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Research Article

PSEUDOMONAS SEPTICEMIA IN *LABEO ROHITA* (HAM.) AND *CYPRINUS CARPIO* (LINN.) IN ANDHRA PRADESH–NATURAL OCCURRENCE AND ARTIFICIAL CHALLENGE

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ABSTRACT

Objective: The aim of present work is to identify pathogens of diseased *Labeo rohita* and Cypri*nus carpio* and to check their pathogenicity.

Materials and Methods: Incidences of Pseudomonas septicemia were recorded in the months of October to December, 2011 and January and February, 2012 in *Labeo rohita* and *Cyprinus carpio*. A total of 2,076 fishes were screened only affected fishes were brought to the laboratory in living condition. Diseased fish samples were collected randomly every week at regular interval from local fish farms and fish markets, Bhimavaram, Andhra Pradesh, India. Standard microbiological methods were used for isolation and characterization of bacteria associated with diseased fishes.

Results: The clinical symptoms of affected fishes includes erosions at the bases of fins, hemorrhages and skin lesions on the surface of the body, loosening of scales, mucous secretion and in advance stages in some fishes shallow to deep ulcers were observed. Bacteriological examination of infected fishes showed that the occurrence of four species of Pseudomonads namely *Pseudomonas anguilliseptica*, *P. fluorescens, P. aeruginosa,* and *Pseudomonas* sp. Among four species *P. anguilliseptica and P .fluorescens were* consistently obtained from external lesions and hemorrhages and internal organs like liver, kidney, spleen and gills of diseased fishes. Artificial challenge studies indicated that *P. anguilliseptica* and P. *fluorescens* were highly pathogenic to both species of fishes. While *P .aeruginosa* and *Pseudomonas* sp. were fails to fulfil the Koch postulate. The obtained LD_{50} values of *P. anguilliseptica* for *L. rohita* and *C. carpio* were $2\times10^{4.64}$ CFU ml^{-1} and $2\times10^{4.75}$CFU ml^{-1}, respectively while in case of *P. fluorescens* the LD_{50} values for *L. rohita* and *Cyprinus carpio* was $10^{4.5}$and $10^{4.86}$CFU ml^{-1}, respectively.

Conclusion: *P. anguilliseptica* and *P. fluorescens* were pathogenic fishes

Keywords: Pseudomonas septicemia, *Labeo rohita*, *Cyprinus carpio.*

INTRODUCTION

Diseases are more frequent in modern aquaculture system. In intensive aquaculture system, ponds are fertilized with inorganic chemicals, supplement artificial feed, stocking fish at high densities. These all innovative practices increase the fish production from particular ecosystem and at the same time cause stress conditions to fish. The fish is prone to various infections. The bad water quality, high organic load, contaminated feed and unhygienic conditions are some of pre-disposing factors for an outbreak of bacterial diseases in aquatic animals. Pseudomonads are frequently associated with fish diseases. Among the Pseudomonas species recovered from diseased fish, *Pseudomonas anguilliseptica* is considered the most significant pathogen for culture fish [22] and [3]. *Pseudomonas fluorescens* is a dominant component of freshwater ecosystem [2] *P. fluorescens* has been associated with septicemia and ulcerative conditions in wide range of fishes. It has been considered as a fish spoilage organism [14] as well as a primary, but poor pathogen [18] .*P .fluorescens* is normally found in water, soil and on the body of fishes. It is an aquaculture pathogen that can infect many fish species, including Indian major carps, common carp and Japanese flounder [9, 20]. Pseudomonas infection in fish lead to development of so, called Red skin disease, which occur throughout the year particularly when fish is injured by inappropriate handling, physical injury during transportation. Due to lack of effective means of control the disease often leads to high mortality, thus causing heavy losses [24]. Therefore, in the present paper attempts have been made to identify the causative organisms and to determine their pathogencity.

