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BIOCONTROL OF β - HAEMOLYTIC *AEROMONAS HYDROPHILA* INFECTION IN *LABEO ROHITA* USING ANTAGONISTIC BACTERIUM *PSEUDOMONAS AERUGINOSA* FARP72

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ABSTRACT: *Pseudomonas aeruginosa* FARP72 was isolated from the skin mucus of freshwater catfish (*Clarias batrachus*) as a potent antagonistic bacterium. Efforts have made to assess the antagonistic activity against potential fish and human bacterial pathogens *in vitro*. Antagonistic effects were confirmed by cross-streaking method against pathogenic bacteria such as *Aeromonas hydrophila*, *Bacillus subtilis*, *Bacillus amylolequifasciens, Escherichia coli*, *Edwardsiella tarda*, *Klebsiella pneumoniae*, Enterohaemorrhagic *Escherichia coli* (EHEC), *Pseudomonas aeruginosa*, *Salmonella* Typhi, beta haemolytic *Streptococcus, Staphylococcus aureus* ATCC12598 and *Vibrio cholera*. The LD_{50} value of *Pseudomonas aeruginosa* FARP72 and β-haemolytic *Aeromonas hydrophila* N_{10}P were calculated to be >5.65×10^{10} cells/ fish and 2.37×10^{8} cells/fish, which proved them to be non-pathogenic and moderate pathogenic respectively. In biocontrol experiment, the *Labeo rohita* stocks which received a first 15 min dip in *Pseudomonas aeruginosa* FARP72 suspension containing $1x10^{7}$cells/ml followed by another dip in β- haemolytic *Aeromonas hydrophila* suspension containing $1x10^{5}$ cells/ml for 15 min and subsequently cohabited with healthy *Labeo rohita* showed 37% mortality; while 75% of the fish died when treated only with β- haemolytic *Aeromonas hydrophila* suspension containing $1x10^{5}$ cells/ml in 30 days of post-challenge. The results suggest that *Pseudomonas aeruginosa* FARP72 can be a substitute to the commercially available and indiscriminately used common antibiotics, reducing the attachment of *Aeromonas hydrophila* and development of infection and disease outbreaks. Judicious use would help overcome the problems of developing antibiotic resistant pathogenic strains, side effects of antibiotics etc. without causing any environmental pollution, reducing stress and better disease management and ensuring its practical approach for betterment of animal life.

INTRODUCTION: The world aquaculture production of food fish is increased by 6.2% from 59 million tonnes in 2010 to 62.7 million tonnes in 2011 [1].

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To offer high productivity intensive farming has become the best option for the aqua-farmers. Invariably, the high stocking density and high feed input came in to play which results in greater amount of feed wastage, organic build up and accompanies several disease problems, in turn, decreases the growth rate, and the high organic load stimulates proliferation of several opportunistic pathogens [2, 3]. Due to this negative balance, aquaculturists often face mass mortality or disease occurrence of their stocks.

The bacteria *Aeromonas hydrophila is* a widely distributed pathogenic bacteria especially in warm water throughout the world causing MAS (motile *Aeromonas* septicemia) and epizootic ulcerative syndrome (EUS) of different fishes both farmed and wild [4,5]. The use of antimicrobials and indiscriminate use of the commercial antibiotics in disease prevention can bring about the emergence of drug-resistant microorganisms and leave antibiotic residues in the body and in the environment; chemotherapy may kill or inhibit the normal micro flora in the digestive tract which is beneficial to the health. Therefore, alternative strategies to prevent opportunistic infections in aquaculture are strongly needed. The use of antagonistic bacteria to control potential pathogens is an alternative method and is gaining acceptance [6, 7].

Keeping this in view, the present study was taken up with the following objectives: Isolation of antagonistic bacterium from freshwater fish skin mucus and assess the inhibitory activity against selected human and fish bacterial pathogens *in-vitro*, to test the pathogenicity of the potent antagonistic bacterium and pathogenic bacterial strain isolated from diseased fish and to assess the efficacy of potent antagonistic bacterium as biocontrol agent to inhibit the fish bacterial pathogen, *Aeromonas hydrophila in-vivo.*

MATERIALS AND METHODS:

Experimental fish for bacterial challenge and biocontrol assays: The experimental fish *Labeo rohita* of two size groups, viz., 12.02±0.94 cm and 21.21±5.01g, and 3.89±0.69 cm and 2.67±0.71g were procured from commercial fish breeders of Naihati (Lat 22°54'10" N and Long 88°25'01'' E), North 24 Parganas district, West Bengal for bacterial challenge (pathogenicity) and biocontrol assays, respectively. The fish, on receipt, were disinfected with 5 ppm potassium permanganate ($KMnO_4$) solution and acclimatized for 10 days in circular FRP tanks of 500 L capacity containing bore-well water with continuous aeration. All fishes were fed a balanced pellet diet at the rate of 2% of their body weight and maintained under optimal condition.

Pathogenic bacterial strains: The details of human and fish bacterial pathogens used in the study and their sources are presented in **Table 1**.

