Original article

Efficacy of bi-valent whole cell inactivated bacterial vaccine against Motile Aeromonas Septicemia (MAS) in cultured catfishes (*Heteropneustes fossilis, Clarias batrachus* and *pangasius pangasius*) in Bangladesh

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ABSTRACT

The Motile Aeromonas Septicemia (MAS) is an important disease of cultured catfishes (*Heteropneustes fossilis, Clarias batrachus* and *Pangasius pangasius*), caused by different species of Aeromonas bacteria which have been documented to be higher death rates ($\leq$70%) in Bangladesh since 2016. Present study was conducted to develop bi-valent vaccine using *A. hydrophila* and *A. veronii*, and to validate their efficacy via intra-muscular (IM) and oral-routes of immunization in selected species of fishes. Brood fishes of the three species were immunized with three doses of inactivated vaccine (10^7 CFU /2.3 mg/ml). Hematological parameters of brood fishes and antibody levels (IgM) of broods, their larvae and eggs were determined by ELISA. Additionally, Relative Percent Survivability (RPS) and the IgM levels of the larvae after challenge with virulent *A. hydrophila* and *A. veronii* were also evaluated. Findings of this study showed that the lymphocytes, monocytes, granulocytes counts and antibody (IgM) titre of brood fishes, larvae and eggs from the vaccinated fishes were found significantly higher ($p< 0.05$) compared to the unvaccinated control groups. Alternatively, antibody levels (IgM) in the larvae of vaccinated group of brood fishes fed with antigen coated feed was exhibited to be remarkably higher ($p< 0.05$) than the antigen non-fed group. The RPS of larvae of Shing (91.24 ± 2.00%), Magur (88.09 ± 2.88%) and Pangas (93.17 ± 1.52%) was found to be higher in the larvae at 20-day age of vaccinated group compared to non-vaccinated brood fishes group. Findings of this study indicated that the active immunization of brood fishes followed by oral immunization of their larvae feeding with antigen coated feed showed synergistic effect in protecting cultured Shing, Magur and Pangas fishes from frequent attack with *Aeromonas* spp at any age of their lifetime.

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1. Introduction

Bangladesh has been cultivating stinging and walking catfish since 2004 (Sultana et al., 2005). However, Pangas fish was first cultivated in Bangladesh in 1989 that was imported from Thailand. The first successful induced breeding of this species was achieved at BAU and BFRI in 1991 (Hossain, et al., 2018). All these three catfish species are recognized as highly valued local catfishes in Bangladesh. The culture and rearing of these catfishes have a vital role to the income generation activities of the home-state fish farmers (Sultana et al., 2005). In the fiscal year (FY) 2017–18, total species-wise annual production estimated as Shing and Magur, and Pangas catfish fishes were 84,282 tons (2.33%) and 453,383 tons (12.52%), respectively in Bangladesh (DoF, 2018). These catfishes are teleost having non-keratinized stratified squamous epithelial cells which cover their whole-body surfaces, fins and barbells (Zhao et al., 2008; Esteban, 2012). Usually, skin of fish serves a vital function in maintaining homeostatic conditions in the environment, even protection from the outside environment, communication, sense perception, osmoregulation, discharge of body waste, temperature regulation including antimicrobial activities (Elliott and Shotts, 1980; Bordas et al., 1996; Esteban, 2012). In aquatic condition, it assists as the first attachment point for a variety of microbes (Bordas et al., 1996; Esteban, 2012). Therefore, microorganism attachment to the skin frequently causes sores and ruptures, allowing infections to penetrate and multiply within the body. Thus, skin sores and ruptures have a negative effect on normal growth and production, in addition to mass mortality of the catfishes.

The bacteria under the genus *Aeromonas* are abundantly distributed in aquatic environments (Palumbo et al., 1992), and have the ability to producing diseases viz., Motile Aeromonas Septicemia (MAS) and Epizootic Ulcerative Syndrome (EUS) in various freshwater fish species (Shao et al., 2004). Mainly, *A. hydrophila* and *A. veronii* can cause disease in wide-ranging animal species, including mammals, and act as a common infection source in warm water fish cultivation throughout the world (Austin and Adams, 1996; Thune et al., 1993; Yu et al., 2004).

Aeromonas spp were frequently captured as a causal agent of a variety of fish species in farming and wild freshwater in Bangladesh (Rahman and Chowdhury, 1996; Sarker et al., 2000). This species was documented as the cause of infection of ulcer-like disease of diverse farmed fishes, such as, pathogenic *A. veronii* and *A. hydrophila* bacteria were isolated and identified in the EUS-affected Shing (*H. fossilis*). Given the situation, the present study was tailored as prevention and control options for the MAS in cultured catfishes through development of an effective bi-valent vaccine using local isolates (*A. hydrophila* and *A. veronii*). Further, the study evaluated the serum antibody (IgM) levels of brood stinging and walking catfishes and Pangas fish, including their eggs and larvae and thereby, validated the efficacy of the vaccine. In brood catfishes, haematological parameters were also examined. The efficacy of the bi-valent vaccine was further monitored in the larvae with a particular emphasis on RPS. The study aimed to evaluate the potency of bi-vaccine through active immunization via IM route in brood catfishes followed by active immunization to their larvae through oral feeding of feed-based bi-valent vaccine.

2. Materials and methods

2.1. Isolation and identification of bacteria

The samples collected from liver, kidney and skin of dead and moribund cultured stinging catfish (*H. fossilis*), walking catfish (*C. batrachus*) and Pangas (*P. pangasius*) catfish from the outbreak year spanning from 2016 to 2018 at different geographical locations of Bangladesh for isolation and identification of *A. veronii and A. hydrophila*. These were confirmed via both basic to advance assays which include evaluation of morphological and biochemical characteristics, and confirmed by using API 20NE and API 20E biochemical identification strips (bioMérieux, Marcy l'Etoile, France) as per method used by Rahman et al. (2002), and finally, conducted the PCR assay (Hayati et al., 2015; Kingombe et al., 1999).

2.2. Preparation of bi-valent bacterial vaccine with the isolated bacterial spp. From catfishes

In this study, *A. hydrophila* and *A. veronii* bacteria were used for development of vaccine which isolated from infected cultured Shing, Magur and Pangas fishes. The bacteria were stored in Brain Heart Infusion Broth (BHIB) containing 50% buffered glycerol at -80ºC. Later, *A. hydrophila* and *A. veronii* bacterial isolates were cultured onto Trypticase Soy Agar (TSA, HiMedia, India) at 37 °C for 24–48 h in the process of bi-valent vaccine production (Nawaz et al., 2010). Subsequently, the cultured bacteria were purified and confirmed via different biochemical assays in accordance to the Bergey's Manual of Determinative Bacteriology (Holt, 1977). A large propagation of the bacterial isolates of *A. hydrophila* and *A. veronii* were carried out into the Brain Heart Infusion Broth (BHIB) for preparation of the bi-valent vaccine, and further incubated at 37 °C for 48 h. The Total Plate Count (TPC) method was applied for estimation of bacterial concentration by using Plate Count Agar (Oxoid, UK) medium described by Townsend and Naqui, (1998). The final concentration of *A. hydrophila* and *A. veronii* bacteria were counted separately as 10^{12} CFU/ml and maintain final volume of 500 ml. Each of the propagated bacteria was then inactivated by adding around 2.5% of 37% formalin for 72 hours at 20°C and left at 4°C for overnight. The pellet of the concentrated bacterial cells was obtained after centrifugation of the suspension for 30 min at 8000 rpm. The bacterial pellets were rinsed thrice with 1X PBS, and again centrifuged to eliminate remaining formalin residue in the suspension, before an antibiotic (Ceftriaxone 10 mg/ml) was added. Finally, PBS was added with the pellet to obtain a 2.3 mg/ml concentration. Inactivation of the *A. hydrophila* and *A. veronii* bacteria were done by inoculating the bacterial suspension into BHIA medium and incubated at 37 °C, and confirmed that bacteria had been destroyed properly and had no contamination. A non-mineral oil adjuvant (MONTANIDETM IMS-1312, SEPPIC, France) was used to improve vaccine antigenicity and fluid stability as per the method described by Stone (1993). The adjuvant was mixed with an inactivated antigen at a ratio 1:1 for 10–20 min to obtain the expected safety and efficacy balance. The inactivated bi-valent bacterial suspension for each dose of vaccine was reached to a final concentration of protein that is equivalent to 1×10^7 CFU/ml was measured OD value at 260 nm and 280 nm by UV-Spectrophotometer as per method stated by Layne (1957).

