte (lym), monocyte (mon), neutrophil (neu), eosinophil (eos), serum glutamate oxaloacetate transaminase (sgot), serum glutamate pyruvate transaminase (sgpt), not significant (n.s.), significant (\*), highly significant (\*\*), very highly significant (\*\*\*)

## Discussion

Studies on bacterial isolate suggested that the mass mortality that occurred in the stinging catfish farm at Burdwan, West Bengal, India was associated with a bacterial infection caused by *B. cereus* (97%).

According to Tongyoo *et al.*, 2005, *B. cereus* (DQ120941) displayed considerable virulence factors which agglutinated fish erythrocytes. The production of extracellular protease by *B. cereus* is associated with Epizootic Ulcerative Syndrome (EUS) in Siamese tiger fish (*Datnioides microlepsis*).

In the “untreated” sg, the WBC level initially increased from day 5 onwards, reaching a maximum on day 15, while in the “treated” sg it correspondingly decreased.

In both the “untreated” and “treated” sg, the RBC counts did not significantly change from control values. Hb level of both “untreated” and “treated” fish was lower ( $p<0.05$ ) than the control values on day 10 and 15. In *Ictalurus punctatus*, the decreased Hb content was due to swelling of RBC, and poor mobilization of Hb from the spleen and other haemopoietic organs (Scott and Rogers, 1981). These facts support the present findings that any stress can lead to a significant decrease in Hb content, thereby leading to anemia. In pearl spot, *Etroplus suratensis*, the same trend was reported. *E. suratensis* showing symptoms of EUS became anemic followed by significant reduction in Hb and PCV (Pathiratne *et al.*, 1994; Pathiratne and Rajapashe, 1998) values. In this study, LYM<sub>s</sub>, which defend the attack of pathogens, decreased in the “untreated”

sg and increased subsequently in the “treated” sg. The MON in the “untreated” sg increased from that in the control sg, but did not differ from that in the “treated” sg. The serum protein level came down significantly in “untreated” fish from that of control fish. The highest importance is attached to the decline of albumin fractions, involved in hypoproteinaemia. Low level of albumin may be the result of losses from the skin lesions, an increased catabolism in acute inflammation or reduced synthesis due to hepatopathy, or may be related to renal damage. The “Untreated” sg reflected a marked decrease in the albumin content of up to 15%, the serum protein content of up to 50% and the ratio of Albumin: Globulin of up to 12%, in comparison to their respective normal values. Since albumin exercises significant antihaemolytic and antibacterial effects and the  $\gamma$ -globulin are carriers of immunoglobulins, their decrease favors infection.

Total and free cholesterol were also somewhat lower in the “untreated” sg. The decrease in lipid fraction following infection could be a result of one or more factors: (1) decrease in the rate and amount of lipid synthesis; (2) increase in the clearance of lipids from serum; (3) less release of lipids from tissues and organs into the blood; and (4) utilization of cholesterol during corticosteroidogenesis. SGOT and SGPT are the other two important enzymes found in all tissues. They catalyze the transfer of the amino group (-NH<sub>2</sub>) from glutamic acid to either oxaloacetic acid or pyruvic acid. Data obtained showed remarkable increase in both the enzymes following *B. cereus*

infection in *H. fossilis*. Marked excess of SGOT is common in myocardial damage (Varley, 1976). The increase in the serum SGOT activity may be an indication of considerable clinical damage and histopathological changes caused by the infection in the liver. This is because SGOT, LDH and SGPT activities in fish serum are known to be very useful as an index for diagnosis of liver function (Everall et al., 1992; Mughal et al., 1993; Shakoori et al., 1994; Ahmad et al., 1995). Interestingly in the "treated" sg, reversible changes occurred, leading to the recovery of the studied parameters to near normal.

It is reported that the plant extracts can control several bacterial diseases of fishes (Bhargava et al. 1986; Thakre and Anjaria, 1986; Harikrishnan et al., 2003, 2005; Rajendiran et al., 2008). The present study showed that dip treatment of *A. mexicana* extract helped complete recovery from *B. cereus* infection and restoration of altered hematological parameters of *H. fossilis*. Further studies are needed to determine the healing power of this herb in mixed infections as well as under laboratory trials since under field conditions diseases usually attain a syndrome level and often the primary pathogen causing a disease is uncertain.

Injection of the isolated bacterium into the healthy fish resulted in high mortality of the fish and developed signs comparable to those found in diseased fish in the farm and so, no test for viremia was done. The biochemical and 16S rDNA sequencing verified that the bacterium reisolated from experimentally infected fish was the same as that used for reinfection. This reisolation of identical bacterium from an

experimentally infected fish presenting signs of disease fulfilled Koch's postulates.

