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Evaluation of pH Inactivated Vaccine for *Aeromonas hydrophila* Infection in the Nile Tilapia (*Oreochromis niloticus* L.)

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ABSTRACT

The study investigated the efficacy of a pH inactivated vaccine against *Aeromonas hydrophila* in the juvenile Nile tilapia (*Oreochromis niloticus* L.). The inactivated vaccine was developed by treating the bacterial suspension with sodium hydroxide to raise the pH to 10.0, followed by readjustment to neutral pH, and finally formaldehyde treatment. Vaccination was administered via intraperitoneal injection, and fish were subsequently challenged with live *A. hydrophila* to assess the vaccine's protective efficacy. Clinical observations revealed no mortality during the acclimatization phase, however, signs of infection emerged in non-vaccinated group after bacterial exposure. The computed 100% relative percentage survival (RPS) in the vaccinated and challenged group underscores the vaccine's effectiveness. Thus, the study concludes that the pH inactivated vaccine provides significant protection against *A. hydrophila*, presenting a viable strategy for enhancing disease resistance in aquaculture settings.

INTRODUCTION

Tilapia (*Oreochromis* spp.) is a leading aquaculture product that is marketed globally, with the Philippines ranking among the top producers of this fish. This freshwater species has gained immense popularity due to its adaptability, rapid growth, and mild flavor, making it a staple in various culinary traditions. The country's favorable climate and abundant water resources have facilitated the establishment of extensive tilapia farming operations. As a result, the Philippines plays a significant role in meeting the increasing global demand for tilapia, contributing to both local consumption and international exports. With ongoing advancements in aquaculture practices, the

Philippines is poised to enhance its production capabilities and maintain its competitive position in the global tilapia market **(Miao & Wang, 2020)**. Various culture techniques for tilapia have gained popularity, including pond culture, cage culture, monosex male tilapia culture, saltwater culture, rice-fish culture, and aquaponics **(Romana *et al.*, 2020)**. As of 2023, tilapia production from the aquaculture sector reached 307,878.28 metric tons, accounting for approximately 7.2% of the total aquaculture production in the Philippines **(PSA, 2024)**.

Aquaculture, particularly tilapia farming, is a significant source of protein and income globally, yet it faces substantial challenges from various diseases. Among these, bacterial infections are particularly detrimental, with pathogens such as *Aeromonas hydrophila*, *Streptococcus iniae*, and *Edwarsiella tarda* causing severe economic losses due to high mortality rates and reduced growth performance in tilapia populations **(Reyes *et al.*, 2021; Reyes *et al.*, 2022)**. The intensification of aquaculture practices has exacerbated these issues, creating conditions that favor the proliferation of these pathogens, especially in environments with high nutrient concentrations and water temperatures **(Reyes *et al.*, 2021; Reyes *et al.*, 2022; Haenen *et al.*, 2023)**.

A. hydrophila is typically treated as an opportunistic or secondary pathogen but it can also become a primary pathogen, causing mass mortalities of fish and huge economic losses to the aquaculture industry **(Fang *et al.*, 2004; Shahi *et al.*, 2013)**. The increasing prevalence of *A. hydrophila* infections poses significant challenges to the aquaculture industry, particularly in the farming of the Nile tilapia. This bacterium is a major pathogen responsible for motile aeromonad septicemia (MAS), which can lead to high mortality rates and substantial economic losses for fish farmers **(Aly *et al.*, 2015)**.

To combat diseases in aquaculture, vaccination strategies have been developed, such as inactivation of the bacteria using formalin, chloroform, phenol, heat, sonication and lysis with sodium dodecyl sulphate (SDS) or with sodium hydroxide at pH 9.5 **(Austin, 1984)**. The advantages of using vaccines over traditional disease management approaches are manifold. Vaccination provides long-lasting immunity, reducing the incidence of disease outbreaks and enhancing overall fish health. It allows for a more sustainable aquaculture practice by decreasing reliance on antibiotics and mitigating the risks associated with their overuse **(Ma *et al.*, 2019)**.

