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# Pathogenicity Test of Bacterial and Fungal Fish Pathogens in *Cirrihinus mrigala* Infected with EUS Disease

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**Abstract:** The study was conducted for 8 weeks to determine pathogenic effect of different fish pathogens in *Cirrihinus mrigal* (*C. mrigala*) which was infected with EUS disease. The pathogenic organisms (bacteria and fungi) collected from the infected part of the disease fish. Isolation and screening of microbes were carried out with the help of a number of biochemical tests. A total of eight bacterial isolates were obtained from the diseased fish. Out of eight, six bacteria viz., *Streptococcus* grp Q1, *Aeromonas hydrophilla*, *Shigella* spp., *Streptococcus faecalis*, *Cellobiosococcus sciuri*, *Micrococcus luteus* were found to be pathogenic. The fungus, *Aphanomyces invadens* was also isolated from the diseased fishes. The pathogenicity of disease causing organisms was tested through *in vitro* and *in vivo* experiments in different treatments. The result of experiment was found to be significant at level of $p \leq 0.05$.

**Key words:** Fish, *C. mrigal*, bacteria, fungi, disease

## INTRODUCTION

Fish disease is the vital problem of the fish industry. Fish require optimal hydrobiological parameters for growth and survival. Poor pond management practices and higher stocking rate often result into outbreaks of diseases which lead to mass mortality in fish (Kumar *et al*., 1986; Dey, 1989; Sharma *et al*., 2011). With the increasing fish culture activities, several bacterial diseases, causing morbidity and mortality in fish. Some of the important bacterial pathogens are *Flavobacterium* sp., *Photobacterium damsela* subspecies *pisicida* (Aoki *et al*., 1995, 1996; Iqbal *et al*., 1999); *Vibrio damsela*, *V. alginolyticus*, *V. cholerae*, *V. vulnificus*, *Pasturella piscida*, *Providencia rettgeri*, *Aeromonas hydrophila*, *A. salmonicida*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Flexibacter columnaris*, *Edwarsiella tarda*, *Enterococcus*, *Staphylococcus aureus* and *Micrococcus* sp. (Dey, 1989; Kumar, 1989; Mukherjee *et al*., 1991; Roberts, 1997; Sharma *et al*., 2012) which have been identified as the most commonly occurring bacterial agents of fish diseases. The aim of the study was to find out the pathogenic effect of different pathogens (bacteria and fungi) which causing disease in fish.

## MATERIALS AND METHODS

***In vitro* pathogenicity test:** *In vitro* pathogenicity test of different bacteria was done by streaking pure culture of isolated bacteria on blood agar plate. The plates were incubated in B.O.D. at 30±2°C for 24 h. The pathogenicity of bacteria was confirmed by determining α-β zone of growing bacteria on the plates (Ryan and Ray, 2004). To further confirm or ascertain and for cross-checking of the earlier results, *in vitro* and *in vivo* pathogenicity test were performed.

***In vivo* pathogenicity tests:** The healthy individuals of mrigal fish weighing 20 g were taken and acclimated at 25°C for one week in flat bottomed circular 30 l tubs. The tubs were filled with dechlorinated tap water which was 70% removed daily and was also properly aerated with the help of aerators. The fish were fed a normal recommended commercial diet. Only the healthy fish which were showing normal activities selected for further experimention. *In vivo* pathogenicity test was carried out following Keskin *et al*. (2004).

**Experimental design:** Nine fish were kept in 15 L flat bottom circular tubs filled with a well aerated and dechlorinated tap water. Each tub was cleaned daily by siphoning fish fecal matter and food remains and 70% of its water was refilled to ensure clean water in the tubs. In this experiment pure culture of the isolated and identified bacteria was inoculated at 250 μL of bacterial suspension into the intra peritoneal cavity of mrigal fish each weighing 20 g fish with known viable counts (Table 1). The fungus was inoculated through motile spores. The motile spores of the fungus were dissolved or submerged

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into 1 mL of PBS solution. The concentration/μL of the spores was determined with the help of haemocytometer. The solution was then diluted up to 100 spores/250 μL of PBS. It was inoculated into the intraperitoneal cavity of fish. The control fish were inoculated only 250 μL of physiological buffer saline.