MATERIALS AND METHODS

For the purpose of present study, a total of 2,076 fishes were screened .Only the infected specimens of *Labeo rohita* and *Cyprinus carpio* were brought to the laboratory in living condition and kept in large aquaria of the size 90x45xx45 cm, filled with clean water. Diseased fishes were collected randomly every week at regular interval from local fish farms and fish markets, Bhimavaram, West Godavari district, Andhra Pradesh. Samples for bacteriological examinations were collected by inserting sterile platinum inoculating loop into the lesions and ulcers. The inocula were sampled from the surface as well as deeper portion of lesions and internal organs particularly from liver, kidneys, spleen and gills. They were streaked on separate agar plates having Tryptone Soy Agar (TSA), Brain Heart Infusion Agar (BHIA),Nutrient agar and Selective Pseudomonas isolation agar and incubated at 37ºC for 2 to 4 days. Identification of bacteria was carried out on the basis of morphological and biochemical tests. The biochemical tests were carried out following the methods of Austin and Austin [3]Bullock [5] Roberts [17] and Buchanan and Gibbons [4].The isolated bacterial cultures were subjected for Gram's staining and tested for Oxidative/ Fermentative reaction, Oxidase activity, Catalase activity, indol production and production of acid from various carbohydrates.

Experimental infection trails were conducted to test pathogencity of isolated bacterium on the same species of fishes from which they were originally isolated. To check the pathogencity of isolated bacteria active, healthy, disease free *L. rohita* (n-216) and *C carpio* (n-216) average length 14-16cm and average weigh 60-80 gm were procured from local fish farm, Bhimavaram, Andhra Pradesh. India. Fishes were acclimatized in laboratory condition for one week in aquaria filled with non-chlorinated fresh tap water. Then fishes were divided into different groups and experiment was conducted in triplicate set. Each group comprises 12 fishes. Prior to experiment, one day before, feeding was stopped. During experiment fish were fed with commercial feed once in a day. Water was exchanged thrice in a week and residual feed was removed every two days by siphoning.

Determination of LD_{50}

For the determination of LD_{50} dose of bacteria, health, disease free *Labeo rohita* and *Cyprinus carpio* were inoculated intramuscularly @0.2 ml $fish^{-1}$ with bacterial cell suspension (0.4% TSA) consists of 2×10^2 to 2×10^6 an d 2×10^2 to 2×10^5 cfu $/ml^{-1}$ of live *P. fluorescens* , respectively. *Pseudomonas*. aeruginosa and Pseudomonas sp. were inoculated @0.2ml $fish^{-1}$ with bacterial cell suspension consists of 2×10^2 to 2×10^5 cfu/ml^{-1} was inoculated to both species of fishes. The

value of LD_{50} was calculated using formula of Reed and Muench [15].The control fish received 0.2ml of 0.4% TSA broth without bacterium. Challenged fish kept in aquaria filled fresh tap water. Mortality in challenged fishes was recorded after 20 hr of bacterial inoculation. Re-isolation of inoculated bacteria were done by tacking sample from liver, kidney, spleen, gills and blood and streaked on PIA and TSA plates to check the presence and absence of bacterium.

Antibiogram Studies

The isolated bacteria tested for their antibiotic sensitivity test by using Disk –Diffusions technique [8].

RESULTS

In the present study, only two species of fishes namely *Labeo rohita* and *Cyprinus carpio* were found suffering with disease. No other species of fishes are found to be affected with disease. The incidences of disease reported were found only in the months of October, November, December, 2011and January and February, 2012. The maximum percentage of infection was recorded to be 13.3 in January, 2012, while the minimum 6.25 in October, 2011 (Fig.1). In case of fish infection, the highest percentage of infection (5.9) was found to be observed in *Labeo rohita* while the lowest (3.8) in *Cyprinus carpio* (Fig. 2).