One of the emerging and promising approaches of producing inactivated vaccines is through pH manipulation. This approach allows for large-scale vaccination of fish with reduced stress associated with handling. The manipulation of pH during the preparation of these vaccines can enhance their efficacy by optimizing the stability and immunogenicity of the inactivated bacterial antigens. Researches have shown that inactivated vaccines for *A. hydrophila* can significantly improve the immune response in vaccinated tilapia, resulting in higher survival rates following exposure to the pathogen **(Silva *et al.*, 2009; Dubey *et al.*, 2016; Bogwald & Dalmo, 2019; Bashir *et al.*, 2023)**.

The integration of pH manipulation techniques into vaccine development represents a promising avenue for improving vaccine efficacy and ensuring better health outcomes for tilapia populations. Thus, this study was conducted to assess the efficacy of pH-inactivated vaccine for *A. hydrophila* in the Nile tilapia.

MATERIALS AND METHODS

1. Preparation of *Aeromonas hydrophila*

Molecularly identified *A. hydrophila* was sub-cultured in a 1L flask containing casein soybean digest broth and was incubated for three days to promote growth. To confirm its purity, the bacterium was streaked on *Aeromonas* selective agar. The purified bacterium was again sub-cultured in broth until use.

2. Acquisition and conditioning of the Nile tilapia

The Nile tilapia juvenile (49.96±1.51 g; 10.20±2.35cm) obtained from the Freshwater Aquaculture Center-Central Luzon State University (FAC-CLSU) were transported to the Wet Laboratory, and each fish was placed in 50L capacity aquarium with constant aeration. Before the experiment, the fish were fed twice a day (8:00 am, 4:00 pm) with commercial feeds mixed with antibiotics tetracycline (50mg/ kg) at 1% of body weight to ensure that the fish were free from *A. hydrophila*. The conditioning of the fish lasted for 7 days.

3. Description of experimental treatments

The description of experimental treatments is shown in Table (1). Each treatment was replicated 10 times, and the experimental units were arranged in a complete randomized design (CRD). Following the acclimation period, every fish was randomly assigned in an aquarium filled with tap water.

Table 1. Description of experimental treatments

Treatment	Description
T1 (Negative control)	Non-vaccinated (only NSS injection); unchallenged
T2 (Positive control)	Non-vaccinated (only NSS injection); challenged
T3	Vaccinated (pH inactivated vaccine); unchallenged
T4	Vaccinated (pH inactivated vaccine); challenged

4. Production of inactivated vaccine via pH manipulation

The vaccine was prepared according to **Nguyen *et al.* (2018)** with slight modifications. Briefly, *A. hydrophila* suspension was treated with 1 M sodium hydroxide

(NaOH) to raise the pH to 10.0 and was kept at room temperature for 6 hours. Then, the suspension was readjusted to pH 7.0 with 1 M hydrochloric acid (HCl) using a pH meter. To avoid contamination during pH manipulation, duplicated flasks with the same *A. hydrophila* concentration were prepared, and the volume of each reagent needed to alter the desired pH was calculated for one flask and was applied to the other. Then, sterile Normal Saline Solution (NSS) was added to the suspension prior to 0.3% formaldehyde addition, and was incubated at 4°C, for 24 hours. Then, the bacterium was washed three times with NSS by centrifugation at 3,350 rpm, for 10 minutes at 4°C. The bacterial concentration was adjusted to 0.5 McFarland standard (1.5 x 10^8 CFU/mL). To confirm the inactivation, 0.1mL of the bacterial suspension was streaked onto *Aeromonas* selective agar and the plates were incubated at 28°C, for 72 hours. Finally, the vaccine was stored at 4°C until use.

