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Determination of Lethal Dose of *Aeromonas hydrophila* RTMCX1 and *in vitro* Efficacy of Oxytetracycline Hydrochloride in Golden Mahseer, *Tor putitora* (Hamilton, 1822)

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ABSTRACT

Background: *Aeromonas hydrophila* is considered as a major bacterial pathogen to fish. It's wide host susceptibility has caused huge economic losses in aquaculture. In the present study, the lethal dose (LD_{50} 96 h) of *Aeromonas hydrophila*, RTMCX1 and its control through oxytetracycline hydrochloride was appraised in fry of golden mahseer, *Tor putitora*.

Methods: LD_{50} 96 h of *A. hydrophila*, RTMCX1 in fry of golden mahseer was calculated through Probit model analysis. The total microbial load in muscle tissue of infected fry was estimated using standard microbial technique. The effect of oxytetracycline hydrochloride treatment was assessed at varied concentrations; 20, 40, 60 and 80 mg L^{-1} in experimentally infected fry at 10^8 CFU mL^{-1} of *A. hydrophila*.

Result: LD_{50} 96 h of *A. hydrophila*, RTMCX1 in fry of golden mahseer was calculated as 10^8 CFU mL^{-1}. The total microbial load in muscle tissue of infected fry was ranged from 10^3-10^7 CFU mL^{-1}. The post infection by the test bacterium demonstrated 25% of cumulative mortality in golden mahseer fry. The abnormal behaviour of the experimental fry was become normal and feed intake increased (scale 3) within 96 h of successive oxytetracycline hydrochloride bath treatment 1 h every day at 20 to 80 mg L^{-1}. Post 96 h oxytetracycline hydrochloride treatment at 40-80 mg L^{-1} also demonstrated significant survival percentage (75-80%) in fry as compared to the group treated with 20 mg L^{-1} and positive control ($p<0.05$). The present study determined the LD_{50} of *A. hydrophila* at 10^8 CFU mL^{-1} in golden mahseer fry and its effective control through administration of oxytetracycline hydrochloride bath treatment 1 h at 40 mg L^{-1} for 4 consecutive days.

Key words: *Aeromonas hydrophila*, Dose, Golden mahseer, LD_{50}, Oxytetracycline hydrochloride.

INTRODUCTION

Aeromonas hydrophila is considered as one of the major opportunistic bacterial pathogens and causes huge economic loss in global aquaculture, signifying its distribution in different aquatic habitats; freshwater, estuarine and marine (Samayanpaulraj *et al*. 2019). The disease outbreak in different fish species by *A. hydrophila* is mainly manifested with the development of clinical signs such as hemorrhages, lesions, red-colored patches on the body surfaces around anal and urogenital pore, excessive mucus secretion, necrosis, lesions, distended abdominal cavity and ulcerations in both wild and cultured conditions (Pavanelli *et al*. 2002; Shahi *et al*. 2013). Other than the clinical signs, the infection of *A. hydrophila* also resulted in necrotic hemorrhage in liver, kidney, spleen and abdominal cavity filled with ascites content (Barja and Esteves, 1988; Schlotfeldt and Alderman, 1995).

There are reports of bacterial diseases in golden mahseer (Shahi and Mallik, 2014; Shahi *et al*. 2018) and the major bacterial pathogen being identified is *A. hydrophila*. The infection of *A. hydrophila* in gills and epithelial tissue has led to mortality of golden mahseer (Kumar *et al*. 2016). Thus, the determination of lethal dose (LD_{50}) of *A. hydrophila* in golden mahseer may establish disease control measures. Moreover, this may also help to study the efficacy of any aquaculture product, drug, antibiotic or chemical formulation for the prophylactic and therapeutic applications. Among the several antibiotics, oxytetracycline hydrochloride is active against a broad range of gram-positive and gram-negative bacteria. It is widely used to control furunculosis, coldwater bacterial disease in salmonids, hemorrhagic septicaemia in carps, gafkemia in lobsters and *Pseudomonas* disease in catfish (Serrano, 2005). US Food and Drug Administration

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(USFDA) has also approved bath and oral medication of oxytetracycline for the treatment of the bacterial infection in certain fish species *i.e*. channel catfish, salmonids and lobster in aquaculture. Though, the efficacy and stability trials for FDA regulated oxytetracycline hydrochloride has been established for the temperate foodfish, the varied fish culture practices, food and feeding habit, the water quality parameters, agro-climatic conditions and microbial infections may affect the effectiveness of oxytetracycline hydrochloride application. There is lack of information regarding lethal dose (LD_{50} 96 h) of *A. hydrophila* and use of optimum concentration of oxytetracycline hydrochloride to control the infection in golden mahseer.

