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Research Article

Isolation and virulence characterization of *Pseudomonas fluorescens* isolated from symptomatic catfishes of sub-Himalayan region.

Arvind Kumar Sharma[1]* and Preeti Saxena[2]

1 Maharaja Agrasen Mahavidyalaya, Bareilly, India
2 Gangashee IMahavidyalaya, Nawabganj, Bareilly, India.

ABSTRACT

Occurrence of Bacterial Haemorrhagicsepticaemia (BHS) has been recorded in juvenile / mature *Clarias batrachus* during the summer months (April – July) of the years 2004 – 2006. The symptomatic fishes were exhibiting distended abdomen, haemorrhages, necrotization and ulceration on the general body surface, lethargic behaviour, low appetite, erosion of fins, swollen vents, presence of ascetic fluid in the abdomen and empty intestine filled with exudate fluid. Bacteriological examination revealed the appearance of colonies of *Pseudomonas fluorescens* (PF) in the form of convex / flat rounded with smooth edges and yellowish to fluorescent green on to agar medium. They proliferate at 30 ± 5ºC temperature but did not develop at 45ºC. Out of fifteen isolates PF4 was more virulent. Biochemical characterization of the PF isolates explicated that they were oxidative in nature, oxidase & catalase positive, gelatin, nitrate, arginin, and citrate degradative, mannitol, inositol, sorbitol and arbinose positive but lysine, ornithin, urease, H2S, indole and MR-VP responses negative. Experimental infection trial revealed 100% mortality in CB challenged with bacterial suspension (PF4) containing 6.1 x 10^5, 6.1 x 10^4, 6.1 x 10^3 cfu/ml and LD50 value of this isolate was enumerated to 6.1x$10^{3.5}$ cfu/ml fish-1 in case of CB.

KEY WORDS:*Pseudomonas fluorescens*, catfishes,bacterial suspension

Introduction

The production of fish is generally encumbered due to increasing frequency of infectious diseases in aquaculture which cause high mortality in fish resulting into a great economic loss (Adams *et al.*, 1997) to fish industries. There are about 40-60 bacterial pathogens involved in various diseases but few of them are recognized as potent fish pathogens. Pseudomonads and Aeromonads have been found associated with most devastated disease i.e. EUS of wild and culturable species of the fish (Qureshi *et al.*, 2000) and other aquatic organisms (Austin and Austin, 1999). Almost all culturable species of fishes including carps and catfishes are prone to these pathogens because they are

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***Address for Correspondence** Department of Zoology, Maharaja Agrasen Mahavidyalaya, Bareilly, India
E-mail: arvindsharmamicro@gmail.com

Normal bacterial flora of fish is a direct reflection of the bacterial populations of the water in which they thrive (Sakata *et al.*, 1980; Rintamaki *et al.*, 1997; Hakalathi and Voltonen, 2003 and Gao *et al.*, 2005). Pathogenic microbes are opportunistic pathogens which invade the tissues of a host (fish) and use them as aliment and render them to be susceptible to infection and cause a variety of ailments.

Pseudomonas fluorescens (PF) is important pathogen, ubiquitously present in aquatic system and involved in Bacterial Haemorrhagic Septicaemia (BHS) in fish (Prasad and Qureshi, 1994) as well as in other aquatic animals (Austin and Austin, 1999). This bacterium has also been found persistently associated with EUS infected fishes (Qureshi *et al.*, 2000) which has proved to be devastated disease of reservoirs. It has also been reported to be involved in several ulcerative diseases of carps (Prasad, 1992) and catfishes (Markovick *et al.*, 1996).

P. fluorescens is a Gram-negative, aerobic rod measuring about 0.5 x 3 µm in size, motile with single polar flagellum belonging to the family Pseudomonadaceae. This family

includes other genera which together with certain other bacteria, constitute the members of Pseudomonads. The bacteria of this family are common inhabitant of soil and water. They have been found to be associated with plankton and owing to their pathogenic nature, PF has attracted to the aquaculture microbiologists. It gets entered in to the host body through injury and causes hyperplasia in gill lamellae followed by rottening in soft tissue and colonization of bacteria in the villi of intestine resulting into a variety of systemic infections in freshwater fishes (Prasad, 1992; Prasad and Qureshi, 1995 and Prasad, 2002).