TABLE 1: DETAILS OF THE HUMAN AND FISH BACTERIAL PATHOGENS AND THEIR SOURCES

Test bacterial strains	Source
Klebsiella pneumoniae CNMC *Pseudomonas aeruginosa* CNMC *Staphylococcus aureus* CNMC *Escherichia coli* CNMC	Calcutta National Medical College and Hospital, Kolkata
Edwardsiella tarda SDDL	Shrimp Disease Diagnostic Laboratory, Tamil Nadu Fisheries University, Madhavaram, Chennai
Staphylococcus aureus ATCC 12598 *Vibrio cholerae* FCRI Enterohaemorrhagic *Escherichia coli* FCRI *Salmonella* Typhi FCRI	Fisheries College and Research Institute, Thoothukudi
Escherichia coli FPT *Staphylococcus aureus* FPT1 *Pseudomonas* sp. FPT *Bacillus pumilus* FPT *Bacillus amyloliquefaciens* FPT2 *Staphylococcus aureus* FPT	Department of Fish Processing Technology, FFSc, Kolkata
Aeromonas sp. AAH β- haemolytic *Streptococcus* sp. AAH *Aeromonas hydrophila* HS_3 AAH β- haemolytic *Aeromonas hydrophila* $N_{10}P$ AAH	Department of Aquatic Animal Health, FFSc, Kolkata

Antagonistic bacterial strain *Pseudomonas aeruginosa* FARP72: Total 41 potent antagonistic bacteria were isolated, purified and identified from 25 fresh water fish samples. Out of which the broad spectrum of inhibitory activity was elucidated by Gram negative *Pseudomonas aeruginosa* FARP72 isolated from *Clarias batrachus*.

Inhibitory activity of antagonistic bacterium *Pseudomonas aeruginosa* FARP72 against fish and human pathogens *in-vitro*: *In-vitro* inhibitory activity of the antagonistic bacterium *Pseudomonas aeruginosa* FARP72 from fish mucus was tested against eighteen opportunistic bacterial human and fish pathogens by modified cross streak technique [8, 9]. The severity of inhibition was determined by parallel streaking method [10]. The inhibitory activity of the producer (antagonistic) strain was determined on the basis of growth inhibition of the test strain and the severity of inhibition was rated as "++" for total inhibition, "+" for feeble / thin growth and "–" for no inhibition and comparison with control plates.

Pathogenicity and biocontrol experiments

Preparation of bacterial cell suspensions of *Pseudomonas aeruginosa* FARP72 and β-haemolytic *Aeromonas hydrophila* N_{10}P: Antagonistic *Pseudomonas aeruginosa* FARP72 and pathogenic β-haemolytic *Aeromonas hydrophila* N_{10}P (collected from diseased fish sample), maintained on Tryptone soya agar (TSA) slant was streaked on to a TSA plate and incubated at 30±2°C for 24 h to get young discrete colonies. One or two young colonies were aseptically picked, transferred to 10 ml TSB and incubated at 30±2°C for 24 h. This 24 h old culture was then transferred to 300 ml Tryptone soya broth (TSB) and reincubated at 30±2°C for 48 h. The cells were harvested by centrifugation at 7500 rpm for 20 min at 25°C in a cooling centrifuge (Remi, Mumbai). The cell pellets were washed twice by centrifugation with sterile physiological saline and finally resuspended in 30 ml sterile physiological saline and used immediately. A portion of the cell suspension was suitably diluted up to 10^{-9} in sterile saline and the number of cells/ml of suspension was determined by drop count method [11] on TSA after incubation at 30±2°C for 48 h.

Determination of water quality parameters: The water samples for determining water quality parameters were collected in glass containers of 300 ml capacity. The pH, temperature, dissolved oxygen (DO), conductivity, total dissolved solids (TDS) and ammonia were measured by Hanna Multi-parameter water analysis kit, Ecuador and Thermo Scientific multi-parameter water analysis instrument, Beverly, USA.

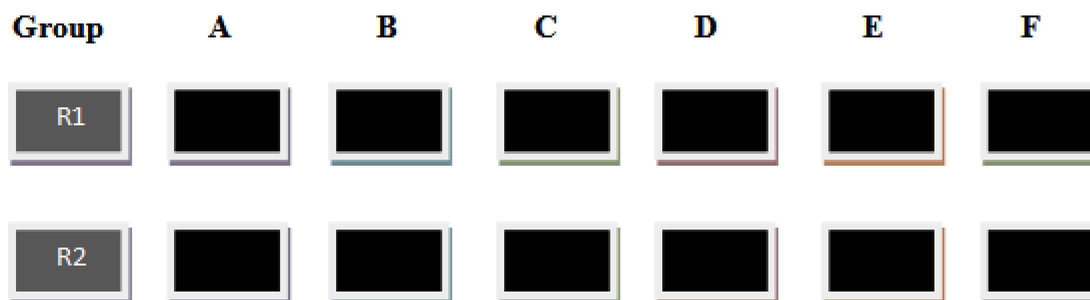
Experimental set up: All the experiments were carried out in the wet laboratory of the Department of Aquatic Animal Health. The ambient temperature was generally in the range of 28 – 36°C and the laboratory was well ventilated so as to allow sufficient fresh air. All the experimental fish were first disinfected by placing in 5 ppm $KMnO_4$ solution for 15 min. The morbid fish and morts were removed immediately and the healthy ones were stocked at the rate of 100 fish/tank in circular FRP tanks of 500 L capacity containing aged bore-well water for 10 days with continuous aeration. All fish were fed a balanced basal dry pellet feed twice daily at the rate of 2% of their body weight and maintained under optimal condition. The wastes and faecal matter were siphoned out twice in a week and 50% water exchange was done in three days interval.

Bacterial challenge tests: The growth conditions provided to the experimental fish during challenge tests are given in **Table 2**.