2.3. Experimental design for active immunization of healthy brood catfishes

Healthy brood Shing (185±5g), Magur (245±5g) fishes were obtained from local fish farm Ma Motsho Fisheries and Hatchery, Gouripur, Mymensingh and Pangas (1530±5g) fishes obtained from Bangladesh Fisheries Research Institute (BFRI), Mymensingh 2201, Bangladesh where they maintain biosecurity of their farms strictly. The fishes were acclimatized under laboratory conditions for 14 days before the vaccination trials and 20 each fish was randomly screened for the selective bacteria. After confirmation of the absence of selective bacteria (*A. hydrophila* and *A. veronii*) the fishes were selected for vaccination. In this trial, a total of twenty brood Shing, Magur and Pangas fishes from each species were taken and

divided into two groups, namely, vaccinated and control that included seven females (♀=7) and three males (♂=3) for each group. For each experimental group, ten fishes were placed into one of four glass aquaria for both Shing and Magur fish. However, two separate cisterns (20ft × 5ft × 4ft) were used for Pangas fish. Moreover, three treatment groups were assigned for the larvae under this experiment. The larvae were obtained from immunized brood fish followed by feeding a feed-based bi-valent vaccine (T1), the larvae from immunized brood fish with non-fed vaccine group (T2) and other larval group that was obtained from the non-immunized control group (T3). The brood Shing, Magur and Pangas fishes were immunized three times with 0.3 ml of bi-valent vaccine (1×10^7 CFU/ml) by IM route on day 0, 14 and 28, however, un-vaccinated brood fishes were kept as control. The brood fishes were kept under the average water temperature of 26.244 ± 0.301 °C, dissolved oxygen: 5.311 ± 1.33 mg/L, pH: 7.74 ± 0.109 and ammonia level 0.211 ± 0.084 mg/L during whole the experimental period as per standard protocol (Shoko et al., 2014). The brood catfishes were fed with commercial feed (floating fish feed, CP Bangladesh Co. Ltd, Bangladesh) considering 5% of their body weight, two-times in a day preferably morning (8 am) and evening (3 pm) throughout the study period.

2.4. Collection of blood samples from the control and vaccinated brood Shing, Magur and Pangas fishes for serum and haematological analysis

The blood samples were collected separately from each group at 7, 21 and 35 days of post-immunization (dpi). The brood fishes were anesthetized with clove oil (0.5 ml/L) and subjected to collect blood sample directly from the heart of each fish group by injecting a 21-gauge needle attached with a 3 ml syringe. Two distinct types of tubes, viz., one contained 3% EDTA (Ethylene Diamine Tetra Acetic Acid) and other without EDTA were used for blood sample collection. Subsequently, blood samples without EDTA-containing tubes were taken for the evaluation of serum antibody (IgM) levels, however, EDTA-containing samples were dispatched to the designated laboratory immediately to examine haematological parameters. The blood samples were centrifuged for 10 min at 1000 rpm and thus the serum samples were obtained, later taken in eppendorf tubes marked with unique identification number, and preserved at −20 °C for determination of antibody (IgM) as per standard method described by Sukenda et al. (2017). The antibody (IgM) titre was estimated using the ELISA labelled by Nurani et al. (2020). The tubes containing EDTA-blood samples were used for haematological parameter evaluations which included total leukocytes, lymphocytes, monocytes and granulocyte counts by staining the blood smear with modified Wright's method.

2.5. Preparation of larval feed with bacterial antigen

Inclusion of ingredients such as 9961s-0.8 mm commercial floating pellet fish feed (CP Bangladesh Co. Ltd) skimmed milk and fresh egg yolk for feeding the larva were accomplished in this study. The floating fish feed (50% w/w), skimmed milk (25% w/w) and fresh egg yolk (25% w/w) were mixed properly with killed bacterial cells (10^{12} cells/ml/g of feed) as per to the procedure depicted by Ismail et al., (2017). The commercially available feed binder gel (1% Nutrigel; SK + F Bangladesh Ltd.) was used to coat the antigen with the feed thoroughly under an air stream at ambient temperature for 10–15 min.

2.6. Collection of fertilized eggs and fish larvae from brood catfishes

The gonads were collected from male fish of Magur, and used for conjoining the sperm with the eggs collected from female fish, further mixed in a separate container artificially, and allow until fertilization (30–45 min). Likewise, the eggs from female pangas fish and the sperm from adult male were collected independently, then artificially mixed and kept in a bowl for fertilization (30–45 min) of the eggs. In contrast, both brood female and male Shing fishes were allowed to live together for 2–3 days after hormone (S-GnRHa, Ovaprim® 0.5 ml/kg per kg of fish) injection in a cistern (Hafeez-ur-Rehman et al., 2015). Usually, the male Shing released their sperm on the eggs of female before fertilization. The fry from fertile eggs of Shing hatched out distinct to the eggs of Magur and Pangas. The eggs of brood Shing and Magur fishes were collected from Ma Motsho Fisheries and Hatchery, Gouripur, Mymensingh. However, the Freshwater Station of Bangladesh Fisheries Research Institute (BFRI), Mymensingh 2201, Bangladesh, supplied the fertile eggs of brood Pangas fishes. The fertile eggs of Shing, Magur and Pangas were incubated in different cisterns at 27 ± 1 °C until hatching out of their fry. The larvae of Shing (n = 400), Magur (n = 400) and Pangas (n = 400) fishes were stocked in 120L aquarium, separately. Each aquarium was equipped with an individual recirculation filter arrangement at 27 ± 1 °C heating system before conducting this study.

2.7. Immunization of catfish larvae through feeding of antigen coated feed

The larvae of the T1 group derived from Shing, Magur and Pangas fishes were fed with coated feed-based bi-valent vaccine from day 4 to 20 targeting to develop immunity against A. *hydrophila* and A. *veronii* bacterial antigen. However, the larvae of the T2 and T3 groups those were obtained from immunized and non-immunized brood of Shing, Magur and Pangas fishes and fed the floating feed without bacterial antigen. The larvae of all treatment groups (T1, T2, and T3) were fed considering 25% of their body weight four to five times daily until they reached at the age of 20 days.