In natural habitat PF is not particularly distinctive as a pseudomonad but it does have a combination of physiological traits that are noteworthy and may relate to its pathogenesis (Saharia and Prasad, 2001). This bacterium can proliferate even in simple nutrients consisting acetate and ammonium sulphate therefore, its laboratory culture is very easy. Its optimum growth temperature is 30 $\pm$ 5^{0}C and can grow at temperatures ranging between 4 to 40^{0}C. It may tolerate to high concentrations of salts and dyes, weak antiseptics and many commonly used antibiotics. These attributes of this bacterium undoubtedly contribute to its ecological success as a ubiquitous distribution and opportunistic pathogen. Its colonies appear like fried egg with convex shape, smooth and flat edged periphery. The smooth colonies are presumed to play a role in colonization and virulence. PF has been found associated with septicaemic and ulcerative conditions in wide range of fish species (Prasad and Qureshi, 1995; Prasad, 2002; Spiers *et al*., 2000; Saharia and Prasad, 2001 and Tryfinopoulou *et al*., 2002).

This bacterium produces three other soluble proteins, a cytotoxin (25 kDa) and two haemolysins which are essential for invasion. The cytotoxin is a pore-forming protein. It was originally named leukocidin due to its effect on neutrophils and proved to be cytotoxic for most of the eukaryotic cells. One of the haemolysins is a phospholipase and the other is a lecithinase. They act synergistically to break down lipids and lecithin. The cytotoxin and haemolysins contribute to invasion through their cytotoxic effects on eukaryotic cells (OCED, 1997).

Pseudomonads are reported to be involved in Tail & Fin rot disease in tilapia, *Tilapia mojambicus* (El-Attar &Moustafa, 1996), Bacterial Gill Disease in salmonids, *Salmo salar* (Ostaland *et al*., 1999), Bacterial Septicaemia in common carps, *Cyprinuscarpiocommunis* (Prasad, 1992 and Prasad & Qureshi, 1994), integumental lesions in coho salmon, *O. kisutch* (Pavanelli *et al*., 2000), internal and external lesions in *Rhanidaquelen* (Shama *et al*., 2000), skin disease with association of *Acinetobaterboumanii*&*Stenotrophomonasmaltopilia* in European eel, *Anguilla anguilla* (Ugar *et al*., 2002) and high mortality of eggs of white fish, *Coregonus sp*. (Wedekind, 2002).

Materials and Methods

Isolation of *Pseudomonas fluorescens*

To isolate *Pseudomonas fluorescens* (PF), inocula were obtained by inserting inoculating loop in the infected regions and organs (liver and kidney) of moribund *Clariasbatrachus* exhibiting overt symptoms of Bacterial HaemorrhagicSepticaemia (BHS) - such as distended abdomen, haemorrhages on the general body surface, lethargic behaviour and low appetite. Attempts were also made to isolate bacteria from the organs / infected regions of diseased fish in which such organs were cut and put in to saline (0.3%) solution, homogenized, centrifuged (Remi R-8CDX) at 3000 rpm for 15 min and supernatants were used for the isolation of PF. They were cultured on selective media - *Pseudomonas* Isolation Agar (PIA, Hi-Media, Mumbai) and *Pseudomonas* Isolation Agar Fluoricine (PIF, Hi-Media, Mumbai) and complex media (Nutrient / Trypticase Soya Agar). Inoculated plates were incubated at 30 $\pm$ 5^{0}C for about 18–24 h and development of creamish / yellowish colonies was noted and photographed as and when required.

Identification of *Pseudomonas*

Culture obtained on selective medium was also grown (streaked) on non-selective medium to get pure colonies of desired bacterium. Colonies of targeted bacterium were further grown on selective medium (*Pseudomonas* isolation agar) and subjected to following biochemical tests to make comparison with the controlled one (ATCC-13525).