In the present study, the LD_{50} 96 h of *A. hydrophila*, RTMCX1 is determined in golden mahseer fry. The trial is also designed and performed based on the FDA regulations to study the efficacy of oxytetracycline hydrochloride bath treatment against *A. hydrophila*, RTMCX1 infection at varied antibiotic concentrations; 20, 40, 60, 80 mg L^{-1} with reference to gross pathology, behavioral characteristics, feed intake and fry survival percentage.

MATERIALS AND METHODS

The present experiment was conducted at ICAR-Directorate of Coldwater Fisheries Research, Bhimtal, India during 2021-2022. The healthy golden mahseer fry (average weight 0.20±0.001 g; length 28±0.021 mm) were collected from the mahseer hatchery unit of the directorate. They were acclimatized in wet-laboratory conditions and fed with a basal feed containing 40% protein @ 2-3% of their body. The basal feed was prepared by admixing appropriate amount of vitamin and mineral mixture with 8% crude lipid and 40 % crude protein. After acclimatization, the fry were distributed into glass aquarium for the experimental infection by immersion method and oxytetracycline bath treatments at different concentrations; 20, 40, 60 and 80 mg L^{-1} for 1 h every day for 4 consecutive days.

Previously identified *A. hydrophila*, RTMCX1 (JX390650) (Shahi *et al*. 2013) preserved in bacterial freezing medium (N400-25PK, AMRESCO) at -80°C, was streaked on BHIA (Brain Heart Infusion Agar, HiMedia) plates and incubated at 28°C for 18-24 h. After incubation, aseptically a single discrete colony was picked up and inoculated into two 100 mL Erlenmeyer flasks, containing 50 mL TSB (Tryptone Soya Broth, HiMedia, India) each. The flasks were incubated in a shaking incubator at 28°C for 18 to 24 h. After incubation, the culture in conical flask was centrifuged at 2737 × g for 5 min. The bacterial pellet was re-suspended in Phosphate buffer saline (PBS) and again centrifuged at 2737 × g for 5 min for washing. The same process was repeated thrice for the complete removal of broth contents in the culture. In 3 mL of PBS, the bacterial pellet was re-constituted as McFarland standard suspension of 10^4, 10^6 and 10^8 CFU mL^{-1} for experimental bath infection of fry to determine the lethal dose (LD_{50} 96 h) of *A. hydrophila*, RTMCX1.

To determine the LD_{50} 96 h of *A. hydrophila*, RTMCX1 the golden mahseer fry (240 no.) were equally distributed in 6 tanks (each tank containing 40 no. of fry) in replicate with one control. The fry were kept on starvation 24 h prior to the experimental infection and then they were subjected to bath challenge by *A. hydrophila*, RTMCX1 suspensions at 10^4, 10^6 and 10^8 CFU mL^{-1}. The mortality percentage of fry was calculated at 24, 48, 72 and 96 h post challenge period (Abbott, 1925). Then LD_{50} 96 h was determined by using Probit model analysis in SPSS 22.

Post 96 h infected fry (n=18) randomly collected from the individual treatment group (10^4, 10^6 and 10^8 CFU mL^{-1}) were pooled separately and processed aseptically to determine the microbial load. One gram muscle tissue was collected from pooled sample and macerated with 99 mL physiological saline (0.85% NaCl) and then serially diluted for plating on Rimler-Shotts medium. The microbial load was counted (CFU mL^{-1}) for each concentration of *A. hydrophila*, RTMCX1 24 h post incubation at 28°C.