In this study, parasites such as *Trichodina* sp. were occasionally found in some of the affected fish in the farm (data not shown). However, gross lesions were somewhat different from those observed in *Trichodina* infection. Thus the parasite did not seem to be the major factor causing mass mortality.

Present study indicates that the bacterium was highly sensitive to norfloxacin, ciprofloxacin and oxolinic acid compared to other antibiotics. This portrays the feasibility of the antibiotics tested in this study in controlling the *B. cereus* infections. However, chloroform: methanol extract of *A. mexicana* seeds can be used as a potent alternative to control *B. cereus* infection as evidenced by recovery of dip treated fish from infection (disappearance of signs) and restoration of different hematological indices.

## References

- Ahmad, F., Ali, S.S. and Shakoori, A.R., 1995.** Sublethal effects of danitol (Fenpropothrin), a synthetic pyrethroid on the Chinese grass carp, *Ctenopharyngodon idella*. *Folia Biologica*, 43, 151–159.
- Alderman, D.J. and Michel, C., 1992.** Chemotherapy in aquaculture today. In: Chemotherapy in aquaculture from theory to reality (ed. by C. Michel and D.J. Alderman), Office International Des Epizooties, Paris. pp. 3-4.
- Austin, B. and Austin, D.A., 1993.** Bacterial fish pathogens. Eliss Horwood 2<sup>nd</sup> ed. New York, 384P.

- Bauer, A.W., Kirby, W.M., Sheris, J.C. and Turck, M., 1966.** Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology*, 45, 149–158.
- Bayo, A.M., Li, T., Lupiani, B. and Hetrick, F., 1992.** *Bacillus cereus*, a pathogen for striped bass. Eastern Fish Health and American Fisheries Society Fish Health Section Workshop, 16-19 June 1992, Auburn University, Auburn, AL. 6P.
- Behrens, A.L. and Karber, L., 1953.** Determination of LD<sub>50</sub>. Screening in Pharmacology. Academic Press, NY, USA, 60P.
- Bhargava, J.N., Thakur, D.R., Dwivedi, M.P. and Tripathi, S.N., 1986.** Determination of maturity indices in July Elberta peach fruit. In: Advances in Research on Temperate Fruits T.R. Chadha, V.P. Bhutani, J.L. Kaul. (Eds). Solan, India, pp. 315–318.
- Bhattacharjee, I., Chatterjee, S.K., Chatterjee, S.N. and Chandra, G., 2006.** Antibacterial potentiality of *Argemone mexicana* solvent extracts against some pathogenic bacteria. *Memorias Instituto Oswaldo Cruz*, 101(6), 645–648.
- Chah, K.F., Eze, C.A., Emuelosi, C.E. and Esimone, C.O., 2006.** Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants. *Journal of Ethnopharmacology*, 104, 164–167.
- Everall, N.C., Mitchell, C.G. and Robson, J.N., 1992.** Effluent causes of the syndrome in wild adult Atlantic salmon *Salmo salar* from the river Don in Aberdeenshire. *Diseases of Aquatic Organisms*, 12, 199–205.
- Grisez, L. and Ollevier, F., 1995.** Vibrio (*Listonella anguillarum*) infections in marine fish larviculture. In: Lavens, P., Jasper, RoeLands, I. (Eds.), Larvi 91. Fish and Crustacean Larviculture Symposium. European Aquaculture Society, Gent, 497P.
- Harikrishnan, R., Rani, N.M. and Balasundaram, C., 2003.** Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. *Aquaculture*, 221, 41–50.
- Harikrishnan, R., Balasundaram, C. and Bhuvaneswari, R., 2005.** Restorative effect of *Azadirachta indica* aqueous leaf extract dip treatment on haematological parameter changes in *Cyprinus carpio* (L.) experimentally infected with *Aphanomyces invadans* fungus. *Journal of Applied Ichthyology*, 21, 410–413.
- Houston, A.H., 1990.** Blood and circulation. In: Methods for fish Biology. C. B. Schreck and P. B. Moyle (Eds). American Fisheries Society, Maryland, pp. 273–334.
- Khanna, S.S., Singh, H.R., 2003.** A text book of fish biology and fisheries. Narendra Publishing House, Delhi, India.
- Larsen, H.N. and Snieszko, S.F., 1961.** Modification of the microhematocrit techniques with trout blood. *Transactions of American Fish Society*, 90, 139–142.