Staining

For this purpose a smear was prepared on a clean glass slide by taking inocula from pure colony of test bacterium, heat fixed and stained with crystal violet (30 sec.), Gram's iodine (30 sec.), decolourizer (30 sec.) and safranin (30 sec.). Washing with distilled water was followed with each step for 30 sec. The slides were dried and examined under microscope (Olympus 100X) by using immersion oil. Appearance of bacterial cell in blue colour (violet) was considered as Gram positive and pink colour as Gram negative.

Shape and colour of colonies

Developed colonies were visualized for their shape and appearance of particular colour in them on selective medium and photographed as and when required.

Motility

In this case agar medium was stab inoculated with a straight wire inside the glass tubing to a depth of about 4mm

avoiding the surface of the medium outside the glass tubing and incubated at 30 ± 5^{0}C for 24h. Development of the bacterial growth on the surface of medium outside the inner glass tube was considered as motile activity of bacteria.

Temperature

Pseudomonas fluorescens was subjected to grow in the broth of selective medium and incubated at different temperature ranging between 15-40^{0}C and simultaneously their incubation periods were extended from 24h to 168h depending upon the incubation temperature used.

Growth at Different pH

Test tubes filled with BHI broth having different pH were inoculated with 18 – 24 h old pure culture of PF and incubated at 30 ± 5^{0}C for 24 h. After that turbidity was checked. For confirmation such broth cultures were serially diluted and spread over selective media and further incubated at 30 ± 5^{0}C and appearance of colonies was visualized.

Biochemical Tests

To identify up to genus and species level of the isolated bacteria, selected biochemical tests were accomplished along with control in sterilized media and reagents procured from Hi Media, Mumbai.

Oxidation - Fermentation

Method of Hugh and Leifson (1953) was used with slight modification. Briefly, inoculated tubes were stabbing with a straight wire and one of the tubes was added a layer of molten soft paraffin to a depth of about 1 cm and incubated at 30 ± 5^{0}C and examined daily for about 14 d. Development of green colour was considered as positive reaction and yellow colour denoted as negative reaction.

Oxidase Test

Oxidase test was carried out as per Cowan and Steel (1995) by using filter paper. The filter papers were impregnated with 1% Oxidase reagent (tetra methyl Para phenylenediaminedehydrochloride). The impregnated papers were immediately placed on clean glass slides and a loop full of test bacteria was smeared on them with the help of inoculating loop. Appearance of deep purple colour within 30 sec was considered as positive for Oxidase test.

Indole Test

For this purpose 0.5 ml of Kovac's reagent was added to 5 ml of broth culture grown in 2% peptone water at 35^{0}C for 12 h. Appearance of red colour after shaking was indicated the presence of indole production (positive reaction).

Methyl Red (MR) test

About 2-3 drops of 0.04% methyl red solution were added to 5 ml culture grown in glucose phosphate peptone water for 18 – 24 h at 35^{0}C. Development of bright red colour denoted as positive reaction and yellow colour denoted as negative reaction.

VogesProskauer (VP) test

Following MR test about 0.6 ml of β naphthol was added and after shaking 0.2 ml of 40% aqueous solution of KOH was added mixed well and observed up to 4 h for the development of pink colour which was considered as a positive reaction.

H_2S Production

Triple sugar iron agar (TSIA) was inoculated by stabbing the isolate in to the bottom and streaked on the slop surface. Tubes were incubated at 35^{0}C for 7 d forappearance of blacking in the tubes was considered as positive test.

Catalase Test

Smears of young colonies of each isolate were prepared on clean glass slides and few drops of Hydrogen Per Oxide (H_2O_2) were mixed individually. Production of bubbles immediately after mixing the H_2O_2 was considered as positive for catalase test.

Gelatin Degradation Test

A stab culture of the test bacteria was made in gelatin containing medium and incubated at 35^{0}C for 4d in an up right position. The tubes were kept in refrigerator for half an hour before determining whether the gelatin was still capable of solidifying or not.