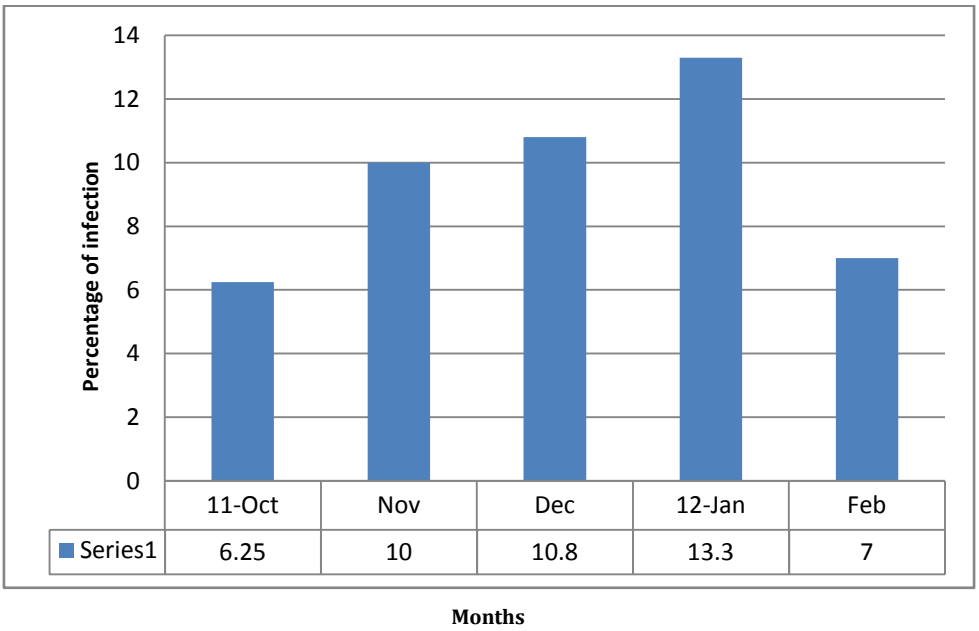

Fig. 1: Monthly percentage of infection (Monthly percentage of infection was calculated as No. of fish infected÷ No. of fish screened×100)

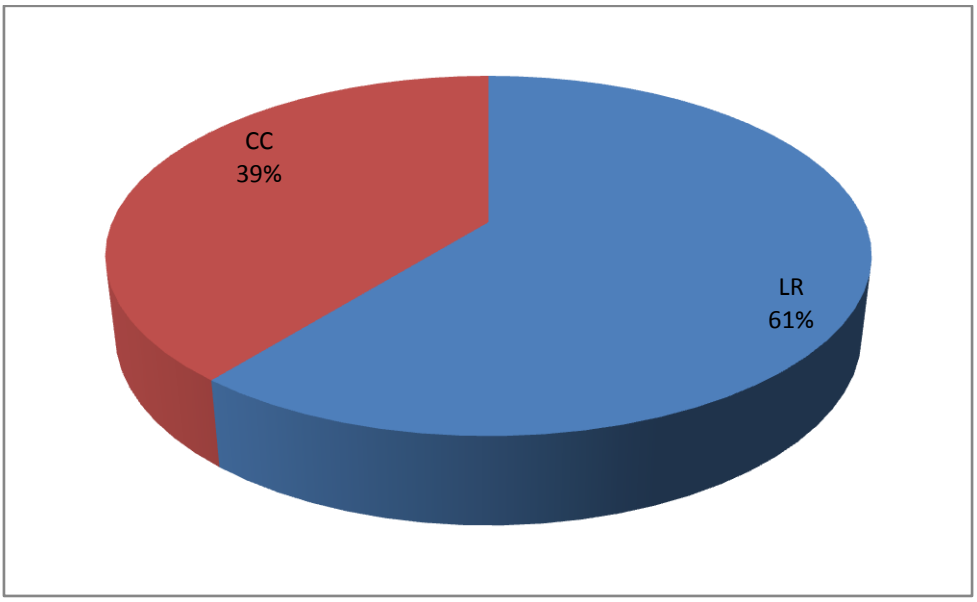

Fig. 2: Species wise percentage of infection (Species wise percentage of infection was calculated as No. of specimens of particular species infected÷ Total no. of fish screened×100)

LR: *Labeo rohita;* CC: *Cyprinus carpio*

Bacteriological examinations of diseased fishes showed that the occurrence of four species of bacteria viz. *Pseudomonas anguilliseptica, P. fluorescens*, *P. aeruginosa* and *Pseudomonas sp* .in diseased fishes. The details of bacteria isolated from fishes are presented in table-1.