2.8. Hatched fish larvae were subjected to a challenge test after being fed with antigen-coated and uncoated feed

An immersion challenge test was carried out on larvae obtained from brood Shing, Magur and Pangas fishes for determining the efficacy of the bi-valent vaccine which included: vaccinated three times through the IM route (T1 and T2) and non-vaccinated (T3) control groups. The larvae from all three treatment groups (T1, T2 and T3) were immerged into each A. *hydrophila* and A. *veronii* at a dilution of 10^7 CFU/ml for 30 min after fed with/without antigen-coated feed. The challenge test of larvae was done at day 5, 10, 15 and 20 after hatching. The A. *hydrophila* and A. *veronii* were subjected to re-isolate and confirmed from dead of the larvae. The status of cumulative mortality from treatment groups (immunized and non-immunized fish of each group) was evaluated during a period of 14-days. The efficacy of the vaccine was established by calculating the Relative Percent Survival (RPS) according to the standard procedure (Amend, 1981).

2.9. Preparation of homogenate with eggs and larvae of immunized brood fishes fed with/without antigen-coated feed

The sampling method of eggs and larvae homogenates was performed according to Hanif et al. (2004). The egg samples were obtained from three groups [(three times vaccinated (T1 and T2) and un-vaccinated (T3) control groups] of brood fishes. After hatching, the larvae from brood fishes fed with (T1)/without (T2 and T3) antigen-coated feed were collected at day 5, 10, 15 and 20. Sample of eggs and larvae was homogenized in PBS-T (PBS + 0.05% Tween-20) solution with a proportion of 1:4, and the mixture was then centrifuged for 10 min at 5000 rpm, and

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the supernatant was collected. An aliquot of all samples was utilized to determine the antibody (IgM) levels. The IgM level of eggs and larvae were measured using the ELISA as described by Nurani et al. (2020).

2.10. Statistical analysis

The statistical calculation was done via the SPSS version 24.0, IBM®Co., USA. The results of three replications were presented as mean and standard errors (M ± SE). The RPS was analyzed statistically by comparing among the trial groups (immunized and non-immunized groups with a challenge test using the same pathogenic bacteria). One-way ANOVA including Duncan's Multiple Range Test (DMRT) employed to assess variation in haematoimmunological parameters and RPS among the groups. The level significance at $p < 0.05$ was considered for every analyses.

3. Results

3.1. Haematological parameters of vaccinated brood Shing, Magur and Pangas fishes

The leukocytes, lymphocytes, monocytes including granulocytes counts were found to be significantly greater ($p<0.05$) via IM route of vaccination (day 0, 14 and 28) in Shing brood fish group than un-vaccinated group (control). The numbers of leukocytes, lymphocytes, monocytes and granulocytes in vaccinated Shing brood fish group were found statistically significant ($p<0.05$) after seven days of each vaccination (day 7, 21, and 35). After post-immunization of 35 days, the leucocytes, monocytes, lymphocytes, and granulocytes counts were found to be significantly higher ($p<0.05$) in vaccinated group than in the un-vaccinated control group. The counts of leucocytes, lymphocytes, monocytes and granulocytes were as $22.172\pm2.27\times10^3$/µl, $21.211\pm1.97\times10^3$/µl, $1.196\pm0.03\times10^3$/µl and $6.428\pm0.27\times10^3$/µl and that were found to be highest values in the Shing brood fish respectively. In this trail, lymphocytes, leucocytes, monocytes, and granulocytes counts were found be significantly higher ($p<0.05$) in vaccinated group at day 35 of post-vaccination compared to the un-vaccinated group in trail brood Shing fish (Table 1). The leukocytes, lymphocytes, monocytes including granulocytes counts were found to be significantly greater ($p<0.05$) via IM route of vaccination (day 0, 14 and 28) in brood Magur fish group than un-vaccinated group (control). The numbers of leukocytes, lymphocytes, monocytes and granulocytes in vaccinated brood Magur fish group were found statistically significant ($p<0.05$) after seven days of each vaccination (day 7, 21, and 35). After post-immunization of 35 days, the leucocytes, monocytes, lymphocytes, and granulocytes counts were found to be significantly higher ($p<0.05$) in vaccinated group than in the un-vaccinated control group. The counts of leucocytes, lymphocytes, monocytes and granulocytes were as $16.751\pm0.79\times10^3$/µl, $15.322\pm0.98\times10^3$/µl, $0.378\pm0.01\times10^3$/µl and $6.788\pm0.14\times10^3$/µl

which were found to be highest values in the brood Magur fish, respectively. In this trail, lymphocytes, leucocytes, monocytes, and granulocytes counts were found be significantly higher ($p<0.05$) in vaccinated group at day 35 of post-vaccination compared to the un-vaccinated group in trail brood Magur fish (Table 2). The leukocytes, lymphocytes, monocytes including granulocytes counts were found to be significantly greater ($p<0.05$) via IM route of vaccination (day 0, 14 and 28) in brood Pangas fish group than un-vaccinated group (control). The numbers of leukocytes, lymphocytes, monocytes and granulocytes in vaccinated brood Pangas fish group were found statistically significant ($p<0.05$) after seven days of each vaccination (day 7, 21, and 35). After post-immunization of 35 days, the leucocytes, monocytes, lymphocytes, and granulocytes counts were found to be significantly higher ($p<0.05$) in vaccinated groups than in the un-vaccinated control groups. The counts of leucocytes, lymphocytes, monocytes and granulocytes were as $109.468\pm5.95\times10^3$/µl, $82.384\pm4.18\times10^3$/µl, $1.196\pm0.03\times10^3$/µl and $26.271\pm0.40\times10^3$/µl which were found to be highest values in the brood Pangas fish, respectively. In this trail, lymphocytes, leucocytes, monocytes, and granulocytes counts were found be significantly higher ($p<0.05$) in vaccinated groups at day 35 of post-vaccination compared to the un-vaccinated group in trail brood Pangas fish (Table 3).

3.2. Serum antibody (IgM) level of brood Shing, Magur and Pangas fishes vaccinated with A. Hydrophila and A. Veronii antigens through IM route of immunization

Antibody titer of the immunized (A. hydrophila and A. veronii antigens) and non-immunized brood Shing, Magur and Pangas brood fish's serum were estimated by using the indirect ELISA. The vaccinated brood Shing fish group presented significantly higher ($p<0.05$) antibody levels compared to the control group. In the vaccinated brood Shing fish the antibody level estimated 0.095±0.004, 0.186±0.003 and 0.215±0.019 at day 7, 21 and 35, respectively. On day 35 of post-vaccination, the brood Shing fish that was vaccinated together with A. hydrophila and A. veronii antigen presented considerably higher ($p<0.05$) serum IgM level (0.215±0.019) than in the unvaccinated control group. In this study, it was confirmed that the serum antibody (IgM) levels of brood Shing fish immunized with bi-valent vaccine via IM route were noticed to be significantly higher ($p<0.05$) compare to the un-vaccinated control groups, and showed gradual acceleration of IgM levels in every 7 days after each immunization (Fig. 1). The vaccinated brood Magur fish group presented significantly higher ($p<0.05$) antibody levels compared to the control group. In the vaccinated brood Magur fish the antibody level documented 0.088±0.012, 0.184±0.003 and 0.211±0.013 at day 7, 21 and 35, respectively. On day 35 of post-vaccination, the brood Magur fish that was vaccinated together with A. hydrophila and A. veronii antigen presented significantly higher ($p<0.05$) serum IgM level

Table 1
Haematological parameters (mean ± SD) of Shing fish at every 7 days after first and second vaccination and at day 35th of third vaccination with bi-valent (A. hydrophila and A. veronii) vaccine.