Nitrate Test

0.5 ml of 1:1 mixture of nitrate test reagent was added to the culture grown in nitrate broth for 24h at 35^{0}C. Production of pink red colour denoted as positive nitrate production test.

Citrate Test

Citramide agar base plates were inoculated with bacterial growth on non selective medium and incubated at 30 ± 5^{0}C for 24 h yellowish green colour colonies confirms the presence of *Pseudomonas*.

Decarboxylation

Four decarboxylase test media base rich in arginin, lysine, ornithin β galactosidase and control were filled in four sterilized test tubes separately. After plugging with cotton they were autoclaved and after solidification of media 200 ml of liquid paraffin was poured on the solidified surface thereafter, bacterial inocula were taken with the help of straight wire from the plate culture and inoculated in to three test tubes separately through paraffin layer. They were

incubated at 30 ± 5^{0}C and examined daily for four days. Development of yellow colour was taken as an indication of acid production from glucose while development of violet colour was taken as an indication of decarboxylation.

Urease Production

The tubes of urease slant were inoculated by heavy inocula of 18-24h old pure culture of test bacterium and spread all over the slant. These tubes were incubatedwith loosened caps at 35^{0}C for 6 days. Intensive red or cerise colour indicated the positive result and no change in colour indicated the negative result.

Starch Hydrolysis

Starch agar filled plates were inoculated with *P. fluorescens* and incubated at 35^{0}C for 24 - 120 h. Old culture plates were flooded with Lugol's iodine solution. Development of blue colour was an indication that starch has not been hydrolyzed while the appearance of clear colour less zone was an indication of starch hydrolysis.

Virulence characterization (LD_{50})

For each virulent bacterial isolates (PF1 and PF4) 10 Clariasbatrachus (CB) were injected intra muscularly (im) with 0.2 ml of bacterial suspension (prepared in 0.3% NaCl/ BHI broth) containing 6.1 x 10^5, 6.1 x 10^4, 6.1 x 10^3 and 6.1 x 10^2cfu ml-1 of *P. fluorescens* (PF4) and 2.67 x 10^8, 2.67 x 10^7 and 2.67 x 10^6 PF6 isolate. A group (n = 10) was maintained as control by injecting 0.2 ml of 0.3% NaCl / BHI solution. Experimental fishes were kept in aquaria filled with non-chlorinated tap water and temperature was maintained at 25 ± 2^{0}C. The cumulative mortality was noted after 24 h and recorded upto 7, 14, 21 & 28 d and LD50 was calculated as per Reed and Muench (1938). Reisolation of test bacteria was also accomplished on the selective medium by taking inocula from liver and kidney of experimentally infected fishes.

Result

Bacteriological Isolation

Bacterial isolates were obtained from diseased *Labeorohita* and *Clariasbatrachus* exhibiting overt manifestations of Bacterial HaemorrhagicSepticaemia (BHS) collected from Baur Dam, Nanak sagar, Timuria Dam, Haripura Dam, Sharda sagar, Kalagarh Dam, Dhaura Dam and Zhed fish farm during the summer months of 2004-2006. Most of the isolates were isolated from necrotized region and internal organs (liver, kidney, intestine etc.) of symptomatic fish (Table - 1). Overall fifteen isolates (PF1 – PF15) of *Pseudomonas fluorescens* were isolated and examined for their characteristic features through selected biochemical tests and compared with the known strain of Pseudomonas fluorescens (Aqua. Bt. YA/ PFI), identified and divided into three groups.

Diseased fishes exhibited the following symptoms on the basis of which BHS was identified: Haemorrhages, necrotization, (Figs.- 1a, e & f), erosion of fins and swollen vent were highly marked in diseased specimen. Ascetic fluid was filled in the abdomen of infected fishes. Small to large ulcers were also noticed on the general body surface (Figs.- 1b, c and d). Intestine was free from food but filled with exudate fluid.

Table –1: Isolation of *Pseudomonas fluorescens* from different organs of diseased fish collected form various sampling stations. (**Hypophthalmichthys moilitrix*).