- Martin-Bettolo, G.B., 1980.** Present aspects of the use of medicinal plants in traditional medicine. *Journal of Ethnopharmacol*, 2, 5–7.
- Mughal, A.L., Iqbal, M.J. and Shakoori, A.R., 1993.** Toxicity of short term exposure of sublethal doses of a synthetic pyrethroid, fenvalerate, on the Chinese grass carp, *Ctenopharyngodon idella*. Proceedings Seminar on Aquatic Development in Pakistan. pp. 49–74.
- Pathiratne, A., Widanapathirana, G. S. and Chandrakanthi, W. H. S., 1994.** Association of *Aeromonas hydrophila* with epizootic ulcerative syndrome (EUS) of freshwater in Sri Lanka. *Journal of Applied Ichthyology*, 10, 204–208.
- Pathiratne, A. and Rajapashe, W., 1998.** Hematological changes associated with epizootic ulcerative syndrome in the Asian cichlid fish *Etroplus suratensis*. *Asian Fisheries Science*, 11, 203–211.
- Pychynski, T., Malanowska, T. and Kozlowski, M., 1981.** Bacterial flora in branchiolar necrosis of carp (particularly *Bacillus cereus* and *Bacillus subtilis*). M ed. Weter. 37, 742–743 (in Polish, summaries In English and Russian).
- Rajendiran, A., Natarajan, E. and Subramanian, P., 2008.** Control of *Aeromonas hydrophila* infection in spotted snakehead, *Channa punctatus*, by *Solanum nigrum* L., a medicinal plant. *Journal of World Aquaculture Society*, 39, 375–383.
- Scott, A.L. and Rogers, W.A., 1981.** Hematological effects of prolonged sublethal hypoxia on channel catfish *Ictalurus punctatus* (Rafinesque). *Journal of Fish Biology*, 18, 591–601.
- Shakoori, A.R., Iqbal, M.J., Mughal, A.L. and Ali, S.S., 1994.** Biochemical changes induced by inorganic mercury on the blood, liver and muscles of fresh water Chinese grass carp, *Ctenopharyngodon idella*. *Journal of Ecotoxicology Environmental Monitoring*, 4, 81–92.
- Smith, P., Hiney, M.P. and Samuels, O.B., 1994.** Bacterial resistance to antimicrobial agents used in fish farming. *Annual Review of Fish Diseases*, 4, 273–313.
- Srinivasan, D., Nathan, S., Suresh, T. and Perumalsamy, O., 2001.** Antibacterial activity of certain Indian medicinal plants used in folkloric medicine. *Journal of Ethnopharmacology*, 74, 217–220.
- Swain, P., Nayak, S.K., Sahu, A., Mohapatra, B.C. and Meher, P.K., 2002.** Bath immunization of spawns, fries and fingerlings of Indian major carps using a particulate antigen and determination of age, dose and duration of antigen exposure. *Fish Shellfish Immunol*, 13, 133–140.
- Talwar, P.K. and Jhingran, A.G., 1991.** Inland fishes of India and adjacent countries. Oxford and IBH Publishing Co., New Delhi, India. 541P.
- Thakre, A.M. and Anjaria, J.V., 1986.** Antimicrobial and infected wound healing response of some traditional drugs. *Indian Journal of Pharmacology*, 18, 171–174.
- Tongyoo, N., Thongsaeng, P., Kumphakarm, R. and Moungmai,**

- R., 2005.** *Bacillus cereus* and *B. Pumilus* associated with epizootic ulcerative syndrome in Siamese Tiger fish (*Datnioides microlepis*). 31st Congress on Science and Technology of Thailand at Suranaree University of Technology, 18 – 20 October.
- VanKampen, E.J. and Zijlstra, W.G., 1961.** Recommendations for haemoglobinometry in Human Blood. *Brazilian Journal of Hematology*, 13, 71.
- Varley, H., 1976.** Practical clinical biochemistry, 4th edn. Heinmann, Indian Pauls Press, New Delhi.
- Williams, S.T., Sharpe, M.E. and Holt, G., (eds.) 1986.** Bergey's manual of systematic bacteriology. Vol.I. Williams & Wilkins, Baltimore. MD. USA.
- Yu, J.H., Han, J.J., Park, K.S., Park, K.H. and Park, S.W., 2009.** *Edwardsiella tarda* infection in Korean catfish, *Silurus asotus*, in a Korean fish farm. *Aquaculture Research*, 41, 19-26.
- Zar, J.H. 1999.** Biostatistical analysis, fourth ed. singapore: Pearson Education (Singapore) (Pte) Ltd., New Delhi (Indian Branch), India. pp. 1-663.