TABLE 2 - GROWTH CONDITIONS PROVIDED TO THE EXPERIMENTAL FISH DURING CHALLENGE TESTS

Parameter	Value
Glass aquarium capacity (in liter)	50
Water volume (in liter)	30
Stocking density (number / aquarium)	10
Water temperature (ºC)	26.80-30.20
pH	7.94-8.09
Dissolved oxygen (ppm)	3.68-5.88
Total dissolved solids (ppm)	1476-1867
Water conductivity (ms/cm)	3.01-3.80
Total ammonia (ppm)	0.051-0.059

Challenge test with antagonistic bacterium, *Pseudomonas aeruginosa* FARP72: The antagonistic *Pseudomonas aeruginosa* FARP72 strain at levels of 5.65x10^{10} cells/fish, 5.65x10^9 cells/fish, 5.65x10^8 cells/fish and 5.65 x10^7 cells/fish were used in experimental challenge through intramascular injection [12] .Twelve glass aquaria (60 cm L x 45 cm B x 30 cm H) of 50 L capacity were used for the experiment. Prior to use, the glass aquaria were scrubbed, cleaned with chlorinated water (200 ppm), flushed thoroughly with freshwater, dried for 5 days before use and arranged in three tier iron stands. The glass aquaria were grouped in to six groups namely A, B, C, D, E and F with two aquaria for each group as given below. All glass aquaria were filled with clean water to a volume of 30 L each and conditioned for three days. After three days, the glass aquaria were stocked with experimental fish at the rate of 10 fish/aquarium tank from the acclimatized stocks. The aquaria were labelled and covered with nylon netting for adequate protection. The fishes were acclimatized in aquaria for three days. After acclimatization in the glass aquaria, each fishes were injected with 0.1ml of bacterial suspension at different concentrations as required. Positive control fish received 0.1ml of 0.85% physiological saline instead of bacterial suspension. Negative control fishes received no injection. The challenged fishes of each group were maintained in the respective aquaria for 20 days. Mortality, external signs of infection and behavioural abnormalities were recorded daily. The experiment was carried out in duplicate. The median lethal dose (LD_{50}) was calculated [13].



EXPERIMENTAL SETUP FOR CHALLENGE TEST WITH ANTAGONISTIC BACTERIUM, PSEUDOMONAS AERUGINOSA FARP72:
Group A: Fish challenged with 10^{10}cells/fish; Group B: Fish challenged with 10^9cells/fish; Group C: Fish challenged with 10^8cells/ fish; Group D: Fish challenged with 10^7cells/ fish; Group E: Fish injected with 0.85% physiological saline and served as positive control; Group F: Fish received no injection and served as negative control.

Challenge test with pathogenic β-haemolytic *Aeromonas hydrophila* N_{10}P: The fish pathogenic β-haemolytic *Aeromonas hydrophila* at levels of 6.37x10^{10} cells/fish, 6.37x10^9 cells/fish, 6.37x10^8 cells/fish and 6.37x10^7 cells/fish were used in experimental challenge through intramascular injection [12]. The experimental design and protocol are as described in previous section.

Pathogenicity test by Cohabitation challenge:

Cohabitation challenge: In order to examine the water-borne route for transmission of *Pseudomonas aeruginosa* FARP72 and β- haemolytic *Aeromonas hydrophila* from infected to non-infected fish, cohabitation challenge experiments [14] were done using 'Skin wounded' and 'Non-injured' fish (2.67±0.71g; 10 fish/treatment in duplicate) as recipients. Eight glass aquaria (60 cm L x 45 cm B x 30 cm H) of 50 L capacity were used for the experiment. The glass aquaria were scrubbed, cleaned with chlorinated water (200 ppm), flushed thoroughly with freshwater and dried for 5 days before use. The glass aquaria were grouped in to four groups namely A, B, C and D with two aquaria for each group as given below. All glass aquaria were filled with clean water to a volume of 30 L each and conditioned for three days. After three days, the glass aquaria were stocked with experimental fish at the rate of 50 fish/ aquarium tank from the acclimatised stocks. The aquaria were labelled and covered with nylon netting for adequate protection. The fishes were acclimatised in the aquaria for three days. After acclimatisation in the glass aquaria, scales of ten fishes from each aquarium were scrapped off gently with a scalpel from caudal peduncle to the pectoral fin, i.e., in the opposite direction (skin wounded).

Ten fishes from each of the first two aquaria were dipped in β-haemolytic *Aeromonas hydrophila* suspension containing $1x10^5$ cells/ml for 15 min (Group A). Similarly the ten fishes from each of the other two aquaria were dipped in *Pseudomonas aeruginosa* FARP72 suspension containing $1x10^7$ cells/ml for 15 min (Group B).

Ten skin wounded fish received a 15 min dip in 0.85% physiological saline instead of bacterial suspension and served as positive control (Group C). All the fish after dip treatment were transferred to the respective aquarium containing 40 healthy (non-injured) fish. Negative control fish (all non-injured) were kept undisturbed (Group D). One day before the treatment and on the day of treatment the fish were starved and 24 h post treatment fish were fed at the rate of 2% of body weight twice daily.

EXPERIMENTAL SETUP FOR COHABITATION CHALLENGE:

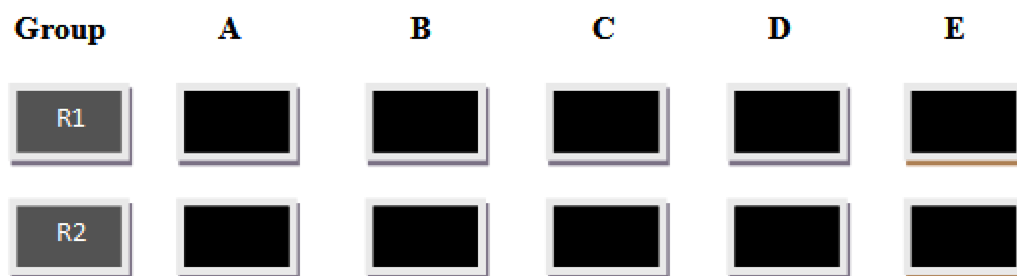
Group A: Ten skin wounded fish dipped in β-haemolytic *Aeromonas hydrophila* suspension, $1x10^5$ cells/ml for 15 min and introduced in to the respective aquarium containing healthy fish; Group B: Ten skin wounded fish dipped in *Pseudomonas aeruginosa* FARP72 suspension, $1x10^7$cells/ml for 15 min and introduced in to the respective aquarium containing healthy fish; Group C: Ten skin wounded fish dipped in 0.85% physiological saline for 15 min and introduced in to the respective aquarium containing healthy fish. This served as positive control; Group D: Non injured fish received no dip and served as negative control.