To study the safety and efficacy of oxytetracycline hydrochloride bath treatment, the LD_{50} 96 h value of *A. hydrophila*, RTMCX1 at 10^8 CFU mL^{-1}, was selected for the experimental infection of the fry. The healthy fry were distributed randomly into glass aquaria of dimension; Length (L)×Breadth (B)×Depth (D); 0.43×0.3×0.3 m each) marked as negative control, positive control with PBS, positive control with bacteria, treatment group-1 (20 mg L^{-1}), treatment group-2 (40 mg L^{-1}), treatment group-3 (60 mg L^{-1}) and treatment group- 4 (80 mg L^{-1}) in triplicates. Each glass aquarium was stocked with 40 no. of fry for bath infection by *A. hydrophila,* RTMCX1 suspensions at 10^8 CFU mL^{-1} for 1 h. The treatment of fry with oxytetracycline hydrochloride was initiated 96 h post challenge by the test bacterium. The desired concentrations of oxytetracycline hydrochloride; 20, 40, 60 and 80 mg L^{-1} weighed and dissolved in 0.1N HCl in 4 different separate beakers (500 mL each) and aerated vigorously. The fry were subjected to bath treatment of oxytetracycline hydrochloride 1 h every day at antibiotic concentrations of 20, 40, 60 and 80 mg L^{-1} for 4 consecutive days.

Fish behavioral changes and the external sign of infection in fry were recorded daily during the entire experimental period. The unconsumed feed from different experimental tanks was collected separately after 1 h of broadcasting of the feed every day, then air-dried and measured carefully. Depending on the feed consumption, the feeding behaviour of fry was rated using a scale ranged from 0-4 as follows:

Scale 0=No feed consumed, Scale 1=25% feed consumed, Scale 2= 50% feed consumed, Scale 3=75% feed consumed and Scale 4=100% feed consumed.

The cumulative mortality (%) in experimentally infected golden mahseer fry was recorded at 24 to 96 h, whereas survival (%) of fry was recorded post 96 h oxytetracycline hydrochloride treatment for 10-d.

The Probit model analysis was performed to determine the LD_{50} value using Statistical package for social science (SPSS), version 22. The values of water quality parameters were expressed as mean±SD. Data on cumulative mortality (%) were analyzed and expressed as mean±standard error and graphically represented using GraphPad, Prism Version 5.01. The average fry survival percentage for oxytetracycline hydrochloride treatments were compared using One-way ANOVA and considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Fry mortality percentage and LD_{50} 96 h of *A. hydrophila*, RTMCX1

Mortality of the golden mahseer fry was recorded 96 h post bath challenge with *A. hydrophila*, RTMCX1. Initially, golden mahseer fry demonstrated tolerance to 10^4 CFU mL^{-1} of *A. hydrophila*, RTMCX1, whereas 24 h post challenge recorded the first mortality; 7 and 10% in the experimental groups infected with 10^6 and 10^8 CFU mL^{-1} of *A. hydrophila*, RTMCX1 respectively. Similarly, 40% fry mortality was observed in the group at 10^8 CFU mL^{-1} of *A. hydrophila* RTMCX1 within 48 to 96 h challenge period. Using Probit model in SPSS 22, the mortality data recorded (Table 1) for 96 h challenge period established 10^8 CFU mL^{-1} as LD_{50} 96 h of *A. hydrophila*, RTMCX1 in golden mahseer fry. No mortality was observed in the control group. LD 50 assay defines the tolerance of a fish species to pathogenic bacteria suspensions at a particular cell count, which ultimately determines the pathogenicity of the bacteria by killing 50% of the fish population over a time period. *A. hydrophila* being ubiquitous and pathogenic in nature, its distribution in different aquatic habitats has posed a serious threat to health management of different fish species. This bacterium has also caused a considerable economic loss both in tropical and subtropical aquaculture practices (de Oliveira *et al*. 2011). According to the resistance of fish to the bacterial infection, the LD 50 of *A. hydrophila* varies from species to species. The earlier reports demonstrated LD 50 of *A. hydrophila* against several fish species *i.e. Salmo trutta fario* (2×10^5 cells mL^{-1}), *Anguilla japonica* (>10^8 cells mL^{-1}), *Plecoglossus altivelis* (8.6×10^4 cells mL^{-1}), *Lepomis macrochirus* (>10^8 cells mL^{-1}), *Onchorhyncus mykiss* (3.2×10^4 to 3.2×10^8 cells mL^{-1}) and *Brycon amazonicus* (6.66×10^{11} cells mL^{-1}) (Santos *et al*.1991; de Oliveira *et al*. 2011). In the present study, the LD_{50} 96 h assay of *A. hydrophila*, RTMCX1 in golden mahseer showed mortality of 50% population of fry at cell suspension of 10^8 CFU mL^{-1}.

