

Detection of sub-lethal concentration (LC₅₀) of *Aeromonas hydrophila* against hybrid lemon fin barb (*Hypsibarbus wetmorei* ♂ × *Barbonymus gonionotus* ♀) early fry

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Abstract The common bacterial pathogens of cultured freshwater fish are mostly aeromonads, especially the pathogenic strains of *Aeromonas hydrophila*. Early fry stages of fish reared in hatcheries face severe disease outbreaks caused by aeromonads. Disease challenge studies with pathogenic bacteria including *Aeromonas* sp. have become a more common tool used in the investigation of the effects of micronutrients and other immunomodulatory additives in fish diets. This study examined the sub-lethal concentration (LC₅₀) of *A. hydrophila* against hybrid lemon fin barb (*Barbonymus gonionotus* ♀ × *Hypsibarbus wetmorei* ♂) early fry for 14 days. Three hundred and seventy-five fry (24 days after hatching) averaging 1.8±0.24 mg were randomly distributed in fifteen 2L plastic tanks. Fish fry were submerged in varying concentrations of *A. hydrophila* (2.5×10⁵, 2.5×10⁶, 2.5×10⁷, 2.5×10⁸ and 1×10⁹ CFU ml⁻¹). During the trial period, early fry was fed with a formulated diet containing the optimum crude protein and lipid contents. The mortality of fry in each tank was recorded daily. The LC₅₀ of *A. hydrophila* for early fry of hybrid lemon fin barb by immersion was found at 6.06×10⁸ CFU ml⁻¹.

Keywords: Hybrid lemon fin barb, early fry, *Aeromonas hydrophila*, LC₅₀

INTRODUCTION

Hybrid lemon fin barb was developed and introduced into the Malaysian aquaculture industry by the Department of Fisheries Malaysia early in 2004 (DOF 2012). It is a cross between the male lemon fin barb *Hypsibarbus wetmorei* and the female silver barb *Barbonymus gonionotus*. The hybrid has the fast growth of silver barb and the high meat quality of lemon fin barb (Suharmili *et al.* 2015), and fetches a good retail price as high as USD 13.67 kg⁻¹ (DOF 2015). Since its introduction, most research has been concentrated on its nutritional requirements while almost no research has been focused on its health management.

Among several pathogenic bacteria, gram negative bacilliform aeromonads cause most of the

diseases in terrestrial animals including humans (Janda 1991; Janda and Abbott 1996) and aquatic animals including fish (Rahman *et al.* 2005). Major causal pathogens in the *Aeromonas* group are *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas caviae* (Janda 1991; Plumb 1994). Gautam *et al.* (1992) reported that motile *Aeromonas* spp. are found in natural aquatic and terrestrial environments with *A. hydrophila* and *A. sobria* showing higher pathogenic characteristics. *Aeromonas hydrophila* has caused severe economic losses in the fish farming industry in Southeast Asia (Austin and Austin 1985; Harikrishnan and Balasundaram 2005; Thampuran *et al.* 1995). On the other hand, cases have been reported of its disease transmission into farms from hatcheries through fry and water supply (Zamri-Saad *et al.*



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2014). Therefore, hatcheries must be free from diseases to prevent economic losses in grow-out fish farms.

As a new candidate in freshwater aquaculture, studies on bacterial diseases of hybrid lemon fin barb have not been reported. *Aeromonas hydrophila* is known as a pathogenic bacteria that commonly cause mortalities in carps (Alsaphar and Al-faragi 2012). *Aeromonas hydrophila* subsp. has also negatively affected the quality of cryopreserved silver barb *B. gonionotus* sperm (Boonthai et al. 2016; Boonthai et al. 2018). Moreover, silver barb is susceptible to diseases caused by *A. sobria* (Sarker et al. 2000). *A. sobria* also causes diseases in several other carps (Sopinska et al. 1997) including caudal peduncle disease in grass carp (Xu et al. 1986). Miles et al. (2001) observed a reduced germlings growth of *A. invadans* in macrophages and serum of silver barb. *A. hydrophila* and *A. sobria* isolates have been reported to show virulence in rainbow trout *Salmo gairdneri* (Paniagua et al. 1990).