External gross and clinical signs of infections, behavioural abnormalities and mortality were recorded daily. Samples from the affected haemorrhagic parts of the body of freshly dead fish were taken and inoculated on to glutamate starch phenol red agar (Hi-Media, Mumbai, India) plates or *Pseudomonas* isolation agar and then incubated at 30±2°C for 24 h. Standard biochemical tests were performed to identify whether or not the infective agent is pre-inoculated β- haemolytic *Aeromonas hydrophila* $N_{10}P$ or *Pseudomonas aeruginosa* FARP72. Cumulative mortality was computed from the daily mortality data.

Biocontrol Experiment: Six glass aquaria (60 cm L x 45 cm B x 30 cm H) of 50-L capacity were used for the experiment. The glass aquaria were scrubbed, cleaned with chlorinated water (200 ppm), flushed thoroughly with freshwater and dried for 5 days before use. The glass aquaria were grouped in to five groups namely A, B, C, D and E with two aquaria for each group as given below. All glass aquaria were filled with clean water to a volume of 30-L each and conditioned for three days. After three days, the glass aquaria were stocked with experimental fish (2.67±0.71g) at the rate of 50 fish/ aquarium tank from the acclimatized stocks. The aquaria were labelled and covered with nylon netting for adequate protection. The fishes were acclimatized in the aquaria for three days. After acclimatization in the glass aquaria, scales of ten fishes from each aquarium were scrapped off gently with a scalpel in from caudal peduncle to the pectoral fin, i.e., in the opposite direction. Ten fishes from each of the first two aquaria were dipped in β-haemolytic *Aeromonas hydrophila* suspension containing $1x10^5$ cells/ml for 15 min (Group A).

Ten skin wounded fishes from each of the two aquaria were received a first 15 min dip in *Pseudomonas aeruginosa* FARP72 suspension containing $1x10^7$cells/ml followed by another dip in β- haemolytic *Aeromonas hydrophila* $N_{10}P$ suspension containing $1x10^5$ cells/ml for 15 min (Group B). Positive control skin wounded fish received a 15 min dip in 0.85% physiological saline instead of bacterial suspension (Group C). Negative control non-injured fish were kept undisturbed (Group D). The challenged fishes of each group were maintained in the respective aquaria for 30 days. All fish were fed a balanced basal dry pellet feed twice daily at the rate of 2% of their body weight.

One day before the treatment and on the day of treatment the fish were starved. External signs of infections, behavioural abnormalities and mortality were recorded daily. The experiment was carried out in duplicate. Cumulative mortality was computed from the daily mortality data.



SETUP FOR BIOCONTROL EXPERIMENT:

Group A: Ten skin wounded fish dipped in β- haemolytic *Aeromonas hydrophila* $N_{10}P$ suspension, $1x10^5$ cells/ml for 15 min and introduced in to the respective aquarium containing healthy fish; Group B: Ten skin wounded fish dipped in *Pseudomonas aeruginosa* FARP72 suspension containing $1x10^7$cells/ml for 15 min and introduced in to the respective aquarium containing healthy fish; Group C: Ten skin wounded fish first dipped in *Pseudomonas aeruginosa* FARP72 suspension containing $1x10^7$cells/ml for 15 min and followed by dip in β- haemolytic *Aeromonas hydrophila* $N_{10}P$ suspension containing $1x10^5$ cells/ml for 15 min and introduced in to the respective aquarium containing healthy fish; Group D: Ten skin wounded fish dipped in 0.85% physiological saline for 15 min and introducted in to the respective aquarium containing healthy fish. This served as positive control; Group E: Non injured fish received no dip and served as negative control.

Statistical analysis: The results of the bacterial counts were processed by log transformation. One way analysis of variance (ANOVA) was followed to test the level of significance among the log transformed bacterial counts of different fish species and locations. Students'‘t’ test was used to determine the significant differences in fish mortality among treatments.

RESULTS AND DISCUSSION:

Antibacterial spectrum of *Pseudomonas aeruginosa* FARP72 against human and fish pathogens: *Pseudomonas aeruginosa* FARP72 was proven to be a potent antagonistic bacterium on the basis of the broad spectrum of activity and zone of inhibition (>5 mm) produced against the 18 test strains by cross streaking method (**Table 3**).

In parallel streaking method, it was observed that the growth of *Pseudomonas aeruginosa* CNMC; *Escherichia coli* CNMC, *Edwardsiella tarda* SDDL; *Staphylococcus aureus* ATCC 12598 *Vibrio cholerae* FCRI; Enterohaemorrhagic *Escherichia coli* FCRI; *Salmonella* Typhi FCRI; *Aeromonas* sp. AAH; *Pseudomonas* sp. FPT; *Bacillus pumilus* FPT; *Bacillus amyloliquefaciens* FPT, *Staphylococcus aureus* S2, FPT; *Escherichia coli* FPT; β-haemolytic *Streptococcus* sp. AAH; β-haemolytic *Aeromonas hydrophila* AAH was totally inhibited; whereas *Klebsiella pneumoniae* CNMC and *Staphylococcus aureus* CNMC were only slightly inhibited by *Pseudomonas aeruginosa* FARP72 strain and represented in **Table 3**.