Estimated microbial load (CFU mL^{-1})

Significant microbial load was recorded in the muscle tissue of *A. hydrophila*, RTMCX1 infected golden mahseer fry at cell concentrations of 10^4, 10^6 and 10^8 CFU mL^{-1}. Microbial count in the muscle was estimated to range from 5.4×10^3 to 2.8×10^7 CFU mL^{-1}. Intraperitoneal infection of *A. hydrophila* in *Channa striatus* showed maximum microbial count in muscle tissue as compared to liver, kidney and spleen (Samayanpaulraj *et al*. 2019). Golden mahseer fry being smaller in size, it deterred the collection and process of the other tissue samples (liver, kidney and spleen) for the estimation of the total microbial load.

Efficacy of oxytetracycline hydrochloride

Bio-safety assay and efficacy study of antibiotics are generally carried out to evaluate the prudent use of antimicrobials at optimum doses that maximizes therapeutic effect and minimizes the development of antimicrobial resistance in aquaculture. The infected fry were kept under observation for 96 h in glass aquarium with a continuous supply of oxygen for disease progression. It was observed that fry were surfacing, grouping at corners of experimental tanks and demonstrating rapid opercula movement during 24-48 h post-infection. The visible signs of disease progression developed 24 h post-bath infection with *A. hydrophila*, RTMCX1. The clinical signs were evident as the appearance of the red patches on the dorsal body surface of fry 40 hpi and haemorrhage in the eye, caudal fins and operculum regions of fry 70 hpi (Fig 1a-b), which were similar to the earlier reports (Plumb, 1999; Darwish *et al*. 2002; Julinta *et al*. 2017). Along with the development of visible symptoms of disease progression, the mortality of fry in the experimental tanks was also recorded 24 hpi. The behavioural characteristics of fry included erratic swimming (swimming upside down) and faster opercula movement. They grouped at the corners and rested at the bottom of the tanks. Some of the fry swam to the surface of the water and showed lethargic movement, while behavioural characteristics of fry in the control groups were observed normal. Post-infection behaviour changes in fish fry were marked with lethargy in movement, reduction in feed intake, fry swimming in circles and random direction with arched bodies, suspended near the water surface and orientated to water flow (Darwish *et al*. 2002; Rach, 2008; Julinta *et al*. 2017). The fry were subjected to the bath treatment regimes of oxytetracycline hydrochloride; 20, 40, 60, 80 mgL^{-1} 96 hpi. It was observed that oxytetracycline hydrochloride bath treatments for 4 consecutive days led to the restoration of

Table 1: Post challenged fry mortality data in LD_{50} 96 h of *A. hydrophila,* RTMCX1.

Aeromonas hydrophila (CFU mL^{-1})	24 hr	48 hr	72 hr	96 hr	Mortality (%)
Control	0	0	0	0	0
10^4	0	0	2	2	10
10^6	3	2	1	0	15
10^8	4	7	3	6	50

n: 40 number of golden mahseer fry challenged for each *A. hydrophila* concentration.

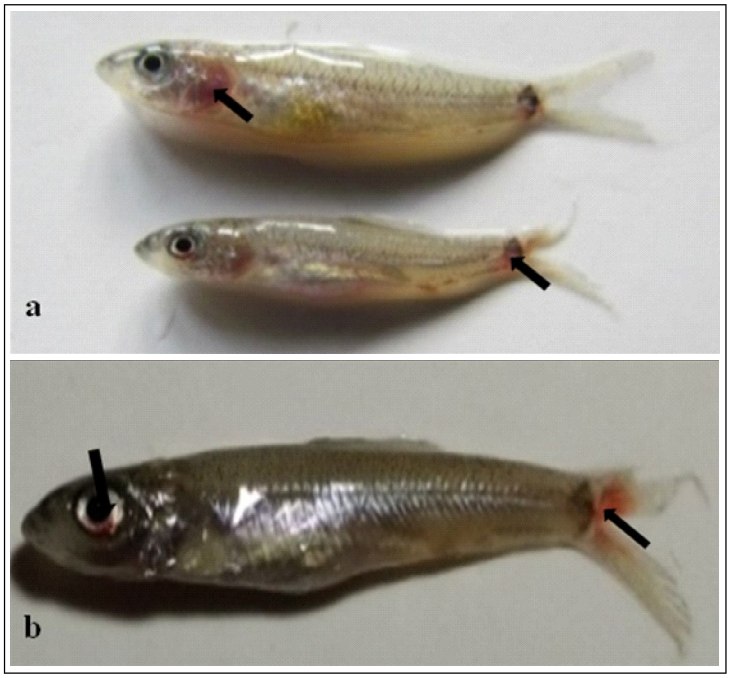

Fig 1(a-b): Post infection clinical signs developed by *A. hydrophila* in golden mahseer fry Red patches on the dorsal body surface of fry 40 hpi and hemorrhagic eye, caudal fins and operculum of fry 70 hpi.