Sl. No.	Place of sampling	Name of fish sampled	***Organs used for bacterial isolation***				
			Gill	Liver	Kidney	Intestine	Body surface
1	Baur Dam	Catla catla	+	-	-	-	+
2	Dhaura Dam	*C. batrachus*	-	+	+	-	-
3	Haripura Dam	*Channa sp.*	-	-	-	-	-
4	Kalagarh Dam	*Puntius sp.*	+	-	-	-	-
5	Nanak Sagar	*C. batrachus*	+	+	+	+	-
6	Sharda Sagar	*H. fossilis* *C. batrachus*	- +	+ +	+ -	- -	- +
7	Timuria Dam	*Labeo rohita*	+	-	-	-	+
8	Zhed Farm	*C. batrachus* Silver Carp*	- +	+ -	+ -	+ -	- +

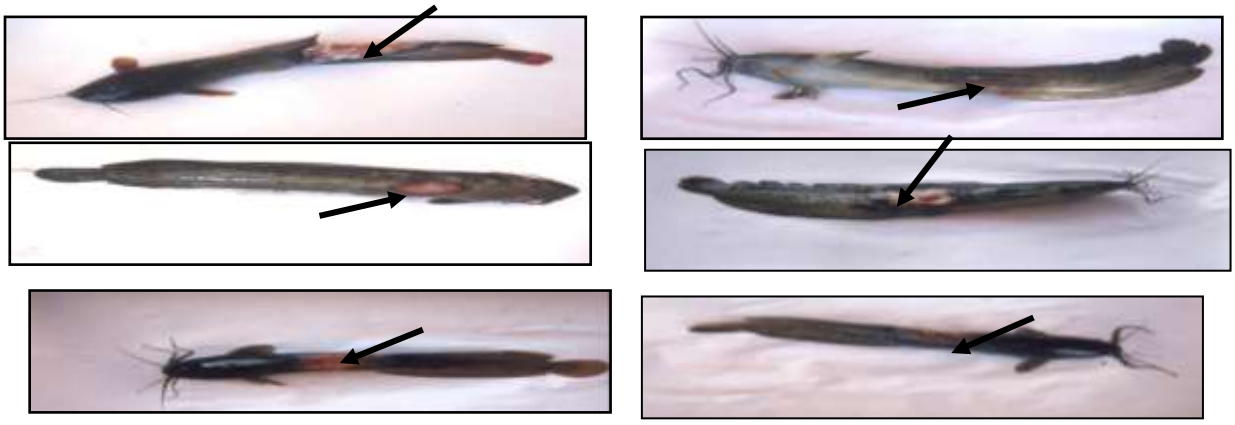

Fig 1a to 1f: Symptomatic *Clarias batrachus* exhibiting symptoms of BHS on the body surface and used for the isolation of *Pseudomonas fluorescens*.

Identification of *Pseudomonas fluorescens*

Pseudomonas flourescens (PF) developed in the form of convex / flat rounded with smooth edges (Figs.- 2a and b) and creamy white to fluorescent green colonies on to agar medium. Colonies appeared like small water droplets which can be removed very easily i.e. they were not firmly attached. All the isolates were Gram-negative rods (Fig. – 2c) and exhibited gliding motility. They developed at optimum temperature of 30 $\pm$ 5^{0}C but did not proliferate at 45^{0}C (Table - 2).

Biochemical tests revealed that all the isolates were oxidative, positive in the oxidase and catalase production, gelatin degradation and nitrate, lysine and citrate utilization. They produced acid from glucose, mannitol, inositol, sorbitol &arbinose and do not produce acid from rhamnose, sucrose, melibiose& amygdalin. But they exhibited negative responses with ornithin utilization, arginindehydrolase, and β galactosidase, H2S, urease production, tryptophane, indole and M.R. - V.P. reactions (Table -2). All the isolates exhibited fluoricin pigmentation.

Table - 2: Selected biochemical characteristics of different isolates of *Pseudomonas fluorescens* isolated from diseased fish and their comparison with standard isolate (Aqua. Bt. YA/ PFI).