Pathogenicity and biocontrol experiment:

Challenge test with *Pseudomonas aeruginosa* FARP72: The results of the pathogenicity test of *Pseudomonas aeruginosa* FARP72 on *Labeo rohita* and the respective LD_{50} value are presented in **Table 4**. The first mortality was observed in 24 h post-injection. Within 24 h, one fish died in the tank containing 10 fish challenged with $5.65x10^{10}$ cells of *Pseudomonas aeruginosa* FARP72/fish, another two fish died after 48 h of injection in the same stock.

The stocks where *Pseudomonas aeruginosa* FARP72 were injected at a level of $5.65x10^9$ cells/fish, $5.65x10^8$ cells/fish, $5.65x10^7$ cells/fish remained active with no serious abnormalities and mortalities except the primary inflammatory response at the place of injection, which was recovered in 3-4 days post-injection period. No mortalities were noticed in stocks which were used as positive control and negative control. After 15 days, 30% fish mortalities were observed in the stock inoculated with $5.65x10^{10}$ cells/fish. The lethal dose at which 50% of the experimental population die (LD_{50}) was calculated on the basis of mortality.

TABLE 3: ANTIBACTERIAL SPECTRUM OF *PSEUDOMONAS AERUGINOSA* FARP72 AGAINST HUMAN AND FISH PATHOGENS BY CROSS STREAK AND PARALLEL STREAK METHODS *IN-VITRO*

Test strains and source	Cross streaking: Zone of inhibition in mm	Parallel streaking: Severity of inhibition
Klebsiella pneumoniae CNMC	2.50	+
Pseudomonas aeruginosa CNMC	8.50	++
Staphylococcus aureus CNMC	2.00	+
Escherichia coli CNMC	13.00	++
Edwardsiella tarda SDDL	14.50	++
Staphylococcus aureus ATCC 12598 FCRI	22.00	++
Vibrio cholerae FCRI	17.00	++
Enterohaemorrhagic *Escherichia coli* FCRI	23.00	++
Salmonella Typhi FCRI	25.00	++
Aeromonas sp. AAH	31.00	++
Staphylococcus aureus FPT1	6.00	+
Pseudomonas sp. FPT	6.50	++
Bacillus pumilus FPT	2.00	++
Bacillus amyloliquefaciens FPT	4.00	++
Staphylococcus aureus FPT2	37.00	++
Escherichia coli FPT	4.00	++
β- haemolytic *Streptococcus* sp. AAH	8.50	++
Aeromonas hydrophila HS_3 AAH	11.00	++
Aeromonas hydrophila $N_{10}P$ AAH	22.00	++

+: Feeble/Thin growth, ++: Total inhibition.

CNMC-Calcutta National Medical College and Hospital, Kolkata; SDDL –Shrimp Disease Diagnostic Laboratory, Tamil Nadu Fisheries University, Madhavaram, Chennai; FCRI - Fisheries College and Research Institute, Thoothukudi; AAH - Department of Aquatic Animal Health, FFSc, Kolkata; FPT- Department of Fish Processing Technology, FFSc, Kolkata

The LD_{50} value of *Pseudomonas aeruginosa* FARP72 was calculated to be $>5.65\times10^{10}$ cells/ fish (Table 3). Samples from the affected parts of the body of freshly dead fish yielded translucent and colourless colonies initially on *Pseudomonas* isolation agar (Hi-Media, Mumbai, India) plates with 24 h of incubation at 30±2°C, and up on subsequent incubation, it yielded diffusible brown pigmentation surrounding the colonies. The isolated bacterium was identified as *Pseudomonas aeruginosa* by standard biochemical tests. Mittal *et al.,* [15] categorized bacterial pathogens as avirulent, which exhibited $LD_{50}>10^8$ cfu/mL. The recovery of fish in 3-4 days post-injection period, the LD_{50} value of $>5.65\times10^{10}$ cells/ fish and the categorization of Mittal *et al.* [15] suggested that the antagonistic bacterial strain *Pseudomonas aeruginosa* FARP72 is avirulent or less pathogenic.

Challenge test with pathogenic β-haemolytic *Aeromonas hydrophila* N10P: Clinical signs, behaviour and mortalities of the challenged fish were recorded after injection. Lethargy, abnormal behaviour, wandering around corners and erect swimming were the first signs observed in challenged *Labeo rohita*. The first mortality observed within 2 h post-injection. All 10 fish died where β-haemolytic *Aeromonas hydrophila* $N_{10}P$ were inoculated @ $6.37\text{x}10^{10}$ cells/fish in 24 h of post-injection; whereas 9 out of 10 challenged fish died in the stock inoculated with $6.37\text{x}10^9$ cells/fish within 24 h. All the fish in third stock containing 10 fish, which were inoculated with $6.37\text{x}10^8$ cells/fish died within 48 h post-injection and $6.37\text{x}10^7$ cells/fish inoculated stock recorded two mortalities after 3rd day. No mortalities were noticed in stocks which were used as positive control and negative control. After 15 days observation, 100% fish mortalities were observed in the stocks inoculated with $6.37\text{x}10^{10}$ cells/fish, $6.37\text{x}10^9$ cells/fish and $6.37\text{x}10^8$ cells/fish. Twenty percent (20%) mortalities were recorded in the stock where fish were inoculated with β-haemolytic *Aeromonas hydrophila* $N_{10}P$ @ $6.37\text{x}10^7$ cells/fish. The lethal dose at which 50% of the experimental population die (LD_{50}) was calculated on the basis of mortalities in all test concentrations over a period of 15 days. The LD_{50} value of β-haemolytic *Aeromonas hydrophila* N10P was calculated as 2.37×10^8 cells/fish (Table 4).