Days	Groups	Leukocytes (10^3/µl)	Lymphocytes (10^3/µl)	Monocytes (10^3/µl)	Granulocytes (10^3/µl)
Day 7	Vaccinated Shing	16.128 ± 1.60[a]	14.998 ± 0.77[a]	0.961 ± 0.02[a]	5.358 ± 0.19[a]
	Control	11.252 ± 1.44[b]	8.936 ± 0.97[b]	0.614 ± 0.08[b]	3.022 ± 0.12[b]
Day 21	Vaccinated Shing	18.366 ± 1.89[a]	17.844 ± 1.51[a]	1.138 ± 0.01[a]	6.292 ± 0.55[a]
	Control	11.594 ± 1.69[b]	8.856 ± 1.02[b]	0.628 ± 0.08[b]	3.131 ± 0.23[b]
Day 35	Vaccinated Shing	22.172 ± 2.27[a]	21.211 ± 1.97[a]	1.196 ± 0.03[a]	6.428 ± 0.27[a]
	Control	12.422 ± 0.88[b]	8.741 ± 1.02[b]	0.651 ± 0.04[b]	3.048 ± 0.17[b]

[a,b]Means within the same column shows a significantly different effect ($p < 0.05$).

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Table 2

Haematological parameters (mean ± SD) of Magur fish at every 7 days after first and second vaccination and at day 35th of third vaccination with bi-valent (A. hydrophila and A. veronii) vaccine.

| Days	Groups	Leukocytes ($10^3/\mu l$)	Lymphocytes ($10^3/\mu l$)	Monocytes ($10^3/\mu l$)	Granulocytes ($10^3/\mu l$)
Day 7	Vaccinated Magur	7.562 ± 0.27[a]	6.821 ± 0.23[a]	0.361 ± 0.01[a]	5.298 ± 0.31[a]
	Control	6.491 ± 0.28[b]	5.672 ± 0.29[b]	0.264 ± 0.04[b]	3.002 ± 0.16[b]
Day 21	Vaccinated Magur	11.522 ± 0.89[a]	10.674 ± 0.61[a]	0.402 ± 0.05[a]	6.292 ± 0.55[a]
	Control	6.424 ± 0.23[b]	5.584 ± 0.22[b]	0.268 ± 0.05[b]	3.131 ± 0.23[b]
Day 35	Vaccinated Magur	16.751 ± 0.79[a]	15.322 ± 0.98[a]	0.378 ± 0.01[a]	6.788 ± 0.14[a]
	Control	6.622 ± 0.33[b]	5.714 ± 0.17[b]	0.252 ± 0.01[b]	3.218 ± 0.48[b]

[a,b]Means within the same column shows a significantly different effect ($p < 0.05$).

Table 3

Haematological parameters (mean ± SD) of Pangas fish at every 7 days after first and second vaccination and at day 35th of third vaccination with bi-valent (A. hydrophila and A. veronii) vaccine.

Days	Groups	Leukocytes ($10^3/\mu l$)	Lymphocytes ($10^3/\mu l$)	Monocytes ($10^3/\mu l$)	Granulocytes ($10^3/\mu l$)
Day 7	Vaccinated Pangas	89.162 ± 3.28[a]	66.472 ± 5.24[a]	0.741 ± 0.13[a]	18.498 ± 2.18[a]
	Control	77.581 ± 3.80[b]	35.166 ± 0.61[b]	0.402 ± 0.09[b]	13.022 ± 0.12[b]
Day 21	Vaccinated Pangas	95.122 ± 2.69[a]	73.472 ± 5.31[a]	0.762 ± 0.12[a]	20.092 ± 4.15[a]
	Control	78.711 ± 3.45[b]	36.322 ± 1.07[b]	0.498 ± 0.05[b]	14.131 ± 1.34[b]
Day 35	Vaccinated Pangas	109.468 ± 5.95[a]	82.384 ± 4.18[a]	0.812 ± 0.03[a]	26.271 ± 0.40[a]
	Control	75.304 ± 5.14[b]	35.754 ± 3.71[b]	0.432 ± 0.06[b]	13.218 ± 0.48[b]

[a,b]Means within the same column shows a significantly different effect ($p < 0.05$).
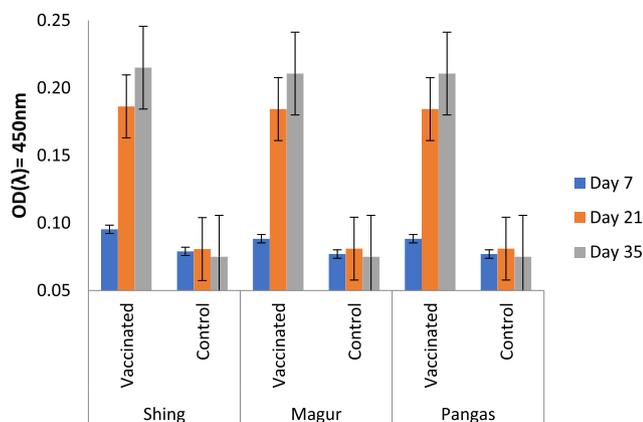
Fig. 1. Level of serum antibody (IgM) in immunized brood Shing, Magur and Pangas fishes at 7, 21 and 35 days of post vaccination.

(0.211±0.013) than in the unvaccinated control group. In this study, it was confirmed that the serum antibody (IgM) levels of brood Magur fish immunized with bi-valent vaccine via IM route were noticed to be significantly higher ($p<0.05$) compare to the un-vaccinated control groups, and showed gradual speeding up of IgM levels in every 7 days after each immunization (Fig. 1). The vaccinated brood Pangas fish group presented significantly higher ($p<0.05$) antibody levels compared to the control group. In the vaccinated brood Pangas fish, the antibody level documented 0.089 ± 0.01, 0.185 ± 0.002 and 0.212±0.014 at day 7, 21 and 35, respectively. On day 35 of post-vaccination, the brood Pangas fish that was vaccinated together with A. hydrophila and A. veronii antigen presented significantly higher ($p<0.05$) serum IgM level (0.212± 0.014) than in the unvaccinated control group. In this study, it was confirmed that the serum antibody (IgM) levels of brood Pangas fish immunized with bi-valent vaccine via IM route were noticed to be significantly higher ($p<0.05$) compare to the un-vaccinated control groups, and showed gradual acceleration of IgM levels in every 7 days after each immunization (Fig. 1).