Sl. No	Tests	Aqua Bt. YA / PFI	PF 1-5	**PF 6-10**	**PF 11-15**
1	Gram reaction	-	-	-	-
2	Shape of colonies	R	R	R	R
3	Colour of colonies	YG	Y	YG	YG
4	Motility	+	+	+	+
5	**Temperature**	**Growth / Survivality of PF**			
	0^{0}C - 3^{0}C	-ve	-ve	-ve	-ve
	4^{0}C -10^{0}C	+/-	+	+	+
	11^{0}C -15^{0}C	++	++	++	++
	20^{0}C -37^{0}C	+++	+++	+++	+++
	38^{0}C - 40^{0}C	+	+	+	+
	41^{0}C - 45^{0}C	-ve	-ve	-ve	-ve
6	**pH**	**Growth / Survivality of PF**			
	5.0 to 5.5	-	-	-	-
	5.6 to 6.0	+	+	+	+
	6.5 to 7.5	++	++	+++	++

	8.0 to 8.5	+	+	+	+
	8.6 to 9.0	-	-	-	-
7	Oxidative / Fermentative	O	O	O	O
8	Oxidase production	+	+	+	+
9	Indole production	-/+	-	-/+	-/+
10	MR test	-	-	-	-
11	Voges Prokauer reaction	-	-	-	-
12	H_2S production	-	-	-/+	-
13	Catalase production	+	+	+	+
14	Gelatin degradation	+	+	+	+
15	Nitrate utilization	+	+	+	+
16	Citrate utilization	+	+	+	+
17	Decarboxylation				
I	Arginin dehydrolase	-	-	-	-
Ii	Lysine decarboxylase	+	+	+	+
Iii	Ornithin	-	-	-	-
Iv	β Galactosidase	-	-/+	-	-/+
18	Urease production	-/+	-	-	-/+
19	Starch utilization	-	-	-	-
20 i	Acid production from Glucose, Mannitol, Inositol, Sorbitol & Arabinose	+	+	+	+
20 ii	Acid production from Rhamnose, Sucrose, Melibiose & Amygdalin	-	-	-	-
21	Flouricin production	+	+	+	+

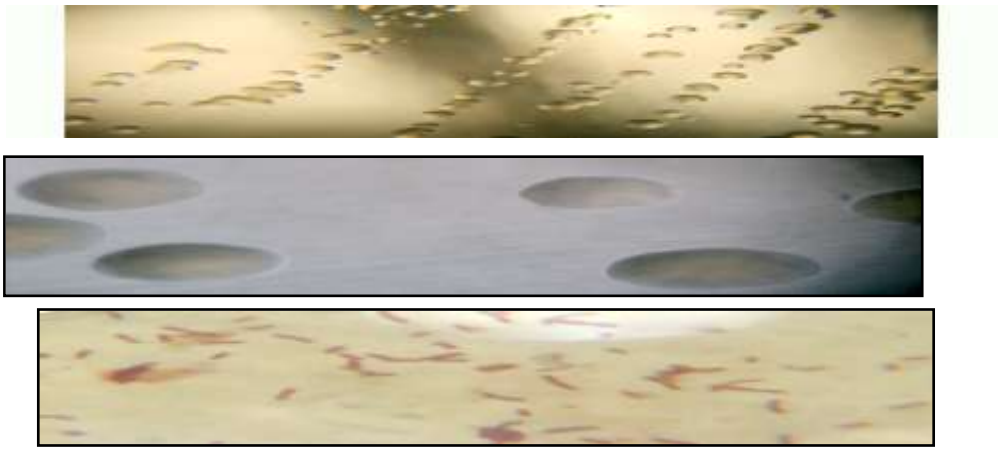

Fig.- 2a to 2c: Showing bacterial colonies (a & b) on agar medium and shape of the Grams stained bacteria 1000 X (c).