5. Administration of vaccine

Prior to injection, the fish were anesthetized by immersion for 3 minutes in 10L of water with 2mL of 2-phenoxyethanol. The vaccine (0.1mL of 1.5 x 10^8 CFU/mL) was administered to the fish in Treatments 3 and 4 via intraperitoneal injection after 7 days of conditioning. Meanwhile, the fish in Treatments 1 and 2 were injected with 0.1mL NSS. After injection, the fish were returned to their respective aquaria.

6. Preparation and intraperitoneal injection of *Aeromas hydrophila*

Stock culture of *A. hydrophila* was sub-cultured in *Aeromonas* selective agar at 28°C, for 72 hours. Then, colonies were selected and inoculated into 300mL Casein soybean digest broth. After that, the bacterium was washed twice with sterile NSS by centrifugation at 3,350 rpm, for 10 minutes at 4°C. The cell pellet was re-suspended in NSS with confirmed concentration of 1.5 x 10^8 CFU/mL.

One week after vaccination, each fish in treatments 2 and 4 were intraperitoneally injected with 0.1mL of *A. hydrohila* preparation. After injection, all the fish were returned to their respective aquaria.

7. Maintenance of the experimental set-up

Dead or moribund fish were removed before feeding the fish twice daily (8:00 am, 4:00 pm) with a commercial diet at 1% of body weight. Waste was siphoned daily and the evaporated water was replaced as needed. Water quality parameters such as temperature, dissolved oxygen, and pH were monitored daily by means of DO meter and pH meter.

8. Observation of the fish

Clinical signs of disease, morbidity and mortality were recorded daily for a period of two weeks.

9. Computation of vaccine efficacy

Vaccine efficacy was assessed by means of the relative percentage survival (RPS), calculated according to **Amend (1981)** formula: RPS = [1-(% mortality in vaccinated fish / % mortality in control fish)] x 100.

RESULTS

1. Inactivation of vaccine

To verify inactivation of the prepared vaccine, 0.1mL of the bacterial suspension was streaked onto *Aeromonas* selective agar in triplicates, and the plates were incubated at 28°C for 72 hours. No bacterial growth was visible in plates; thus, the vaccine is safe to use in the succeeding experiment (Fig. 1).

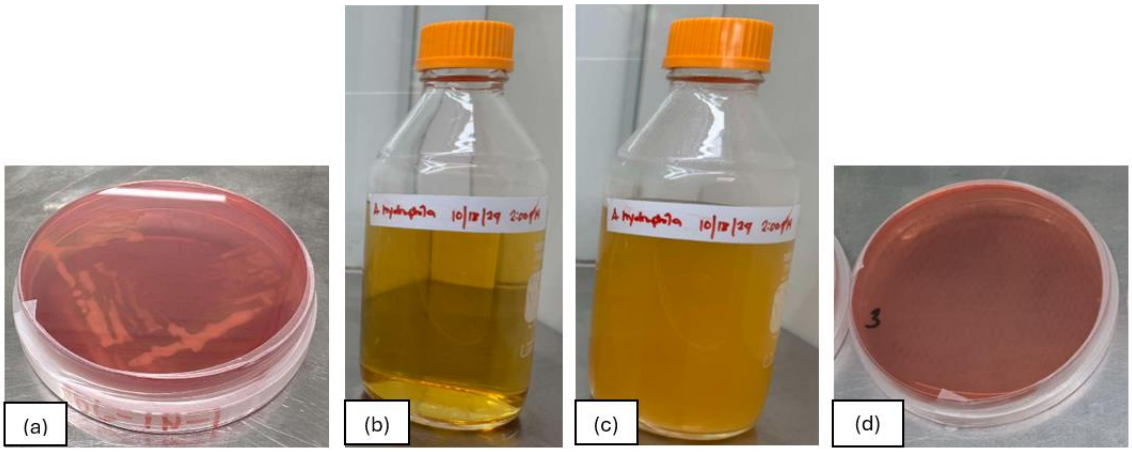

Fig. 1. (a) Preparation of the vaccine: Molecularly identified *Aeromonas hydrophila* streaked in selective agar; **(b)** *A. hydrophila* sub-cultured in a 1 L flask containing casein soybean digest broth; **(c)** *A. hydrophila* suspension after 3 days in a 1 L flask; **(d)** no visible growth of *A. hydrophila* in the selective medium after the pH manipulation