3.3. Antibody (IgM) titer of eggs and larvae homogenates after feeding with/without antigen coated feed

The antibody (IgM) level of egg homogenates from brood Shing fish group that immunized three times with bi-valent A. hydrophila and A. veronii antigen was shown to be remarkably higher ($p<0.05$) than the IgM level of eggs homogenate from un-vaccinated control brood Shing fish. The IgM antibody levels of egg homogenates, larval aged day 5, 10, 15 and 20 were documented as 0.208±0.004, 0.21±0.002; 0.217±0.005, 0.207±0.005; 0.219±0.002, 0.191±0.005; 0.223±0.005, 0.19±0.004 and 0.247±0.006, 0.186±0.003 in treatment T1 and T2 Shing groups, respectively. The antibody (IgM) level of the larvae homogenates of the T1 and T2 treatment group at the age of days 5, 10, 15, and 20 hatching from brood Shing was established to be expressively different ($p<0.05$) from the control groups (T3). The outcomes of this study showed that antibody (IgM) levels of larvae from brood Shing fish which was immunized three times and not fed with antigen-coated feed, titre decreased slowly as the larval age progressed from day 5-20, whereas, the IgM level of larvae from immunized group and fed with antigen-
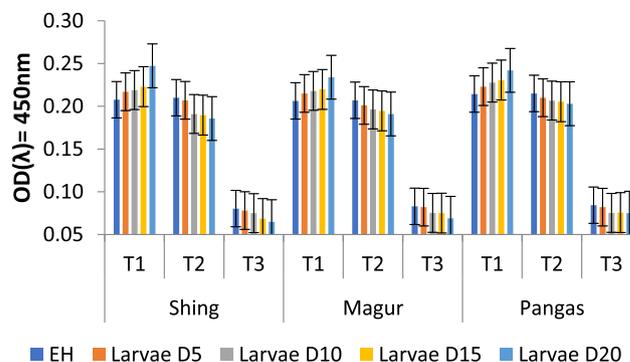
Fig. 2. RPS of larvae produced from the immunized brood Shing, Magur and Pangas fishes and control group after challenged with A. hydrophila and A. veronii at larvae aged 5, 10, 15 and 20 days of hatch. T1, Larvae from immunized brood fish followed by fed with feed-based bi-valent vaccine; T2, Larvae from immunized brood fish with non-fed vaccine.

coated feed, increased gradually. The highest antibody level in the larvae obtained 0.247±0.006 from vaccinated brood Shing fish against bi-valent *A. hydrophila* and *A. veronii* (Fig. 2). The antibody (IgM) level of egg homogenates from brood Magur fish group that immunized three times with bi-valent *A. hydrophila* and *A. veronii* antigen was shown to be remarkably higher ($p<0.05$) than the IgM level of eggs homogenate from un-vaccinated control brood Magur fish. The IgM antibody levels of egg homogenates, larval aged day 5, 10, 15 and 20 were documented as 0.206±0.004, 0.207±0.005; 0.215±0.003, 0.201±0.003; 0.218±0.003, 0.196±0.002; 0.22±0.010, 0.195±0.001 and 0.234±0.029, 0.191±0.005 in treatment T1 and T2 Magur groups, respectively. The antibody (IgM) level of the larvae homogenates of the T1 and T2 treatment group at the age of days 5, 10, 15, and 20 hatching from brood Magur was established to be significant different ($p<0.05$) from the control groups (T3). The outcomes of this study showed that antibody (IgM) levels of larvae from brood Magur fish that was immunized three times and not fed with antigen-coated feed, titre decreased slowly as the larval age progressed from day 5-20, whereas, the IgM level of larvae from immunized group and fed with antigen-coated feed, increased gradually. The highest antibody level in the larvae obtained 0.234±0.029 from vaccinated brood Magur fish against bi-valent *A. hydrophila* and *A. veronii* (Fig. 2). The antibody (IgM) level of egg homogenates from brood Pangas fish group that immunized three times with bi-valent *A. hydrophila* and *A. veronii* antigen was shown to be remarkably higher ($p<0.05$) than the IgM level of eggs homogenate from un-vaccinated control brood Pangas fish. The IgM antibody levels of egg homogenates, larval aged day 5, 10, 15 and 20 were documented as 0.214 ± 0.004, 0.215 ± 0.005; 0.223 ± 0.003, 0.21 ± 0.002; 0.228 ± 0.002, 0.207 ± 0.002; 0.231 ± 0.012, 0.205 ± 0.002 and 0.242 ± 0.029, 0.203 ± 0.002 in treatment T1 and T2 Pangas groups, respectively. The antibody (IgM) level of the larvae homogenates of the T1 and T2 treatment group at the age of days 5, 10, 15, and 20 hatching from brood Pangas was established to be significant different ($p<0.05$) from the control groups (T3). The outcomes of this study showed that antibody (IgM) levels of larvae from brood Pangas fish that was immunized three times and not fed with antigen-coated feed, titre decreased slowly as the larval age progressed from day 5-20, whereas, the IgM level of larvae from immunized group and fed with antigen-coated feed, increased gradually. The highest antibody level in the larvae obtained 0.242±0.029 from vaccinated brood Pangas fish against bi-valent *A. hydrophila* and *A. veronii* (Fig. 2).

3.4. Results of RPS in the challenge test in larvae fed with or without antigen coated feed

The RPS was determined at the larval age of days 5, 10, 15 and 20. The larvae derived from vaccinated brood fishes, the RPS were found in Shing fish was 91.24±2%, at the age of 20 days old larvae feeding with antigen coated feed (T1). The larvae obtained from brood Shing fish group found 66.78±1.52%, 64.62±0.57%; 69±2.64%, 52.76±3.21%; 84.21±2%, 47.71±2.51% and 91.24±2%, 46.35±1% in treatment T1 and T2 Shing groups at the age of days 5-20, respectively. After challenged with *A. hydrophila* and *A. veronii*, the larvae from immunized brood Shing fish fed with antigen-coated feed were found to be significantly distinct ($p<0.05$) than the larvae obtained from non-fed with antigen coated feed and the control groups. The RPS of larvae derived from the immunized Shing fish group that were not fed with antigen-coated feed was shown to fall gradually from day 5-20, nevertheless, the RPS of larvae from immunized Shing group those were fed with antigen-coated feed was found to be risen gradually when challenged the larvae with the *A. hydrophila* and *A. veronii* bacteria (Fig. 3). The RPS was determined at the larval age of days 5, 10, 15 and 20.
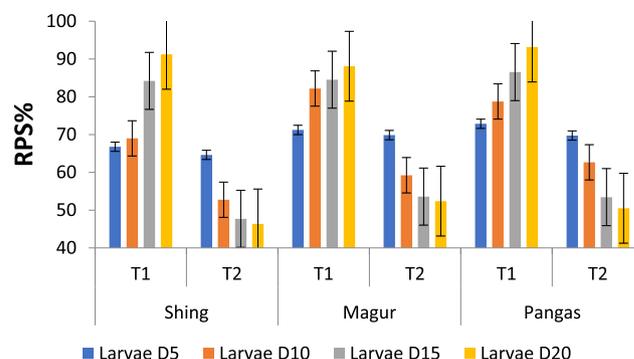

Fig. 3. Antibody (IgM) level of eggs and larvae homogenates at age 5, 10, 15 and 20 days from three times immunized and non-immunized brood fishes. T1, Larvae from immunized brood fish followed by fed with feed-based bi-valent vaccine; T2, Larvae from immunized brood fish with non-fed vaccine and T3, larval group from the non-immunized group (control).