Table 2: Feeding behavior score for golden mahseer advance fry during experimental period.

Treatments	Pre treatment (1-4 days)	Disease progression (5-8 days)	OTC treatment (9-12 days)	Post treatment (13-22 days)
NC	4±0.00	4±0.00	4±0.00	4±0.00
P+PBS	4±0.00	4±0.00	4±0.00	4±0.00
P+AH	4±0.00	1±0.53[a]	1±0.53[a]	1±0.48[a]
20 mgL^{-1}	4±0.00	1±0.53[a]	2±0.48	3±0.34[b]
40 mgL^{-1}	4±0.00	1±0.53[a]	2±0.48	3±0.34[b]
60 mgL^{-1}	4±0.00	1±0.53[a]	2±0.48	3±0.34[b]
80 mgL^{-1}	4±0.00	1±0.53[a]	2±0.48	3±0.34[b]

NC: Negative control; P+PBS: Positive control with phosphate buffer saline; P+AH: Positive control challenged with *A. hydrophila*; RTMCX1, OTC: Oxytetracycline hydrochloride, the superscripts (a and b) signify difference in feed intake (<0.05) between bacterial challenged and post oxytetracycline treated groups.

normalcy in terms of behavioural characteristics of infected fry. Qualitatively, the healing process in fry was noticeable as turning of black colouration of the epidermal layer at the site of infection (Julinta *et al*. 2017).

Feed intake

From acclimatization period to first day of the experimental infection, all the experimental groups of golden mahseer fry exhibited feeding behaviour indistinguishable (score 4). The basal feeds were consumed within 30 minutes of its administration. During 5^{th}-8^{th} day of infection or disease progression period, there was a severe reduction in feed intake (score 1) by the fry bath challenged with *A. hydrophila*, RTMCX1. This was followed by execution of oxytetracycline hydrochloride bath immersion treatment of fry for 4 consecutive days (9^{th}-12^{th}). During this period, there was a slight increase in feed intake by the treated groups (score 2). The marked increase in feed intake by the fry was recorded (score 3) in the post oxytetracycline treatment regimes in all the treated groups (Table 2). Similar observations were also reported *i.e*. reduction in feed intake during *A. hydrophila* infection and early oxytetracycline hydrochloride treatment period (Julinta *et al*. 2017). The 96 h post oxytetracycline hydrochloride treatment restored normalcy in behavioral characteristic of the challenged fry with increased feed intake (Rach, 2008; Julinta *et al*. 2017).

Post infection mortality and fry survival percentage

During 24-96 h bath challenge period, *A. hydrophila*, RTMCX1, induced a cumulative mortality of 20-25% in golden mahseer fry (Fig 2). The exposure to oxytetracycline hydrochloride bath immersion @ 20, 40, 60, 80 mg L^{-1} for 1 h

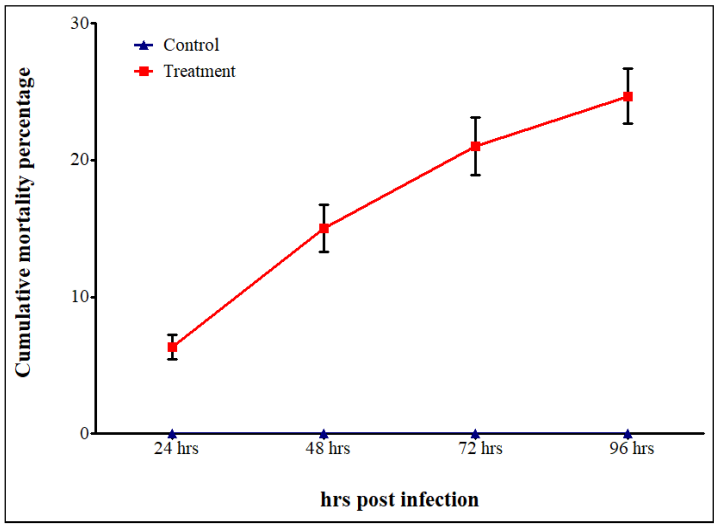

Fig 2: Cumulative mortality (%) of golden mahseer fry 24-96 h post challenged with *A. hydrophila*, RTMCX1 at 10^8 CFU mL^{-1}. Data on cumulative mortality percentage analyzed and expressed as mean±standard error and graphically represented using Graph Pad, Prism Version 5.01.