The pathogen virulence for any fish can be observed by the lethal concentration (LC₅₀) test that gives information on the relations between the fish and the pathogen (Mostafa et al. 2008). The virulence of *Aeromonas* isolates has been observed at 3×10^8 CFU ml⁻¹ in rainbow trout *Salmo gairdneri* (Paniagua et al. 1990) while 1×10^7 CFU ml⁻¹ of *A. hydrophila* is lethal for goldfish *Carassius auratus* (Citarasu et al. 2011). Silver barb *B. gonionotus* subjected to an LC₅₀ value of 2.5×10^8 CFU ml⁻¹ of *Aeromonas sobria* shows signs of ulcerative disease (Sarker et al. 2000). Likewise, Nikapitiya et al. (2018) used a 1×10^9 CFU ml⁻¹ *A. hydrophila* suspension for observing mortality in zebrafish larvae treated with chitosan nanoparticles. Nevertheless, the information on LC₅₀ of aeromonads against cyprinid larvae is scarce. As a new candidate in the Malaysian freshwater sub-

sector, no report on the LC₅₀ value of any kind of pathogen of hybrid lemon fin barb has been published. In this study, hybrid lemon fin barb postlarvae were challenged with several doses of *A. hydrophila* to determine the sub-lethal concentration (LC₅₀) of *A. hydrophila*.

MATERIALS AND METHODS

All larval handling and sampling procedures were in accordance with the approved International Animal Care and Use Committee (IACUC) guidelines number UPM/IACUC/AUP-R017/2020.

Preparation of bacterial culture

Agar culture plates containing *A. hydrophila* were provided by the Fish Health Laboratory, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia. Three liters of 3% Tryptic soy broth (TSB) solution was prepared and sterilized in an autoclave. The cultured *A. hydrophila* bacteria were then inoculated into a few 50ml centrifuge tubes containing sterile TSB and incubated overnight at 30 °C. TSB-bacteria solutions were later poured into pre-sterilized 1L bottles containing 3% TSB. After one-day incubation period, mass-cultured bacteria were distributed into sterile 50 ml tubes and centrifuged at 3000 rpm for 10 minutes. The supernatant of each tube was discarded. The pellets were re-suspended in sterile distilled water by vortex mixing. The resulted, re-suspended bacterial solution was collected into a sterilized glass bottle and the absorbance was recorded spectrophotometrically at 550 nm. The required volume (RV) to achieve the required bacterial concentration in the immersion trial was calculated as follows;

$$RV = \frac{\text{Required bacterial concentration} \times \text{tank volume}}{\text{Absorbance} \times MS}$$

where, tank volume = 2L and MS (McFarland standard) = 1.2×10^9

Identification of lethal concentration (LC₅₀) value

The bacterial concentration in the stock solution was 1.8×10^9 CFU ml⁻¹. The baseline information for applying suitable bacterial concentration in tanks

was prepared according to Sarker et al. (2000). Five treatments representing five consecutive bacterial concentrations were prepared as 2.5×10^5 (C1),

2.5×10^6 (C2), 2.5×10^7 (C3), 2.5×10^8 (C2) and 1×10^9 (C5) CFU ml⁻¹ in 2L tanks following above equation. Twenty-four days old early fry of hybrid lemon fin barb were stocked at the rate of 25 fry per tank. The five treatments were randomly assigned to triplicate groups. The fry were fed a well-balanced diet three times per day at 25% body weight. Table 1 shows the feed and proximate composition of the diet. Mortality of fry was recorded daily for two weeks.

Table 1 Feed and proximate composition (as fed basis) of the diet fed to hybrid lemon fin barb early fry for 14 days

Ingredient	%
Defatted fishmeal ^a	69.54
Vitamin premix ^b	1
Mineral premix ^c	1
Cod liver oil	12
Agar	3
Corn starch	13.26
Choline chloride	0.2
Total	100
<u>Proximate composition</u>	
Moisture	5.04±0.00
Crude lipid	12.03±0.03
Crude protein	50.87±0.22
Ash	17.53±0.11
Crude fiber	0.21±0.02
Nitrogen free extract	14.29±0.14
Energy (kJ g ⁻¹)	17.996

^aFishmeal was defatted with five successive treatments with ethanol: either (1:1;v/v) and contains 71.9% crude protein; 0.1% lipid

^bVitamin premix (g kg⁻¹): ascorbic acid, 50; thiamin mononitrate, 1; niacin, 4.5; riboflavin, 1; pyridoxine, 1; retinyl acetate, 0.6; cholecalciferol, 0.1; choline chloride, 75; Ca-pantothenate, 3; myo-inositol, 5; vitamin K menadione, 1.7; biotin, 0.03; α -tocopheryl acetate (500 IU g⁻¹), 9; vitamin B12, 0.005; folic acid, 0.14.