The larvae derived from vaccinated brood fishes, the RPS were found in Magur fish was 88.09±2.88%, at the age of 20 days old larvae feeding with antigen coated feed (T1). The larvae obtained from brood Magur fish group found 71.23±1%, 69.86±1.15%; 82.19±1.52%, 59.24±0.57%; 84.53±2%, 53.6±2% and 88.09±2.88%, 52.38±1.52% in treatment T1 and T2 Magur groups at the age of days 5-20, respectively. After challenged with *A. hydrophila* and *A. veronii*, the larvae from immunized brood Magur fish fed with antigen-coated feed were found to be significantly distinct ($p<0.05$) than the larvae obtained from non-fed with antigen coated feed and the control groups. The RPS of larvae derived from the immunized Magur fish group that were not fed with antigen-coated feed was shown to fall gradually from day 5-20, nevertheless, the RPS of larvae from immunized Magur group those were fed with antigen-coated feed was found to be risen gradually when challenged the larvae with the *A. hydrophila* and *A. veronii* bacteria (Fig. 3). The RPS was determined at the larval age of days 5, 10, 15 and 20. The larvae derived from vaccinated brood fishes, the RPS were found in Pangas fish was 93.17±1.52%, at the age of 20 days old larvae feeding with antigen coated feed (T1). The larvae obtained from brood Pangas fish group found 72.85±1.15%, 69.75±1.52%; 78.76±2.081%, 62.67±2.51%; 86.55±2%, 53.44±2% and 93.17±1.52%, 50.51±1.52% in treatment T1 and T2 Pangas groups at the age of days 5-20, respectively. After challenged with *A. hydrophila* and *A. veronii*, the larvae from immunized brood Pangas fish fed with antigen-coated feed were found to be significantly distinct ($p<0.05$) than the larvae obtained from non-fed with antigen coated feed and the control groups. The RPS of larvae derived from the immunized Pangas fish group that were not fed with antigen-coated feed was shown to fall gradually from day 5-20, nevertheless, the RPS of larvae from immunized Pangas group those were fed with antigen-coated feed was found to be risen gradually when challenged the larvae with the *A. hydrophila* and *A. veronii* bacteria (Fig. 3).

4. Discussion

Aeromonas bacteria are widely circulated in aquatic conditions that cause Motile Aeromonad Septicemia (MAS) diseases and clinically manifested by red mouth, swollen abdomen, haemorrhages in external surface and near the anus in various freshwater fish species. MAS is a one of the important causes of mortality in cultured catfishes all over the world since 2016–2017. This disease has emerged as a new risk of culture catfishes in Bangladesh (Nahar et al., 2016). During these years many measurements have

been taken to curb the disease epidemics targeted to reduce fish mortality, however, the efforts were found to be futile. Therefore, a bi-valent vaccine is necessitated to be developed to block the transmission pathways of MAS disease in freshwater cultured catfishes, and support to livelihoods of marginal commercial fish farmers in Bangladesh.

The results of this study showed that the leukocytes, lymphocytes, monocytes and granulocytes counts were found statically significant ($p<0.05$) via IM route of vaccination of Shing, Magur and Pangas brood fish on day 0, 14 and 28 in than un-vaccinated control group of brood fishes. Cells count numbers of leukocytes, lymphocytes, monocytes and granulocytes in vaccinated Shing, Magur and Pangas brood fish group were also found statistically significant ($p<0.05$) after seven and 14 days of each vaccination (day 7, 21, and 35). In this study, total leukocytes such as, lymphocytes, monocytes and granulocytes counts were found to be greater in the immunized brood Shing, Magur and Pangas fish species at day 35. The cell count in blood samples taken from the three groups of immunized brood fishes was also exhibited higher than the un-vaccinated control group. In general, the haematological parameters have been applied to screen the immune responses and health status of fish and other aquatic animals. Additionally, the leukocyte count is one of the most significant haematological indicators of non-specific and specific immune responses for the fish species (Misra et al., 2006). Findings of the present study closely agree with a similar study conducted in Malaysia (Monir et al., 2020). They found that the number of total leucocytes ($45.39\pm1.34\times10^3/\mu l$), lymphocytes ($27.63\pm1.40\times10^3/\mu l$), monocytes ($0.46\pm0.03\times10^3/\mu l$) and granulocytes ($7.19\pm0.23\times10^3/\mu l$) were boosted substantially ($p<0.05$) in the feed-based bi-valent vaccinated groups at day 21 post-vaccination compare to that of control group (un-vaccinated). Findings of the study also agree with the finding of Sukenda et al., (2017a). They found that after vaccination of brood stock their total leukocytes found to be increased, indicating that the vaccine can increase the cellular defense response by raising total leukocytes count in the brood stock. The rise is linked to the role of white blood cells (WBC) as a defense mechanism. Purwanti and Suminto (2014) suggested that an increase in leukocyte concentration has a positive impact on antibody production, resulting in a body resistance response to foreign invaders. The serum antibody (IgM) levels of the immunized groups of brood catfishes with bi-valent (*A. hydrophila* and *A. veronii*) vaccine were found higher than the un-vaccinated control groups. The findings of this study trial were found consistent with similar research conducted in Indonesia (Pasaribu et al., 2018). In their study they confirmed that immunization of brood stock using a bi-valent whole cell inactivated bacterial vaccine provides effective protection against infection when the challenge is made with homologous bacteria. Bastardo et al., (2012) indicated that rainbow trout (*O. mykiss*) demonstrated greater survival rate and immune response after immunization with bi-valent vaccines, not withstanding, better than the monovalent vaccines. Thus, the present study findings further recommend that a bi-valent (*A. hydrophila* and *A. veronii*) bacterial vaccine is capable to generate higher protective potentiality to MAS disease of freshwater cultured catfish. Monir et al., (2021) found that fish vaccinated with a feed-based bi-valent vaccine (*A. hydrophila* and *S. iniae*) had a significant ($p<0.05$) increase in IgM levels in both the serum and the skin mucus when tested for both antigens throughout the study compared to the control group. It had also been demonstrated that oral vaccination resulted in a higher level of IgM antibody elicitation in the vaccinated group of fishes. The larvae collected from three times vaccinated brood Shing, Magur and Pangas fishes fed with antigen coated feed challenged with *A. hydrophila* and *A. veronii* and observed greater rate of viability than the larvae not fed with antigen coated feed and the control groups of fishes. The RPS of larvae

from immunized groups that were not fed with antigen-coated feed was found to decline gradually within day 5-20. However, the RPS of larvae from immunized groups that were fed with antigen-coated feed was found to be increased progressively even at challenge with homologous bacteria. The results of the present study highly agree with the findings of Nurani et al., (2020). They also found that the larvae obtained from vaccinated brood fish showed higher RPS value of > 50% until 20 days after hatching and challenged with homologous bacteria. The RPS of the larvae of 5, 10, 15 and 20 days after hatching of fish larvae ranged from 77-89%, 72-82%, 62-72% and 51-65%, respectively. Pasaribu et al., (2018) also stated that the RPS of 5, 10, 15 and 20 days after hatching larvae ranged from 87%, 85%, 77%, and 68%, respectively. Cao et al., (2017) described that, larvae at age 20 days already have difference in the thymus. Firdausi and Nuryati, (2017) found that the larval protection will be increased after re-immunization of *Oreochromis niloticus* at 20 days. This has also been proven that increased rate of fish survivability by re-immunization of fishes (Chettri et al., 2015; Pretto-Giordano et al., 2010). The cumulative mortality in red hybrid tilapia immunized with bi-valent (*A. hydrophila* and *A. veronii*) vaccine was not more than 17.78% and 22.22% in the groups challenged with *S. iniae* and *A. hydrophila*, respectively, according to Monir et al., (2021). The RPS was found significantly ($p<0.05$) higher in the bi-valent vaccinated group (82.22 ±3.85%) compared to the other groups. The antibody (IgM) levels in egg and larvae homogenates of the treatment groups at the age of days 5, 10, 15, and 20 after hatching from immunized brood Shing, Magur and Pangas were found to be higher than the control groups. The antibody (IgM) level of eggs and larvae homogenates obtained from three times immunized brood fishes those were fed with antigen-coated feed (T1) group was found higher compared to antigen non-fed un-vaccinated (T3 group) and also the only vaccinated (T2) group. Result of the study correlated with the findings of the research of Hanif et al., (2004); Sukenda et al., (2018), Nurani et al., (2020). In their studies, they found that the antibody (IgM) levels of larvae produced from vaccinated brood fishes lowered gradually with the increase in larval age which was not sustainable with the active immunization after hatching. On the contrary, the antibody (IgM) levels increased gradually from day 5-20 with the increase of the larval age which were obtained from the vaccinated brood fishes, and fed with antigen coated feed in this study. The immunization of brood fish with whole cell inactivated bacterial vaccine ensued transfer of maternal immunity, which has been extensively documented, like brood fish immunization particularly with *S. agalactiae* vaccine on Nile Tilapia (Nisaa et al., 2016; Sukenda et al., 2017b).