Table 1: Details of bacteria isolated from different organs of diseased fishes

Bacteria isolated from infected fishes											
S. No.	**Name of the Bacteria**	**Name of the fish**									
		Labeo rohita					***Cyprinus carpio***				
		Liver	**Kidney**	**Spleen**	**Gills**	**Blood**	**Liver**	**Kidney**	**Spleen**	**Gills**	**Blood**
1	*P. fluorescens*	+	+	+	+	+	+	+	+	+	+
2	*P. aeruginosa*	-	-	-	+	-	-	-	-	+	-
3	*P. anguilliseptica*	+	+	+	+	+	+	+	+	+	+
3	*Pseudomonas* sp.	-	-	-	+	-	-	-	-	+	-

+: Positive, -: Negative

P. anguilliseptica, *P. fluorescens, P. aeruginosa* and *Pseudomonas* sp. were gram-negative but oxidative rods of approximately 0.9×2.5 to 1×10 µm in size. Out of four bacteria *P. anguilliseptica and P. fluorescens* was found consistently associated with the upper and deeper portion*s* of the ulcers and international organs (liver, kidney, spleen, gills and blood). While *P. aeruginosa* and *Pseudomonas* sp. were isolated from lesions and ulcers and gills diseased fishes and they could not be isolated from internal organs. They were isolated on non-selective media, NA, TSA, BHIA and selective medium, PIA on which they developed as yellowish green colonies with rounded edges and about 1.02 mm in diameter.

P. anguilliseptica was oxidative gram- negative rod, non – fluorescent, Catalase positive, indole positive, MR-VP positive,H_2S production positive (Table-2)

Isolated strain of *P. fluorescens* from fish shows oxidative gram-negative rod , indole negative, H_2S negative, Catalase positive, fluorescent positive, urease negative, methyl red negative and vorges- proskauer negative. While the strain of *P. aeruginosa* was oxidative gram-negative rod, indole production negative,H_2S production positive, urease positive, MR-VP negative. *Pseudomonas sp*. was oxidative gram-negative rod, Catalase negative, MR-VP negative and Indole negative. Other biochemical characteristics of bacteria isolated from Pseudomonas septicemia affected fishes are presented in Table-2

Challenging experimental infection trails indicated that P. *anguilliseptic*a and *P. fluorescens* were highly pathogenic to two species of fishes. It produced disease symptoms in 18 hr after inoculation; mortality was reported after 20 hr of inoculation. Challenged fishes exhibited the symptoms of naturally infected fishes like hemorrhages and red lesions on the surface of body and high mucous secretion were observed in both fishes. In this study 100% mortality recorded @0.2 ml suspension of Pa and Pf 2.0×10^6 cfu ml^{-1} for *L .rohita* and *C. carpio*. The obtained LD_{50} values of *P. anguilliseptica* for *L. rohita* and *C. carpio* were $2\times10^{4.64}$CFU ml^{-1} and $2\times10^{4.75}$CFU ml^{-1}, respectively. While LD_{50} concentration of *P. fluorescens* was calculated to be $2.0\times10^{4.5}$ for *L. rohita* and for *C. carpio* was $2.0\times10^{4.86}$ CFU ml^{-1}.Other two bacteria namely *P. aeruginosa* and *Pseudomonas* sp. were non-pathogenic to *L. rohita* and *C. carpio.* They were fails to fulfil the Koch postulate (Table-3).No bacteria was isolated from inoculated fishes and no mortality was observed during experimental period. In control also no mortality was recorded. Mortality was dose dependant.

Eight drugs namely, Amicacin, Ampicillin Ciprofloxine, Chlorophenicol, Kanamycin, Oxytetracycline, Erythromycin and Vancomycin were tested in *vitro* against four fish pathogens namely *P. fluorescens, P. aeruginosa, and P. anguilliseptica* and *Pseudomonas* sp. It was observed that all the four bacteria were sensitive to Chlorophenicol, Oxytetracycline Kanamycin, and Ciproflaxaine. Chlorophenicol and Oxytetracycline highly effective against all the four bacteria. Ciprofloxine is highly effective against *P. anguilliseptica*, while Kanamycin, Ciprofloxine and Vancomycin shown moderate effect against four species. Erythromycin has less effect on three bacterial species. Amicacin and Ampicillin did not show any effect against all bacteria.