The treatments selected for experiment were:

- Single bacterium species
- Single bacterium species and one single fungus species
- Multiple bacterial species and single fungus species

The symptoms of disease appearance were examined and incubation period of different bacteria in fishes wee record. The longevity period of the fish inoculated with pathogens were also note down. The experiment was continued upto eight weeks and data were recorded to find out the pathogenicity of isolated and identified bacteria. The Colony Forming Unit (CFU) was derived by utilizing following formula:

$$N_{CFU} = N_c \times D_f$$

Where:

$N_{CFU}$ = No. of colony forming units per mL of sample
$N_c$ = No. of colonies (30-300 plate)
$D_f$ = Dilution factor of the plate counted

## RESULTS

***In vitro* pathogenicity test:** The results of *in vitro* test revealed that six out of eight bacteria showed growth and α-β zone of hemolysis on blood agar plate. These were (i) *Aeromonas hydrophilla*, (ii) *Shigella* spp., (iii) *Streptococcus faecalis*, (iv) *Micrococcus luteus*, (v) *Streptococcus* grp Q1 and (vi) *Cellobiosococcus sciuri*. These bacteria seemed to be pathogenic. Other two bacteria viz., *Pseudomonas flourescens* and *Acinetobacter calcoacetius* did not show growth and α-β zone of hemolysis on blood agar plate. Therefore, these seemed to be non-pathogenic.

Table 1: Viable counts of different pathogenic bacteria administrated to the experimental mrigal fish

| Inoculated bacterium | Viable counts (CFU $mL^{-1}$) |
|---|---|
| *Cellobiococcus sciuri* | $1.31\times10^{7}$ |
| *Micrococcus luteus* | $1.51\times10^{7}$ |
| *Acinetobacter calcitios* | $1.63\times10^{8}$ |
| *Streptococcus faecalis* | $5.2\times10^{8}$ |
| *Shigella* spp. | $1.50\times10^{7}$ |
| *Pseudomonas flouresceus* | $1.66\times10^{7}$ |
| *Aeromonas hydrophilla* | $1.44\times10^{10}$ |
| *Streptococcus* spp. | $8.88\times10^{9}$ |

***In vivo* pathogenicity tests:**

**When single bacterium species was inoculated**

**Incubation period of the inoculated bacterial pathogens:** The fish inoculated with *Aeromonas hydrophilla* showed symptoms after 6 days post infection. The fish inoculated with *Shigella* spp. and *Cellobiosococcus sciuri* showed symptoms approximately after 6 days post inoculation, where as the fish inoculated with *Streptococcus faecalis*, *streptococcus* grp Q1 and *Micrococcus luteus* showed symptoms after 7 or 8 days post infection, respectively (Table 2). These results revealed that the fish inoculated with *Aeromonas hydrophilla* showed symptoms earlier as compared to other remaining pathogenic bacteria. The difference among bacteria was significant (C.D., $p \leq 0.05$).

**Effect of inoculated pathogenic bacterium on fish longevity:** The fish inoculated with *Aeromonas hydrophilla* remained alive for 13 days after post inoculation, whereas, the fish inoculated with *Shigella* spp. and *Cellobiosococcus sciuri* remained alive for 15 days after post inoculation. On the other hand, the fish inoculated with *Streptococcus faecalis*, *Streptococcus* grp Q1 and *Micrococcus luteus*, do so for 16, 16 and 17 days after post inoculation, respectively (Table 3).

These results revealed that the fish inoculated with *Aeromonas hydrophilla* showed minimal longevity period as compared to those infected with other remaining bacterial pathogens. The difference among bacteria was significant (C.D., $p \leq 0.05$).