5. Conclusions

This study established that immunization of brood fishes applying whole cell inactivated bi-valent (*A. hydrophila* and *A. veronii*) bacterial vaccine can able to increase the serum antibody (IgM) levels of Shing, Magur and Pangas brood fishes and their eggs and larvae as well. All the three species of brood fish immune cells which are responsible for antibody (IgM) production were found to start increasing after primary vaccination and reach their maximum after third vaccination. The study also confirmed that the IgM levels were the most effective among the brood fishes that were vaccinated intra-muscularly compared to those of non-vaccinated species of brood fish. The larvae obtained from three time's vaccinated brood fishes and fed with antigen coated feed showed long-time survivability. Findings of this study suggested that the active immunization of brood fishes followed by the active immunization of their larvae through feeding with antigen coated feed had a synergistic effect in defending cultured Shing, Magur

and Pangas fishes at any age of their cultivation from MAS disease caused by *Aeromonas* spp. Therefore, it may be concluded that the reduction of mass mortality of cultured catfishes (Shing, Magur and Pangas) will certainly be reduced and their productivity will be increased, thus, providing a healthy habitat for cultured fish.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Ethical approval

The experiment conducted in this study for the challenge test of a newly developed bi-valent whole cell inactivated bacterial vaccine against Motile Aeromonas Septicemia (MAS) for cultured cat fishes (*Heteropneustes fossilis, Clarias batrachus* and *Pangasius pangasius*) were approved by the Animal Welfare and Experimentation Ethics Committee (AWEEC) of Bangladesh Agricultural University, Mymensingh-2202 [AWEEC/BAU/2020 (2)].

References

Amend, D.E., 1981. Potency testing of fish vaccines. Dev. Biol. Stand. 49, 447–454.

Austin, B., Adams, C., 1996. The genus aeromonas. In: Austib, B., Altiwegg, M., Gosling, P.J., Joseph, S.W. (Eds.), Fish Pathogens. Wiley, Singapore, pp. 197–229.

Bastardo, A., Ravelo, C., Castro, N., Calheiros, J., Romalde, J.L., 2012. Effectiveness of bi-valent vaccines against *Aeromonas hydrophila* and *Lactococcus garvieae* infections in rainbow trout *Oncorhynchus mykiss* (Walbaum). Fish Shellfish Immunol. 32 (5), 756–761.

Bordas, M.A., Balebona, M.C., Zorrilla, I., Borrego, J.J., Morinio, M.A., 1996. Kinetics of adhesion of selected fish-pathogenic *Vibrio* strains to skin mucus of gilt-head sea bream (*Sparus aurata* L.). Appl. Environ. Microbiol. 62, 3650–3654.

Cao, J., Chen, Q., Lu, M., Hu, X., Wang, M., 2017. Histology and ultrastructure of the thymus during development in tilapia, *Oreochromis niloticus*. J. Anat. 230 (5), 720–733.

Chettri, J.K., Jaafar, R.M., Skov, J., Kania, P.W., Dalsgaard, I., Buchmann, K., 2015. Booster immersion vaccination using diluted *Yersinia ruckeri* bacterin confers protection against ERM in rainbow trout. Aquaculture 440, 1–5.

DoF, 2018. Yearbook of Fisheries Statistics of Bangladesh, 2017–18. Fisheries Resources Survey System (FRSS), Department of Fisheries. Bangladesh: Ministry of Fisheries, 2018. 35, 23.

Elliott, D.G., Shotts, E.B., 1980. Aetiology of an ulcerative disease in goldfish *Carassius auratus* (L.): microbiological examination of diseased fish from seven locations. J. Fish Dis. 3 (2), 133–143.

Esteban, M.A., 2012. An overview of the immunological defenses in fish skin. Int. Schol. Res. Network. 2012, 1–29.

Firdausi, A.P., Nuryati, S., 2017. The efficacy of vaccination on tilapia seeds of (*Oreochromis niloticus*) using hyperosmotic infiltration method to prevent *Streptococcus agalactiae* infection. J. Veteriner. 18, 634–641.

Hafeez-ur-Rehman, M., Ashraf, M., Abbas, F., Qureshi, I.A., Mehmood-ul-Hassan, M., Iqbal, K.J., Abbas, S., 2015. Effect of different doses of Ovaprim (sGnRHa+

Domperidone) on the egg fecundity and reproductive hormone levels in Channa marulius. J. Anim. Plant Sci. 25 (6).

Hanif, A., Bakopoulos, V., Dimitriadis, G.J., 2004. Maternal transfer of humoral specific and non-specific immune parameters to sea bream (*Sparus aurata*) larvae. Fish Shellfish Immun. 17 (5), 411–435.

Hayati, H.R., Hassan, M.D., Ong, B.L., Abdelhadi, Y.M., Hidayahanum, H.N., Sharifah, R.M., Faten, A.N., Kuttichantran, S., Alsaid, M., 2015. Virulence genes detection of *Aeromonas hydrophila* originated from diseased freshwater fishes. Adv. Environ. Biol. 9 (22 S3), 22–27.

Holt, J.G., 1977. The Shorter Bergey's Manual of Determinative Bacteriology. CABI Publishing, Oxford shire, UK.

Hossain, M.I., Shikha, F.H., Chakrabarty, T., 2018. Studies on the Culture Condition of Pangus (*Pangasius hypophthalmus*) at Different Farms in Trishal Upazila. J. Environ. Sci. Nat. Resour. 11 (1–2), 97–107.

Ismail, M.S., Syafiq, M.R., Siti-Zahrah, A., Fahmi, S., Shahidan, H., Hanan, Y., Amal, M.N.A., Zamri Saad, M., 2017. The effect of feed-based vaccination on tilapia farm endemic for streptococcosis. Fish Shellfish Immunol. 60, 21–24.

Kingombe, C.I.B., Huys, G., Tonolla, M., Albert, M.J., Swings, J., Peduzzi, R., Jemmi, T., 1999. PCR detection, characterization, and distribution of virulence genes in *Aeromonas* spp. Appl. Environ. Microbiol. 65 (12), 5293–5302.

Layne, E., 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Meth. Enzymol. 3, 447–454.

Misra, S., Sahu, N.P., Pal, A.K., Xavier, B., Kumar, S., Mukherjee, S.C., 2006. Pre-and post-challenge immuno-haematological changes in *Labeo rohita* juveniles fed gelatinised or non-gelatinised carbohydrate with n-3 PUFA. Fish Shellfish Immunol. 21 (4), 346–356.