Table 2: Physical-Biochemical tests performed for characterization of bacteria isolated from diseased *Labeo rohita* and *Cyprinus carpio*

S. No.	**Test Conducted**	**Response**			
		P. fluorescens	***P. aeruginosa***	***P. anguilliseptica***	***Pseudomonas* sp.**
1	Colour of colony	Yellowish green	Yellow	Yellow	Yellow
2	Gram staining	-ve	-ve	-ve	-ve
3	Shape	R	R		R
4	Fluorescent	+	-	-	-
5	Motality	+	+	+	+
6	Oxidation/Fermentation	O	O	O	O
7	Catalase test	+	-	+	-
8	Oxidase	+	+	+	+
9	MR-VP	-	-	+	-
10	Indole test	-	-	+	-
11	Oxidase reaction	+	+	+	+
12	Nitrite Reduction	+	+	+	-
13	Ornithine decarboxylase	+	+	-	V
14	Arginine dihydrolase	+	+	-	+
15	β-galatose	+	+	+	V
16	Urease production	+	+	+	+
17	H_2S production	+	+	+	+
18	Production of acid from				
	Glucose	+	+	+	+
	Fructose	+	+	+	+
	Detrose	+	+	-	V
	Galactose	+	-	+	-
	Sucrose	+	-	+	-
	Xylose	+	+	+	-

-: Negative; +: Positive O: Oxidative, V=Variable result

Table 3: **Mortality in *Labeo rohita* and *Cyprinus carpio* challenged to different doses of *P. anguilliseptica***

	Labeo rohita				
Bacteria	**C FU ml^{-1}**	**hr**	**Fish mortality**	**Cumulative Ratio**	**⁒of Mortality**
P. anguilliseptica	2.0×10^{6}	96	12	36/36	100
	2.0×10^{5}	96	09	23/25	92
	2.0×10^{4}	96	07	14/22	63
	2.0×10^{3}	96	06	7/16	43
	2.0×10^{2}	96	01	1/25	40
	Cyprinus carpio				
Bacteria	**CFU ml-1**	**hr**	**Fish mortality**	**Cumulative Ratio**	**Percentage of Mortality**
P. anguilliseptica	2.0×106	96	12	35/35	100
	2.0×105	96	08	23/25	92
	2.0×104	96	07	15/21	71
	2.0×103	96	06	8/16	50
	2.0×102	96	02	2/25	0 8

Table 4: Mortality in *Labeo rohita* and *Cyprinus carpio* challenged to different doses of *P. fluorescens*

	Labeo rohita				
Bacteria	**CFU ml^{-1}**	**hr**	**Fish mortality**	**Cumulative Ratio**	**Percentage of Mortality**
P. fluorescens	2.0×10^{6}	96	12	35/35	100
	2.0×10^{5}	96	08	23/25	92
	2.0×10^{4}	96	07	15/21	71
	2.0×10^{3}	96	06	8/16	50
	2.0×10^{2}	96	02	02/25	08
	Cyprinus carpio				
P. fluorescens	2.0×10^{6}	96	12	35/35	100
	2.0×10^{5}	96	10	23/24	95
	2.0×10^{4}	96	07	13/21	61
	2.0×10^{3}	96	04	06/14	42
	2.0×10^{2}	96	02	02/24	08

Table 5: Antibiogram test on four *Pseudomonas* isolated from diseased fishes

S. No.	**Antibiotic(Cons/Disc)**	***P. fluorescens***	***P. aeruginosa***	***P. anguilliseptica***	**Pseudo*monas sp***
1	Chlorophenicol(30µg)	+++	+++	+++	+++
2	Oxytetracycline(30µg)	+++	+++	+++	+++
3	Ciprofloxine (30µg)	++	++	+++	++
4	Kanamycin(30µg)	++	++	+++	+++
5	Vancomycin(30µg)	++	++	++	++
6	Amicacin(30µ)	-	-	-	-
7	Ampicillin(30µ)	-	-	-	-
8	Erythromycin(30µg)	+	+	-	+