Table 2: Incubation period of different pathogenic bacteria for the appearance of disease symptoms in mrigal (*C. mrigala*)

| Inoculated bacterium | Disease symptoms | Incubation period (in days)[a] of bacteria for the appearance of disease symptoms |
|---|---|---|
| *Streptococcus* grp Q1 | Passive movement of fish | 7.67±1.73 |
| *Aeromonas hydrophilla* | Haemorrhage on gill region | 6.00±0.00 |
| *Shigella* spp. | Haemorrhage on head and lateral side of body | 6.13±1.00 |
| *Micrococcus luteus* | Depigmentation of the body | 8.00±0.57 |
| *Cellobiosococcus sciuri* | Descaling on the lateral side | 6.67±0.57 |
| *Streptococcus faecalis* | Red coloration on body | 7.07±0.57 |
| CD value ($p \leq 0.05$) | | 1.642 |

[a]Mean±SD, N = 27 (9 fishes x 3 replications)

Table 3: Longevity of mrigal (*C. mrigala*) inoculated with different pathogenic bacteria

| Pathogenic bacteria | Longevity (in days)[a] |
|---|---|
| *Streptococcus* grp Q1 | 16.08±1.73 |
| *Aeromonas hydrophilla* | 13.00±1.52 |
| *Shigella* spp. | 15.33±1.15 |
| *Streptococcus faecalis* | 16.00±2.00 |
| *Cellobiosococcus sciuri* | 15.50±1.15 |
| *Micrococcus luteus* | 17.67±1.15 |
| CD value ($p \leq 0.05$) | 2.681 |

[a]Mean±SD, N = 27 (9 fishes x 3 replication)

Table 4: Incubation period of different pathogenic bacteria along with fungus for the appearance of disease symptoms in mrigal (*C. mrigala*)

| Pathogenic organisms | Disease symptoms | Incubation period (in days)[a] of pathogens for the appearance of disease symptoms |
|---|---|---|
| *Streptococcus faecalis*+fungus | Blood eject out from gills | 5.53±0.57 |
| *Aeromonas hydrophilla*+fungus | Fungus cover the body of fish | 4.33±0.57 |
| *Streptococcus* grp Q1+fungus | Haemorrhage on head and lateral side of body | 5.67±1.00 |
| *Cellobiosococcus sciuri*+fungus | Depigmentation and whitening of fish | 5.00±1.15 |
| *Shigella* spp.+fungus | Descaling and haemorrhage on the lateral side | 5.00±1.00 |
| *Micrococcus luteus*+fungus | Haemorrhage nearby tail | 5.87±0.00 |
| CD value ($p \leq 0.05$) | | Non-significant |

[a]Mean±SD, N = 27 (9 fishes x 3 replication)

Table 5: Longevity of mrigal (*C. mrigala*) inoculated with different pathogenic bacteria along with fungus

| Pathogenic organisms | Longevity (in days)[a] |
|---|---|
| *Streptococcus* grp Q1+fungus | 13.62±3.05 |
| *Streptococcus faecalis*+fungus | 12.67±0.57 |
| *Shigella* spp.+fungus | 10.67±1.52 |
| *Micrococcus luteus*+fungus | 13.70±0.00 |
| *Cellobiosococcus sciuri*+fungus | 11.33±1.52 |
| *Aeromonas hydrophilla*+fungus | 8.00±1.00 |
| CD value ($p \leq 0.05$) | 2.34 |

[a]Mean±SD, N = 27 (9 fishes x 3 replication)

**Inoculation of single bacterium species and a single fungus species**

**Incubation period of a bacterium along with a fungus:** The fish inoculated with *Aeromonas hydrophilla* along with fungus showed symptoms after 4 days of post inoculation which was earlier than bacterium alone (Table 4). On the other hand, the fish inoculated with *Shigella* spp. and *Cellobiosococcus sciuri* along with fungus, each bacterial and fungal combination showed symptoms after 5 days of post inoculation, respectively. The fish when inoculated with *Streptococcus faecalis*, *streptococcus* gr Q1 and *Micrococcus luteus* and along with fungus, each bacterial and fungal combination showed symptoms approximately after 6 days of post infection. These results revealed that the fish inoculated with *Aeromonas hydrophilla* along with fungus showed symptoms earlier as compared to other remaining bacterial pathogens alone (Table 5). The difference among bacteria was non-significant (C.D., $p \leq 0.05$).