Monir, M.S.Yusoff, M.S.M., Zulperi, Z.M., Hassim, H.A., Zamri-Saad, M., Amal, M.N.A., Salleh, A., Mohamad, A., Yie, L.J. and Ina-Salwany, M.Y.,, 2021. Immunoprotective efficiency of feed-based whole-cell inactivated bivalent vaccine against Streptococcus and Aeromonas infections in red hybrid tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*). Fish & Shellfish Immunology 113, 162–175. https://doi.org/10.1016/j.fsi.2021.04.006.

Monir, M.S., Yusoff, S.B.M., Zulperi, Z.B.M., Hassim, H.B.A., Mohamad, A., Ngoo, M.S.B.M.H., Ina-Salwany, M.Y., 2020. Haemato-immunological responses and effectiveness of feed-based bi-valent vaccine against *Streptococcus iniae* and *Aeromonas hydrophila* infections in hybrid red tilapia (*Oreochromis mossambicus*×*O. niloticus*). BMC Vet. Res. 16 (1). https://doi.org/10.1186/s12917-020-02443-y.

Nahar, S., Rahman, M.M., Ahmed, G.U., Faruk, A.R., 2016. Isolation, identification, and characterization of *Aeromonas hydrophila* from juvenile farmed pangasius (*Pangasianodon hypophthalmus*). Int. J. Fish. Aquae. 4 (4), 52–60.

Nawaz, M.Saeed A Khan, Ashraf A Khan, Kidon Sung, Quynhtien Tran, Khalil Kerdahi, Roger Steele, 2010. Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiol. 27 (3), 327–331. https://doi.org/10.1016/j.fm.2009.11.007.

Nisaa, K., Zairin, J., Lusiastuti, A.M., Nuryati, S., 2016. Resistance of tilapia (*Oreochrimis niloticus*) fry vaccinated at different gonadal developmental stages toward *Streptococcus agalactiae* infection. J. Veteriner. 17, 355–364.

Nurani, F.S., Sukenda, S., Nuryati, S., 2020. Maternal immunity of tilapia broodstock vaccinated with polyvalent vaccine and resistance of their offspring against *Streptococcus agalactiae*. Aquae. Res. 51 (4), 1513–1522.

Palumbo, S., Abeya, C., Stelma, G., 1992. *Aeromonas hydrophila* group. In: Vanderzant, C., Splittstoesser, F. (Eds.), Compendium of Methods for the Microbiological Examination of Food. Asian Pacific American Heritage Association, Washington, pp. 497–515.

Pasaribu, W., Sukenda, S., Nuryati, S., 2018. The efficacy of Nile Tilapia (*Oreochromis niloticus*) Broodstock and larval immunization against *Streptococcus agalactiae* and *Aeromonas hydrophila*. Fishes 3 (1), 16. https://doi.org/10.3390/fishes3010016.

Pretto-Giordano, L.G., Muller, E.E., Klesius, P., Da Silva, V.G., 2010. Efficacy of an experimentally inactivated *Streptococcus agalactiae* vaccine in Nile tilapia (*Oreochromis niloticus*) reared in Brazil. Aquae. Res. 41, 1539–1544.

Rahman, M., Colque-Navarro, P., Kühn, I., Huys, G., Swings, J., Möllby, R., 2002. Identification and characterization of pathogenic *Aeromonas veronii* biovar *sobria* associated with epizootic ulcerative syndrome in fish in Bangladesh. Appl. Environ. Microbiol. 68 (2), 650–655.

Purwanti, C.S.Suminto, S.A, 2014. The description of blood profile catfish Clarias gariepinus that is fed with a combination of artificial feed and earthworm *Lumbricus rubellus*. J. Aquaculture Manag. and Tech. 3, 50–60.

Rahman, M.M., Chowdhury, M.B.R., 1996. Isolation of bacterial pathogen causing an ulcer disease in farmed carp fishes of Mymensingh. Bangladesh J. Fish 19 (1–2), 103–110.

Sarker, M.G.A., Chowdhury, M.B.R., Faruk, M.A.R., Uddin, M.N., Islam, M.J., 2000. Effect of water temperature on the infectivity of *Aeromonas hydrophila* isolates. Bangladesh J. Fish 23 (2), 99–105.

Shao, J.-Z., Liu, J., Xiang, L.-X., 2004. *Aeromonas hydrophila* induces apoptosis in Carassius auratus lymphocytes in vitro. Aquaculture 229 (1–4), 11–23.

Shoko, A.P., Limbu, S.M., Mrosso, H.D., Mgaya, Y.D., 2014. A comparison of diurnal dynamics of water quality parameters in Nile tilapia (*Oreochromis niloticus*, Linnaeus, 1758) monoculture and polyculture with African sharp tooth catfish (*Clarias gariepinus*, Burchell, 1822) in earthen ponds. Int. Aquat. Res. 6 (1), 1–3.

Stone, H.D., 1993. Efficacy of experimental animal and vegetable oil-emulsion vaccines for Newcastle disease and avian influenza. Avian Dis. 37 (2), 399. https://doi.org/10.2307/1591665.

Sukenda, S., Carman, O., Rahman, R., Hidayatullah, D., Yumaidawati, N.S., 2017a. Vaccination in Nile tilapia broodstock with whole cell vaccine and disease

resistance in its fry against *Aeromonas hydrophila*. Indones. Aquae. J. 16 (2), 268. https://doi.org/10.19027/jai.16.2.268-276.

Sukenda, S., Rahman, R., Nisaa, K., Hidayatullah, D., Vinasyiam, A., 2018. The efficacy of *Streptococcus agalactiae* vaccine preparations, administered to tilapia broodstock, in preventing streptococcosis in their offspring, via transfer of maternal immunity. Aquae. Int. 26 (3), 785–798.

Sukenda, S., Sumiati, T., Nuryati, S., Lusiastuti, A.M., Hidayatullah, D., 2017b. Specific immune response kinetics and mortality patterns of tilapia *Oreochromis niloticus* on post-cocktail vaccination period against the infection of *Aeromonas hydrophila* and *Streptococcus agalactiae*. Omni-Akuatika. 73, 6–15.

Sultana, N., Uddin, K.M., Ahasan, C.T., Hussain, M.G., 2005. Nursery management system of stinging catfish, *Heteropneustes fossilis* and walking catfish, *Clarias batrachus* in Bangladesh. Bangladesh J. Fish. Res. 9 (1), 35–36.

Thune, R.L., Stanley, L.A., Cooper, R.K., 1993. Pathogenesis of Gram-negative bacterial infections in warm water fish. Annu. Rev. Fish Dis. 3, 37–68.

Townsend, D.E., Naqui, A., 1998. Comparison of SimPlate Total™ plate count test with plate count agar method for detection and quantitation of bacteria in food. J. AOAC Int. 81, 563–570.

Yu, H.B., Rao, P.S.S., Lee, H.C., Vilches, S., Merino, S., Tomas, J.M., Leung, K.Y., 2004. A Type III secretion system is required for *Aeromonas hydrophila* AH-1 pathogenesis. Infect. Immun. 72 (3), 1248–1256.

Zhao, X., Findly, R.C., Dickerson, H.W., 2008. Cutaneous antibody secreting cells and B cells in a teleost fish. Dev. Compar. Immunol. 32 (5), 500–508.