Average of two determinations (including disc diameter 6 mm)

-: no inhibition, +: inhibitory zone between5-15mm, ++: inhibitory zone between16-25mm. +++: inhibitory zone between 26-35 mm above

DISCUSSION

Bacterial diseases are major constrain for aquaculture industry throughout the world. The appearance and development of a fish disease is the result of the interaction of pathogen, host and environment. Pseudomonas septicemia is one most serious disease in fishes caused by different species of Pseudomonas species leading heavy loss to fish farmers. Among Pseudomonas species isolated from disease fishes *P .anguilliseptica* is considered the most significant pathogen for cultured fishes [22, 3]. *P. anguilliseptica* is a causative agent of red spot disease, causes severe mortalities in pond cultured fishes in Japan [23]. *P. anguilliseptica* was subsequently isolated from other species of fishes like black seabream and ayu in Japan (Nakai e*t al.,* 1985), salmonids in Finland [25], wild herring in the Baltic sea [12] and is also considered as responsible agent for the winter disease syndrome in seabream fishes. It is recovered as one the emerging pathogen in turbot and black spot seabream (Pag*ellus bogaraveo*) cultured in Spain [11]. *P. anguilliseptica* was also isolated from *O. niloticus* fishes affected with Pseudomonas Septicemia in Bangladesh [21].In the present study four species of Pseudomonas were isolated from fishes suffering with Pseudomonas septicemia. *P. fluorescens* was also associated with various fish diseases [19, 6] It is consistently associated with Epizootic Ulcerative Syndrome of fishes. On the basis of morphological and biochemical tests they were identified as *Pseudomnas anguilliseptica*, *P. fluorescens, and P. aeruginosa* and *Pseudomonas sp*. All the characters shown by four species of bacteria are similar to as described by Buchnan and Gibbons [4] and Austin and Austin [3]. The similar finding were also reported by Khalil *et al.,* [10] who isolated *P. fluorescens* from Pseudomonas septicemia affected fish, *Oreochromus niloticus.* Under present study it has been observed that highest percentage (13.3%) was reported in month of January, 2012.when the temperature is low. The same also reported by workers from Japan. The same was observed by Alicia *et al.,* [1] who reported the occurrence of disease at low temperature during the winter months.

The clinical symptoms shown by affected are hemorrhages, skin lesions, loosening of scales, and erosions at bases of fins and mucous secretion. Hemorrhagic petechia was also observed in internal organs such as liver, kidney and intestine. Similar symptoms were also reported by Alicia et al., [1] Khalil *et al*., [10] and Saleh *et al*., [21].Experimental infection trails shown that *P. anguilliseptica* and *P. fluorescens* were highly pathogenic to L. *rohita* and *C. carpio*. They caused 100% mortality at the concentration of 2×10^{6} CFU ml^{-1} in both fishes. The LD50 values of *P. fluorescens* for *L. rohita* and

Cyprinus carpio was $10^{4.5}$and $10^{4.86}$CFU ml^{-1}, respectively. Under present study eight antibiotics namely Amicacin, Ampicillin Ciprofloxine, Chlorophenicol, Kanamycin, Oxytetracycline, Erythromycin and Vancomycin were tested in *vitro* against four Pseudomonas species isolated from Pseudomonas Septicemia affected fishes. It has been observed that all the four bacteria were sensitive to Chlorophenicol, Oxytetracycline Kanamycin, and Ciproflaxaine.Chlorophenicol and Oxytetracycline highly effective against all the four bacteria. Ciprofloxine is highly effective against *P. anguilliseptica*, while Kanamycin, Ciprofloxine and Vancomycin shown moderate effect against four species. Erythromycin has less effect on three bacterial species. Amicacin and Ampicillin did not show any effect against all bacteria. More or less similar studies were done by Kalil *et al.,* [10] and Pervez [16]

CONCLUSION

Both *P. anguilliseptica* and *P. fluorescens* were pathogenic to fish.

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