2. Clinical signs of infection

One week prior vaccination, no mortality was recorded, indicating a successful acclimatization and response to injected vaccine. In the entire duration of the study, fish in treatments 1 (unchallenged) and 3 (unchallenged and vaccinated) showed no clinical signs of *A. hydrophila* infection, suggesting that these groups remained healthy. However, changes were noted in treatments 2 (challenged) and 4 (challenged and vaccinated) seven days following the bacterial challenge. The observed symptoms include skin darkening and reddish coloration around the pectoral fin area. Additionally, affected fish displayed a loss of appetite, erratic swimming behavior, and exophthalmia (protrusion of the eyes). These clinical signs are illustrated in Fig. (2). After 10 days post-

challenge, mortality was recorded in T2 highlighting the impact of the bacterial infection on this group.

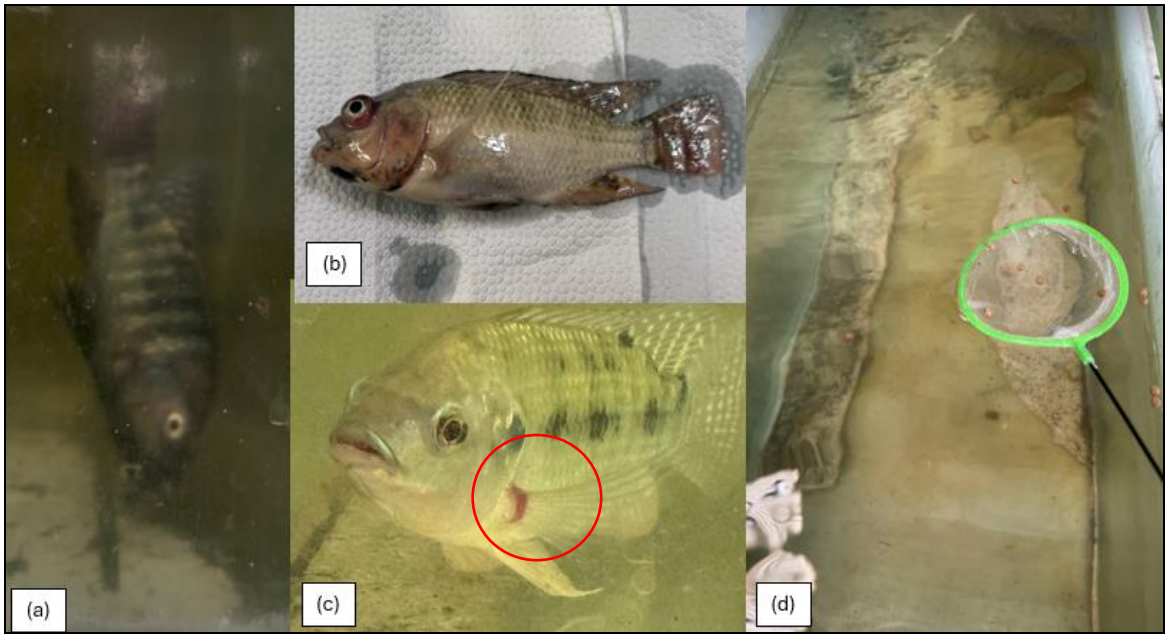

Fig. 2. **(a)** Clinical signs of *Aeromonas hydrophila* infection in treatments 2 and 4: Changes in coloration and erratic swimming; **(b)** Exophthalmia; **(c)** Reddish coloration on the pectoral fin area; **(d)** Loss of appetite

3. Relative percentage survival

The calculated 100% RPS in T4 demonstrated that this vaccinated and challenged group experienced no excess mortality. These findings suggest that vaccination effectively confers protection against mortality when challenged with *A. hydrophila*.