Virulence Characterization (LD_{50})

The cumulative mortality was enumerated in *Clariasbatrachus* (CB) challenged (im) with 16 – 18 h live PF4 (G2 isolate) bacteria (0.2 ml of bacterial suspension at 6.1 x 105 cfu ml-1) within 48 – 72 h at 25 ± 2^0C and accounted to 100 %. Similarly, 80, 60 and 30% cumulative mortalities were noted within 6 – 8, 14 – 16 and 23 – 30 d, respectively in CB challenged with alleviated doses (0.2 ml of bacteria at 6.1 x 10^4, 6.1 x 10^3 and 6.1 x 10^2cfu ml-1) of PF4 (Table – 3 a). Mortalities were enumerated, tabulated and re-isolation of bacteria was confirmed (Koch's postulates) by taking inocula form liver and kidney of challenged fishes and LD_{50} value of PF (G1 isolate) was calculated to be 6.1x 103.5 cfu ml-1. The cumulative mortality of PF6 of G2 isolate was also evaluated in CB subjected to 24 – 48 h old bacteria (0.2 ml of bacterial suspension at 2.67 x 10^8cfu ml-1) and accounted to 50% at 20 - 25^{0}C. Similarly, 40% and 20% cumulative mortalities were recorded within 22 – 24 d and 26 – 30 d, respectively in CB inoculated with (0.2 ml of bacteria at 2.67 x 10^7 and 2.67 x 10^6cfu ml-1) PF6 bacteria (Table – 3b).

Table-3 a: Showing mortality in *Clarias batrachus* subjected to various doses (cfu ml^{-1}) of 16-18 h old *P. fluorescens* (PF4 isolate) at 25 ± 2^0C.

Sl. No.	No. of fishes	Doses (ml/fish)	Hrs/Days	CFU/ml	Mortality
1	10	0.2 ml	Up to 30d	0.3% normal saline	--
2	10	0.2 ml	48-72hrs	$6.1x10^5$	100%
3	10	0.2 ml	6-8 d	$6.1x10^4$	80%
4	10	0.2 ml	14-16d	$6.1x10^3$	60%
5	10	0.2 ml	23-30 d	$6.1x10^2$	30%

Table-3 b: Showing mortality in *Clarias batrachus* subjected to various doses (cfu ml^{-1}) of 16-18 h old *P. fluorescens* (PF 6 isolate) at 20 ± 2^0C.

Sl. No.	No. of fishes	Doses (ml/fish)	Hrs/Days	CFU/ml	Mortality
1	10	0.2 ml	Up to 30d	0.3% normal saline	--
2	10	0.2 ml	20d	$2.67x10^8$	50%
3	10	0.2 ml	22-24d	$2.67x10^7$	40%
4	10	0.2 ml	26-30d	$2.67x10^6$	20%

Discussion

Results of present investigation divulged the occurrence of BHS in *Clariasbatrachus* (15 – 25 cm in length and 20 – 50 g weight) and *Labeorohita* (24 – 41 cm in length and 150 – 500 g in weight) during summer months (April – July) of the years 2004 – 2006. This envisages that elevated water temperature might be ensuing changes in abiotic attributes of water which would have created stress on fish health and make them more vulnerable to BHS. Persistence association of*Pseudomonas fluorescens* (PF) with necrotic regions, liver, kidney and intestine of symptomatic fishes explicates that catfishes and carps are prone to Pseudomonas infection which concurs to the progression made in EUS (Qureshi *et al*., 2000), Carps (Prasad, 1992, 2002 & 2007) and other aquatic organisms (Austin and Austin, 1999). It has also been reported that all freshwater fishes are prone to BHS provided ecological conditions are favourable to pathogens and adverse to fish (Sakata *et al*., 1980; Rintamaki *et al*., 1997; Hakalathi&Voltonen 2003; Gao *et al*., 2005 and Davis 2007) which appears to be true in the present case too. Moreover, diseases caused by this bacterium have also been reported in horse (Sarsola *et al*., 1992), chickens (Lin *et al*., 1993) and marine turtles (Glazebrook and Campbell, 1990). This advocates the opportunistic nature and worldwide distribution of *Pseudomonas sp*.