According to Sarkar and Rashid [16] *Aeromonas hydrophila* caused 60 - 100% mortalities in all the tested fish within 2-11 days at water temperature of 30°C. Santos *et al*. (1991) determined, during seven days, the LD_{50} of *Aeromonas hydrophila* to several

fish species: *Salmo trutta* ($2x10^5$ cells/mL), *Anguilla japonica* ($>10^8$ cells/mL), *Plecoglossus altivelis* ($8.6x10^4$ cells/mL), *Lepomis macrochirus* ($>10^8$ cells/mL) and *Onchorhyncus mykiss* ($3.2x10^4$ - $3.2x10^8$ cells/mL). Oliveira *et al.* [17] indicated that the 96-h LD_{50} value of *A. hydrophila* to Matrinxã (*Brycon amazonicus*) was 6.66 x 10^{11} cells/ mL. The LD_{50} value of *Aeromonas hydrophila* was observed as 0.3× $10^{8.66}$ cells/fish in common carp, *Cyprinus carpio* [18]. It can be interpreted on comparing the results of the LD_{50} value ($2.37×10^8$ cells/fish) of β-haemolytic *Aeromonas hydrophila* $N_{10}P$ with those of earlier studies mentioned above that the present strain can be categorized as a moderately virulent.

TABLE 4: LD_{50} VALUE OF THE ANTAGONISTIC AND FISH PATHOGENIC BACTERIA

Strains	LD_{50} value (cfu/fish)
Pseudomonas aeruginosa P72	$>5.65 \times 10^{10}$
Aeromonas hydrophila $N_{10}P$	2.37×10^8

Pathogenicity test by cohabitation challenge: The results of the mortalities in *Labeo rohita* challenged with β- haemolytic *Aeromonas hydrophila* $N_{10}P$ and *Pseudomonas aeruginosa* FARP72 at different concentrations in cohabitation challenge are represented in **Fig. 1**. Clinical signs, behaviour and mortalities of the fish were recorded daily up to 30 days after challenge and cohabitation. Lethargy, abnormal behaviour, wandering around corners and vertical swimming were the first signs observed in challenged *Labeo rohita*. About 75% of the fish treated with β-haemolytic *Aeromonas hydrophila* $N_{10}P$ suspension containing $1x10^5$ cells/ml died and only 30% mortalities were observed in fish challenged with *Pseudomonas aeruginosa* FARP72 at a level of $1x10^7$ cells/ml.

Significant difference existed in the mortalities of *L. rohita* challenged with β- haemolytic *Aeromonas hydrophila* $N_{10}P$ and *Pseudomonas aeruginosa* FARP72 ($t = 0.002$; df: 2; $P<0.05$). Samples from the affected haemorrhagic parts of the body of freshly dead and moribund fish of the β-haemolytic *Aeromonas hydrophila* $N_{10}P$ infected stock yielded typical yellow colonies on glutamate starch phenol red agar (Hi-Media, Mumbai, India) plates after 24 h of incubation at 30±2°C. The isolated bacterium was identified as *Aeromonas hydrophila* by standard biochemical tests.

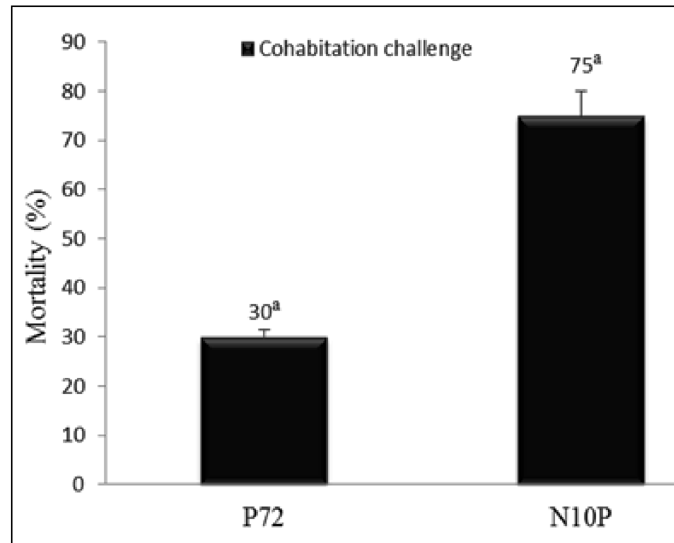


FIGURE 1: MORTALITIES IN *LABEO ROHITA* BY COHIBITATION AND MODIFIED COHABITATION WITH *PSEUDOMONAS AERUGINOSA* FARP72 AND *AEROMONAS HYDROPHILA* $N_{10}P$

The results of the cohabitation experiments thus indicated that the disease can be reproduced under experimental conditions and that injection is not required to cause disease and mortality [19]. The results of the pathogenicity trials by conventional intra-muscular injection method and cohabitation challenge showed variations, possibly due to the use of different size groups and challenge dose levels. The *Labeo rohita* used in the intra-muscular injection experiment were juveniles, whereas the *Labeo rohita* used in cohabitation challenge were fingerlings and both groups might have had different immune status.

Furthermore, the number of fish stocked in the tanks was lower in the first (n=10/tank) compared to the later (n=50/tank). This suggested that the fish mortality was more dependent on age, size, stocking density in tanks and challenge dose and all factors influence the infection pressure [20]. Nevertheless, all these results have proved that *Pseudomonas aeruginosa* FARP72 was an avirulent or less pathogenic bacterium; while the β-haemolytic *Aeromonas hydrophila* $N_{10}P$ was a moderately virulent bacterium.