^cMineral premix (g kg⁻¹): NaCl, 40; KCl, 94; Na₂SeO₃, 0.03; NaF, 1; CaCO₃, 210; Ca (H₂PO₄).H₂O, 500; KI, 0.04; CuSO₄.5H₂O, 3;

FeSO₄.7H₂O, 20; MgOH, 120; MnSO₄.H₂O, 3; CoSO₄, 0.02; ZnSO₄.7H₂O, 3.5.

Data analysis

One-way ANOVA was used to evaluate the significant difference at the $P < 0.05$ level of significance. Tukey's test was used to compare means at $P < 0.05$ level of significance using SAS 9.1 programme. The lethal concentration (LC₅₀) of *A. hydrophila* was calculated by logit analysis through the following equation;

$$LC_{50} \text{ concentration} = 10^{\text{Log value at logit } 0}$$

RESULTS

Fish fry readily accepted the diet before and after the introduction of the bacteria but showed a loss of appetite during the first week. Feed intake of early fry was reduced on Day 2, 3, 4, 5 and 6 and increased from Day 7 onwards. During this time, the movements of fry slowed down significantly and they showed weaknesses in C2, C3, C4 and C5 tanks.

Mortality of fry in treatment C1, C2, C3, C4 and C5 stopped at Day 8, 6, 9, 9, and 4, respectively (Figure 1). The mortality rate of hybrid lemon fin barb fry ranged from 8 to 98.7% on day 9 (Table 2). Fish fry exposed to *A. hydrophila* at 2.5×10^7 and 2.5×10^8 CFU ml⁻¹ showed 13% and 22% mortality, respectively. Lethal concentration (LC₅₀) of *A. hydrophila* by immersion for hybrid lemon fin barb early fry was found at 6.06×10^8 CFU ml⁻¹ (Figure 2).

Table 2 Mortality of hybrid lemon fin barb early fry at different *A. hydrophila* levels after 14 days (means ± SE)

Immersion dose (CFU ml ⁻¹)	Mortality (%)
2.5×10^5 (C1)	8.00±4.00 ^c
2.5×10^6 (C2)	13.33±3.53 ^{bc}
2.5×10^7 (C3)	13.33±3.53 ^{bc}
2.5×10^8 (C4)	21.33±2.67 ^b
1×10^9 (C5)	98.67±1.33 ^a

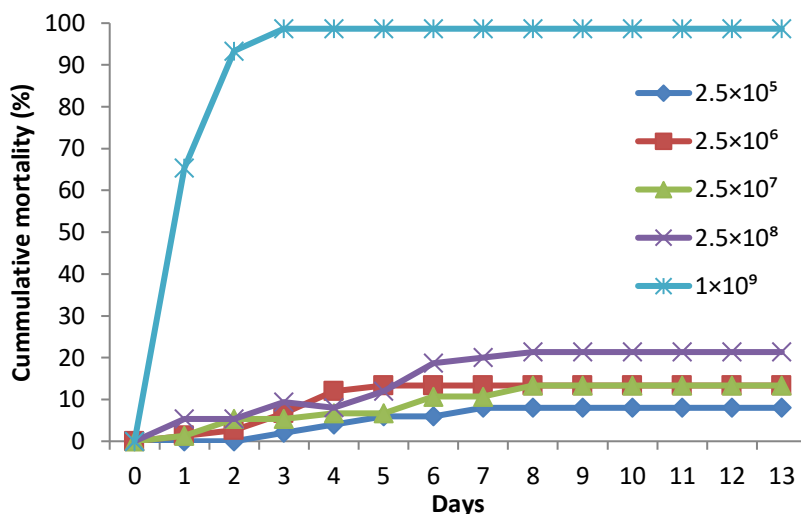


Fig 1 Cumulative mortality of hybrid lemon fin barb early fry at different *A. hydrophila* concentration levels for 14 days