The clinical symptoms such as ulceration and necrotization on the general body surface, haemorrhages erosion of fins, swollen vents, presence of ascetic fluid in the abdomen and empty intestine filled with exudate fluid observed in the present symptomatic *L. rohita* and *C. batrachus* are similar the

observations made in *Cyprinuscarpio* (Prasad and Qureshi, 1995), EUS infected fishes (Mastan and Qureshi, 2001) and *C. batrachus* (Qureshi *et al*., 2000). Involvement of *Pseudomonas* in tail rot and fin rot disease in tilapia, *O. mosembicus* (El-Attar and Moustafa, 1996), Bacterial Gill Disease (BGD) in salmonids, Salmo salar (Bullock, 1972 and Ostaland *et al*., 1999), BHS in common carp, *C. carpio* (Prasad and Qureshi, 1994), integumental lesions in coho salmon, *O. kisutch* (Pavanelli *et al*., 2000) and European eel, *Anguilla anguilla* (Ugar *et al*., 2002) and high mortality of eggs of white fish, *Coregonus Sp.* (Wedekind, 2002) has also been reported and support to the present findings.

Results of bacteriological examination explicated the persistent association of *P. fluorescens* with symptomatic fish suffering from Bacterial HaemorrhagicSeptecaemia (BHS). Isolated PF isolates were identical in their morphological, physiological, biochemical and antigenic attributes. They were Gram-negative rods and exhibiting gliding motility. Development of creamy white colonies of G1 group (PF1 – PF5) and fluorescent green (fluorescent) colonies of G2 and G3 (PF6 – PF10 and PF11 – PF15) isolates on selective medium (*Pseudomonas* Isolation Agar - PIA) and production of fluorescent colour under UV light advocate that G2 and G3 isolates were identical to the positive control *P. fluorescens* (Aqua Bt. YA / PFI). Formation of small water droplets like colonies without firm attachment on the medium (PIA) with smooth edges explicates that they are morphologically similar and require same nutrients for their propagation and concord to the report of Bergen (1981), Palleroni (1992 b and 1992 c), Johnsen *et al*. (1996) and Prasad (1992 & 2007). The gliding motility exhibited by most of the isolates on Pseudomonas Isolation Agar (PIA) andoptimum growth gained at 30 $\pm$ 5^{0}C and negligence or no growth at above 450C manifest that this bacterium prefers to develop at a temperature of 30 $\pm$ 5^{0}C and have conformity with the progression of Rowe and Finn (1991), Palleroni (1992 b & 1992c), Stead (1992), Tryfinopoulou *et al*. (2002), Kojima and Shimizu (2003) and Prasad (2007). Development of colonies at pH 7.2 $\pm$ 0.2 but no growth at pH 5.0 and above pH 8.5 suggests that all the isolates are sensitive to pH variation and accord to the findings of Buchanan and Gibbons (1974) and Kojima and Shimizu (2003).

Experimental infection trials revealed the occurrence of 100% cumulative mortality in CB challenged (im) with 16 – 18 h old live PF4 (0.2 ml of bacterial suspension at 6.0 x 10^{5}cfu ml^{-1}) within 48 – 72 h and 80, 60 and 30% with alleviated doses (0.2 ml of bacteria at 6.0 x 10^4, 6.0 x 10^3 and 6.0 x 10^{2}cfu ml^{-1}) of live PF4 within 6 – 8, 14 – 16 and 23 – 30 d, respectively which indicates that the present isolate of PF belong to high virulence and low virulence groups and temperature plays an important role in the determination of its virulency. It seems that low temperature is not appropriate for the proliferation of this bacterium which is evident from the preceding discussion. It also suggests that to maintain threshold population of this bacterium there is a need of optimum temperature. Foregoing results educe that alleviated bacterial population (cfu ml-1) ensues low mortality in CB. Present findings conform to the contemplations of Esteve (1993), Parco *et al*. (2005), Sharma and Prasad (2005) and Prasad (1992, 2007) who reported that *Pseudomonas fluorescens* might cause mortality at different doses ranging between 105 to 109 cfu ml-1. It has been suggested that rapid change in temperature, over crowding, trauma etc. are the most commonly countered environmental stress factors which predispose the fish to clinical disease (Austin and Austin, 1999) and also support to the present findings.

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