Biocontrol experiment: The results of the biocontrol experiment on the basis of mortalities in cohabited *Labeo rohita* challenged separately or together with β- haemolytic *Aeromonas hydrophila* $N_{10}P$ and *Pseudomonas aeruginosa* FARP72 are represented in **Fig. 2**.

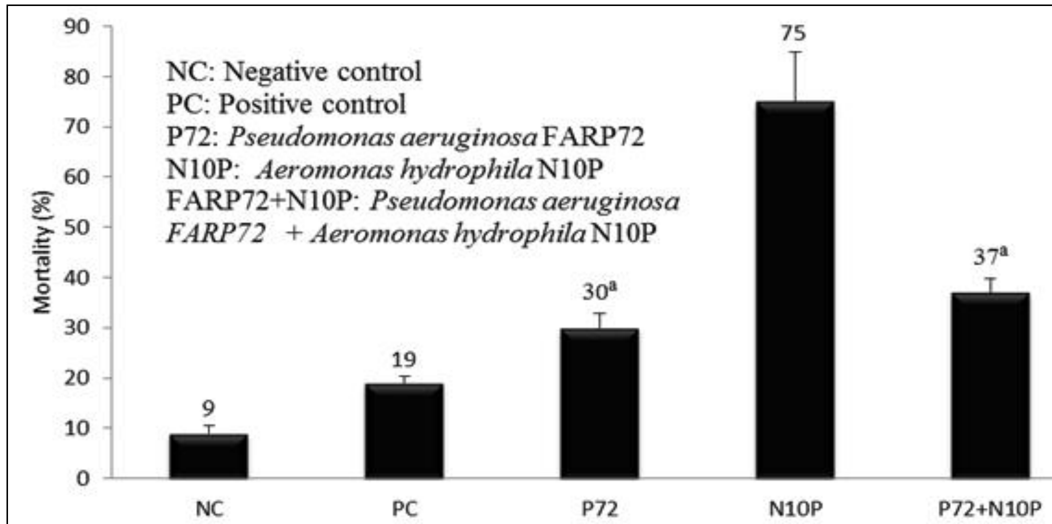


FIGURE 2: *IN-VIVO* CONTROL OF *AEROMONAS HYDROPHILA* $N_{10}P$ BY *PSEUDOMONAS AERUGINOSA* FARP72 IN COHABITATION CHALLENGE EXPERIMENT WITH *LABEO ROHITA*

Clinical signs, behaviour and mortalities of the challenged fish were recorded for 30 consecutive days. The fish which received a first 15 min dip in *Pseudomonas aeruginosa* FARP72 suspension containing $1x10^7$cells/ml followed by another dip in β- haemolytic *Aeromonas hydrophila* $N_{10}P$ suspension containing $1x10^5$ cells/ml for 15 min, showed 37% mortality; while 30% mortalities were observed in fish challenged only with *Pseudomonas aeruginosa* FARP72 at a level of $1x10^7$ cells/ml. On the other hand, 75% of the fish died when treated only with β- haemolytic *Aeromonas hydrophila* $N_{10}P$ suspension containing $1x10^5$ cells/ml.

Positive control skin wounded fish and negative control non-injured fish exhibited 19% and 9% mortalities, respectively during the same period of observation. The cumulative mortalities in *Labeo rohita* are depicted graphically in **Fig. 3**. The mortalities increased with increasing days of post-challenge. The mortality was the maximum in β-haemolytic *Aeromonas hydrophila* $N_{10}P$ challenged fish, followed by mixture of *Pseudomonas aeruginosa* FARP72 and β- haemolytic *Aeromonas hydrophila* $N_{10}P$ challenged fish, *Pseudomonas aeruginosa* FARP72 challenged fish, positive control and negative control fish stocks.