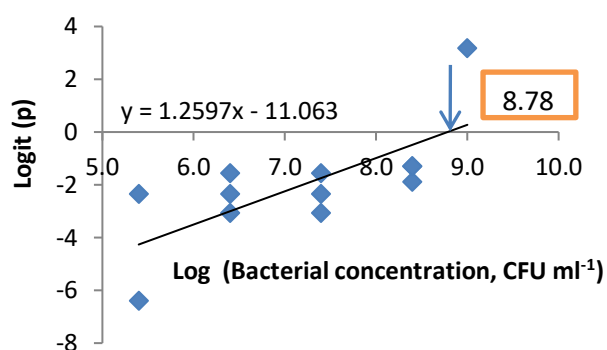


Fig 2 The log value of the sub-lethal concentration of *A. hydrophila* for hybrid lemon fin barb fry. LC₅₀ was found at 6.06×10^8 CFU ml⁻¹ by antilog conversion of the log value at logit '0'.

DISCUSSION

The mortality of fish against different doses of *A. hydrophila* and other pathogens varies with the species of fish and treatment method (Mostafa *et al.* 2008). In the present study, the mortality of hybrid early fry increased by over 25% when they were exposed to more than 2.5×10^8 CFU ml⁻¹ *A. hydrophila*. According to Sabur (2006), several carp species tested against *A. hydrophila* required an injected dose of 2×10^5 - 2×10^6 CFU fish⁻¹ and an immersed dose of $2.2 \times 10^{7-8}$ CFU ml⁻¹ for 40-100%

mortality. Mostafa *et al.* (2008) reported that 40% and 100% mortality in stinging catfish *Heteropneustes fossilis* injected with *A. hydrophila* at 1.3×10^5 and 1.3×10^8 CFU fish⁻¹, respectively. In contrast, isolates of *Edwardsiella tarda* injected into Thai pangas *Pangasius sutchi* in the range of 1.18×10^3 to 4.81×10^4 CFU fish⁻¹ resulted in 33% to 100% mortality in 6-10 days (Alam *et al.* 1999). *Edwardsiella tarda* seems to be more virulent against Thai pangas. In another study, *Pseudomonas fluorescens* at the immersion of $2-6 \times 10^6$ CFU ml⁻¹ caused 40-100% mortality in silver barb (Pal *et al.* 1997). Sarker *et al.* (2000) used an immersion dose of 2.5×10^8 CFU ml⁻¹ *Aeromonas sobria* that causes the ulcerative disease in silver barb *B. gonionotus*.

During the present study, immersion in less than 2.5×10^8 of *A. hydrophila* caused less than 25% mortality in hybrid lemon fin barb fry. Farhana (2014) observed 75% mortality among *Labeo rohita* injected with β -haemolytic *A. hydrophila* N₁₀P after 30 days. A much lower LC₅₀ of 1.5×10^6 CFU ml⁻¹ has been reported for Nile tilapia *Oreochromis niloticus* exposed to *A. hydrophila* by immersion but in a longer 96 h exposure duration (Yambot 1998). Deng *et al.* (2013) reported an LC₅₀ of 10^6 CFU fish⁻¹ for rainbow trout against *A. hydrophila* by injection. Likewise, Shen *et al.* (2001) used three lower sub-lethal concentrations