The association of bacteria with fungi seemed to enhance the speed of pathogenicity. The effect was not merely additive. These organisms seemed to act synergistically in quick expression of the pathogenicity in the reference fishes (Table 4).

**Longevity of mrigal (*C. mrigala*) inoculated with a bacterial-fungus combination:** The fish inoculated with *Aeromonas hydrophilla* along with fungus remained alive for 8 days after post inoculation, however, those inoculated with *Shigella* spp. along with fungus died approximately for 10 days after post inoculation. On the other hand, the fish inoculated with *Cellobiosococcus sciuri*, *streptococcus faecalis*, *streptococcus* grp Q1 and *Micrococcus luteus* remained alive for 11, 12, 13 and 15 days after post inoculation respectively. These results revealed that the fish inoculated with *Aeromonas hydrophilla* along with fungus showed minimum longevity period as compared to those inoculated with other bacterial pathogens (Table 5). The difference among bacteria was significant (C.D., $p \leq 0.05$).

Table 6: Incubation period of pathogens and longevity of mrigal (*C. mrigala*) inoculated with five bacterial types[a] altogether along with fungus

| Treatment | Period (in days) |
|---|---|
| Incubation period (in days)[b] of pathogenic organisms (bacteria+fungus) for appearance of symptoms in mrigal | 3.33±0.57 (Fungus cover the body of fish) |
| Longevity (in days)[b] of mrigal fish inoculated with different bacteria along with fungus | 6.67±1.52 |

[a]*Aeromonas hydrophilla*, *Shigella* spp., *Micrococcus luteus*, *Cellobiosococcus sciuri* and *Streptococcus*, [b]Mean±SE, N = 27 (9 fishes x 3 replication)

**Incubation period of all the bacterium along with fungus:** The fish inoculated with all the six bacterial types (*Aeromonas hydrophilla*, *Shigella spp*., *Micrococcus luteus*, *Cellobiosococcus sciuri* and *Streptococcus* sp.) together with fungus showed symptoms of EUS after three days where as each fish could survive only approximately for six days after post inoculation (Table 6). These results revealed that different bacterial type together and along with fungus speed up the rate of disease occurrence.

## DISCUSSION

**Pathogenicity test:** This test was performed to confirm whether the particular bacterium/ fungus were pathogenic or not. Ryan and Ray (2004) reported α-β zone of hemolysis on blood agar plate of pathogenic bacteria. The Alpha (α) hemolysis is the reduction of hemoglobin to methemoglobin in the medium surrounding the colony, causing a greenish decolorization of the medium where as beta (β) hemolysis is defines as the lysis of red blood cells, resulting in a clear zone surrounding the colony. In the present pathogenicity studies, six bacterial isolates of this study were found to be pathogenic to the mrigal fish as compared by inoculation method, where *Aeromonas*

*hydrophila* was found to be the worst, followed by *Shigella* spp., *Cellobiococcus sciuri*, *Streptococcus faecalis*, *Streptococcus* grp Q1 and *Micrococcus luteus* (Table 2, 3). When these bacteria were inoculated along with fungus then longevity period of fish decreased (Table 4, 5). This indicated that fungi aggravated the incubation period of bacteria and slowed down immune response of the fish. Multiple infections of bacteria along with fungus increased the severity of disease and the longevity period of the fish became only eight days (Table 6). These results directly correlate with the findings of by Sarker *et al.* (1999) who reported that different bacterial mixtures found in the pond water along with pathogenic fungus caused earlier mortality in fishes. In case of pathogenicity test of fungi, *A. invadans* produced characteristic lesion which were very similar to the natural EUS lesions. These results support the findings of Lilley (1997) who reported that the lesions were formed on the body of fish when the latter were infected with fungus.

## CONCLUSION

The conclusion from the above study is that the different bacteria and fungi isolated from the diseased fish have their different incubation period. When these bacteria along with the pathogenic fungi (*A. invadens*) inoculated into the fish then longevity period of the fish were decreased as compared to the individually inoculated bacteria. But when the bacterial mix along with fungi were inoculated into the fish then incubation period of bacteria and longevity period of the fish decrease so much.

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