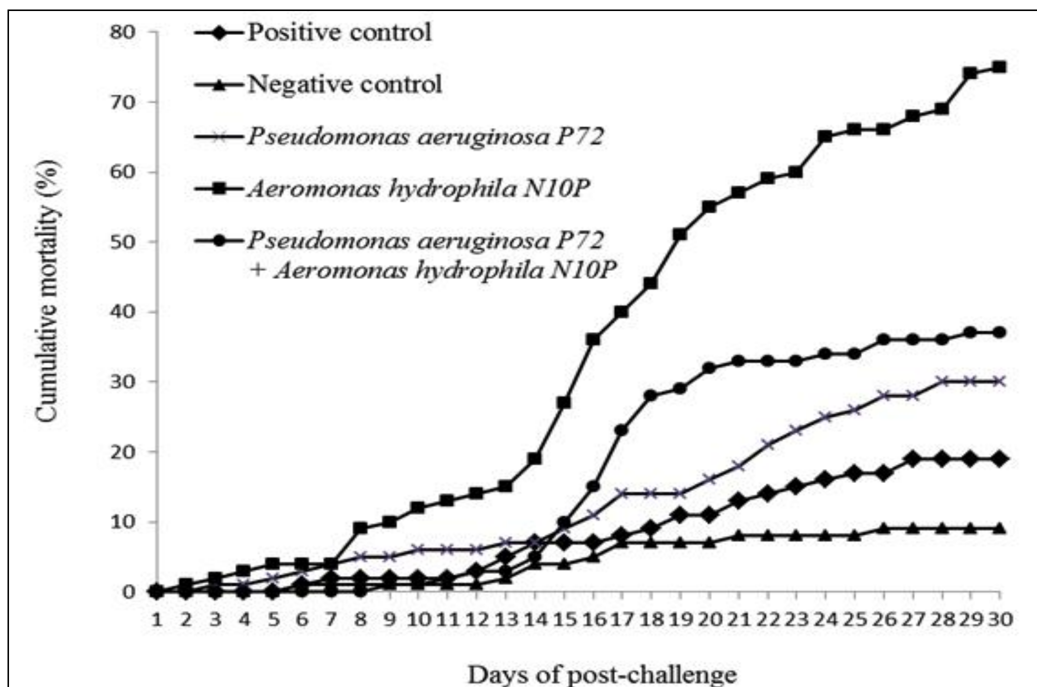


FIGURE 2: BIOCONTROL OF *AEROMONAS HYDROPHILA* $N_{10}P$ BY *PSEUDOMONAS AERUGINOSA* FARP72 IN COHABITATION CHALLENGE: CUMULATIVE MORTALITY IN *LABEO ROHITA*

Significant differences in the mortalities were observed among the *L. rohita*

i) Challenged with β- haemolytic *Aeromonas hydrophila* $N_{10}P$ and *Pseudomonas aeruginosa* FARP72 groups (t = 0.025; df: 2; P<0.05).

ii) Challenged with β- haemolytic *Aeromonas hydrophila* $N_{10}P$ and positive control groups (t = 0.016; df: 2; P<0.05);

iii) Challenged with *Aeromonas hydrophila* $N_{10}P$ and negative control groups (t = 0.011; df: 2; P<0.05);

iv) Challenged with *Aeromonas hydrophila* $N_{10}P$ and both *Pseudomonas aeruginosa* FARP72 and β- haemolytic *Aeromonas hydrophila* $N_{10}P$ groups (t = 0.033; df: 2; P<0.05);

v) Positive control and negative control groups (t = 0.019; df: 2; P<0.05);

vi) Challenged with *Pseudomonas aeruginosa* FARP72 and positive control groups (t = 0.039; df: 2; P<0.05),

vii) Challenged with *Pseudomonas aeruginosa* FARP72 and negative control groups (t = 0.011; df: 2; P<0.05),

viii) Challenged with both *Pseudomonas aeruginosa* P72 and β- haemolytic *Aeromonas hydrophila* $N_{10}P$ and positive control groups (t = 0.016; df: 2; P<0.05),

ix) Fish challenged with both *Pseudomonas aeruginosa* FARP72 and β- haemolytic *Aeromonas hydrophila* $N_{10}P$ and negative control groups (t = 0.006; df: 2; P<0.05).

It is important to note that no significant difference existed in the mortalities of *L. rohita* challenged with *Pseudomonas aeruginosa* FARP72 and *L. rohita* challenged with both *Pseudomonas aeruginosa* FARP72 and β- haemolytic *Aeromonas hydrophila* $N_{10}P$ (t = 0.167; df:2; P>0.05). It has been found that several species of *Pseudomonas* are pathogenic to the fish and others are non-pathogenic and can be used for control of some fish bacterial pathogens [3].

This study evaluated the efficacy of non-pathogenic antagonistic bacterium *Pseudomonas aeruginosa* FARP72 as biological control agent against β-haemolytic *Aeromonas hydrophila* $N_{10}P$.

As seen in Figures 2 and 3, the mortality was maximum in β- haemolytic *Aeromonas hydrophila* $N_{10}P$ challenged fish, followed by mixture of *Pseudomonas aeruginosa* FARP72 and β-haemolytic *Aeromonas hydrophila* $N_{10}P$ challenged fish, *Pseudomonas aeruginosa* FARP72 challenged fish, positive control and negative control fish stocks.

Positive control skin wounded fish and negative control non-injured fish exhibited 19% and 9% mortalities, respectively. In positive control all the skin wounded fish died, may be due to secondary infection. The observed fish mortality in negative control tanks could be attributed to the fluctuations in environmental parameters as a result of monsoon rains during the experimental period.

Several studies reported that the *in-vitro* inhibition effects of bacterial pathogens are difficult to reproduce in *in-vivo* conditions [21, 22]. However, the present work demonstrated that *Pseudomonas aeruginosa* FARP72 was capable of inhibiting human and fish pathogens *in-vitro* and fish pathogenic *Aeromonas hydrophila in-vivo* possibly through the production of antibacterial compounds as demonstrated in this study and competitive exclusion principles. Siderophore mediated competition for iron may also explain the inhibitory activity of the antagonistic strain [23]. The antimicrobial activity of *Pseudomonas* species has been attributed to several antibiotic-like substances such as phenazines [24] and non-nitrogen-containing compounds such as 2, 4-diacetylphloroglucinol [25].

A *Pseudomonas* sp. isolated from a brackish water lagoon showed significant probiotic activity against a number of shrimp pathogenic vibrios, while its safety in a mammalian system was also found satisfactory [27]. In another study, Korkea-aho *et al* [26] recorded lower levels of mortalities in fish fed first with *Pseudomonas* M174 and subsequently challenged with *Flavobacterium psychrophilum*.

CONCLUSIONS: The use of antagonistic bacteria is an important management tool, but its efficiency depends on understanding the nature of competition

between species or strains. It is clear from the results of the studies that colonization of the antagonistic bacterium *Pseudomonas aeruginosa* FARP72 on fish such as *Labeo rohita* would help reducing the attachment of β- haemolytic *Aeromonas hydrophila* and development of infection and disease outbreaks. Still there is a need to continue to screen new antagonistic strains from local aquaculture rearing species to suit the specific requirement. The results of the present study would provide the basis for future research on biological control of fish diseases and its application in aquaculture.

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