(2.13×10^6 , 2.84×10^6 and 6.12×10^6 CFU fish⁻¹) for three isolates of *A. hydrophila* for rice field eel *Monopterus albus* in an antibiotic sensitivity test by the injection. Yildiz & Aydin (2006) reported that the LC₅₀ of *Arcobacter cryaerophilus* in trout *Oncorhynchus mykiss* is 2.25×10^4 cells fish⁻¹ by intramuscular injection while Wang et al. (2015) found a much higher LC₅₀ value of *A. hydrophila* for *Cyprinus carpio* at 4×10^7 CFU ml⁻¹ using intraperitoneal injection. Intramuscular injection of atypical *Aeromonas salmonicida* of $<10^2$ CFU fish⁻¹ has been reported as LC₅₀ for marbled sole *Pleuronectes yokohamae*, spotted halibut *Verasper variegates* and Japanese flounder *Paralichthys olivaceus* (Kumagai et al. 2006). The higher LC₅₀ values for carps could be due to higher disease resistance in carps than in other fish species. On the contrary, *H. fossilis* has a much higher tolerance with 60% mortality after seven days and 100% mortality after five days when challenged with *A. hydrophila* injected at 3.2×10^7 and 3.2×10^8 CFU fish⁻¹, respectively (Mostafa et al. 2008).

Gautam et al. (1992) documented that *A. hydrophila* and *A. sobria* are highly pathogenic compared to others in the *Aeromonas* group in freshwater aquaculture systems. The LC₅₀ value of *A. hydrophila* by immersion for hybrid lemon fin barb early fry recorded during the present study seemed to be higher than other cyprinids. Nevertheless, the value was in agreement with LC₅₀ of *A. hydrophila* for other species such as giant freshwater fish *Arapaima gigas* i.e. 1.8×10^8 CFU ml⁻¹ (Dias et al. 2016) and *Pangasius bocourti* i.e. 2.24×10^8 CFU ml⁻¹ (Doan et al. 2013) and snakehead fish *Channa striata* i.e. 4.1×10^8 CFU ml⁻¹ (Samayanpaulraj et al. 2019). Several bacterial challenge studies on carps against *A. hydrophila* also show high LC₅₀ values. Alsaphar and Al-faragi (2012) reported an LC₅₀ value of 1.37×10^8 CFU fish⁻¹ of *A. hydrophila* by intramuscular injection for common carp *Cyprinus carpio* while Farhana (2014) reported an LC₅₀ value of 2.37×10^8 CFU fish⁻¹ by injection of β - haemolytic *A. hydrophila* N10P for *Labeo rohita*. In contrast, Chen et al. (2018) found that *A. veronii* has a lower LC₅₀ of 3.6×10^4 CFU ml⁻¹ over 96 h in grass carp *Ctenopharyngodon idella*.

Itano et al. (2006) observed that the LC₅₀ of *Nocardia seriolae* for yellowtail *Serbia quinqueradiata* was 1.9×10^2 CFU ml⁻¹ by intraperitoneal injection, 1.7×10^7 CFU ml⁻¹ by oral

administration, 4.3×10^6 CFU ml⁻¹ by intradermal injection, and 1.5×10^4 CFU ml⁻¹ by immersion. Meanwhile, Mekuchi et al. (1995) reported LC₅₀ of *Edwardsiella tarda* (*E. tarda*) for Japanese flounder was 1.7×10^2 CFU fish⁻¹ by intraperitoneal injection, 1.3×10^6 CFU fish⁻¹ by oral administration, 7.1×10^1 CFU fish⁻¹ by intramuscular injection, and 3.6×10^6 CFU ml⁻¹ by immersion. Mammur Rashid et al. (1996) made a similar report of *E. tarda* against Japanese flounder but with higher LC₅₀ values i.e. 4.8×10^4 CFU fish⁻¹ by intraperitoneal injection, 2.2×10^6 CFU fish⁻¹ by oral administration and 1.7×10^7 CFU ml⁻¹ by immersion. In contrast, Mostafa et al. (2008) observed a higher LC₅₀ (9.6×10^6 CFU fish⁻¹) of *A. hydrophila* against *H. fossilis* by intraperitoneal injection. However, the immersed LC₅₀ dose is higher than the injected dose in fish while the LC₅₀ immersed dose for hybrid lemon fin barb was much higher than those of other fish. This LC₅₀ level could be used as a challenge test benchmark for nutritional, antibiotic and other studies for this new hybrid fish against *A. hydrophila*.

CONCLUSION

The sub-lethal concentration of *A. hydrophila* by immersion for hybrid lemon fin barb early fry became effective when it exceeded 10^7 CFU ml⁻¹. Its LC₅₀ level was found at 6.06×10^8 CFU ml⁻¹.

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Conflict of Interest

We declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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