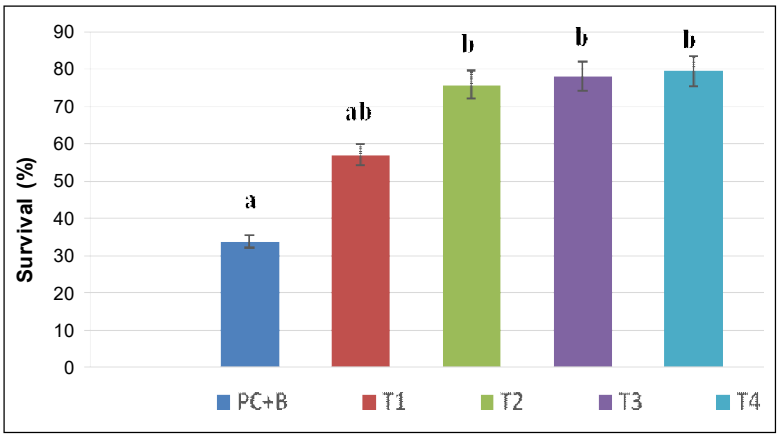

Fig 3: Survival (%) of fry of golden mahseer 96 h post oxytetracycline treatment period. Post 96 h *A. hydrophila*, RTMCX1 (10^8 CFU mL^{-1}) infected golden mahseer fry treated with oxytetracycline hydrochloride at T_1: 20 mg L^{-1}, T_2: 40 mg L^{-1}, T_3: 60 mg L^{-1} and T_4: 80 mg L^{-1}; PC+B: Positive control with bacteria; data expressed as mean±standard error and One-way ANOVA signified higher survival (%) of fry (<0.05) at 40, 60 and 80 mg L^{-1} antibiotic doses as compared to positive control.

increased the survival percentage of fry challenged with *A. hydrophila*, RTMCX1. But, the fry survival percentage (75-80%) was significantly higher (<0.05) in the groups treated with 40, 60 and 80 mg L^{-1} oxytetracycline hydrochloride as compared to 20 mg L^{-1} and the control (Fig 3). No sign of distress was apparent in the fry during oxytetracycline hydrochloride treatment in the study. High mortalities in fry of golden mahseer were recorded during initial infection period (2-5 day), which get stabilized and decreased once the oxytetracycline hydrochloride was administered through bath treatment (Julinta *et al*. 2017; Rey *et al*. 2009) 96 h post challenge. The exposure to oxytetracycline hydrochloride bath treatments @10, 20 and 40 mgL^{-1} for 1 h had significantly increased the survivability of fish fry as compared to the control (Rach *et al*. 2008) that concurred with the findings of the present study in golden mahseer fry, demonstrating higher survival rates in the groups treated with oxytetracycline hydrochloride at 40, 60 and 80 mg L^{-1}.

CONCLUSION

Dose optimization of oxytetracycline hydrochloride may vary from species to species. Thus, the efficacy procedure of antibiotics and dose must be tailored to each particular fish species and culture conditions. The present study determined the LD_{50} 96 h of *A. hydrophila*, RTMCX1 at 10^8 CFU mL^{-1} and optimized the effectiveness of concentrations of oxytetracycline hydrochloride bath treatment against *A. hydrophila* infection in fry of golden mahseer. We should not recommend fish farmers to use higher dose of antibiotics to control the bacterial infection when relatively a lower dose is equally effective to contain the same. Thus, the prudent use of oxytetracycline hydrochloride in bath treatment of

golden mahseer fry 1 h every day @ 40 mg L^{-1} for 4 consecutive days will definitely increase the fry survival percentage against bacterial infection in hatchery conditions.

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Conflict of interest: None.

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