4. Water quality

Table (3) presents the recorded water quality parameters during the experiment. The temperature, dissolved oxygen, and pH readings were stable across all treatments and within the optimal ranges for tilapia.

Table 3. The recorded water quality parameters during the course of experimental set-up

Treatment	Water Quality Parameters		
	Temperature	Dissolved oxygen	pH
T1	24.75 ± 0.57	7.25 ± 0.12	7.49 ± 0.02
T2	24.79 ± 0.69	7.29 ± 0.12	7.49 ± 0.04
T3	24.81 ± 0.78	7.28 ± 0.13	7.49 ± 0.03
T4	24.74 ± 0.61	7.28 ± 0.11	7.50 ± 0.04

DISCUSSION

A. hydrophila poses a significant threat to the aquaculture industry, with infected fish exhibiting symptoms such as enteritis and septicemia, which can escalate mortality **(Semwal *et al.,* 2023)**. Therefore, vaccination may be considered as an alternative way for disease control. Keeping in view the increasing importance of aquaculture vaccines as immunoprophylaxis measure, the main purpose of this present study was to develop and assess pH manipulation-inactivated vaccine for *A. hydrophila* in tilapia.

No mortality was recorded during the vaccination period, indicating a successful acclimatization and response to the vaccination protocol. This finding aligns with existing literature that emphasizes the importance of proper acclimatization in aquaculture, as stress can significantly impact fish health and their response to vaccinations. Successful acclimatization is crucial for enhancing the effectiveness of vaccines, particularly in preventing diseases caused by bacterial infections **(Brudeseth *et al.,* 2013)**. However, changes were seen in Treatment 2 starting from day 7 after the bacterial challenge was administered. Treatment 2, challenged with *A. hydrophila* exhibited clinical signs of infection. This aligns with the research of **Eguia *et al.* (2020)** that tilapia infected with *A. hydrophila* often displays symptoms such as exophthalmia, loss of appetite, and skin lesions, which were also evident in the current study. Similarly, Treatment 4, which involved fish vaccinated with a pH killed vaccine also displayed mild symptoms of diseases after being challenged. Vaccinated fish can sometimes display clinical symptoms after being challenged with pathogens, yet still exhibit effective immunity, as evidenced by the absence of mortality. This situation highlights the complexity of immune responses in fish. Vaccines often stimulate a protective immune response, allowing fish to fight off infections without succumbing to disease. Symptoms such as mild inflammation or stress may arise as part of the immune response but do not necessarily indicate vaccine failure. For instance, studies have shown that fish vaccinated against pathogens like *A. hydrophila* can develop antibodies and activate immune cells while still presenting mild clinical signs after exposure, indicating an effective response **(Mzula *et al.,* 2019)**.

Fish mortality is particularly concerning as it reflects the potential economic losses associated with bacterial infections in aquaculture. High mortality rates due to bacterial diseases can lead to significant financial implications for fish farmers **(DOST, 2024)**. Therefore, implementing effective vaccination programs is essential not only for improving fish health but also for ensuring the sustainability and profitability. Fish mortality at 10% was only recorded in Treatment 2 (non-vaccinated, challenged). Statistical comparison of mortality rates among treatments showed no significant difference. The 100% RPS for Treatment 4 demonstrates good vaccine efficiency, as it quantifies the proportion of vaccinated fish that survive after exposure to a pathogen

relative to unvaccinated controls. In this context, the findings underscore the effectiveness of vaccination in enhancing fish survival rates during bacterial challenges. Previous studies have shown similar results, where vaccinated fish exhibited significantly higher RPS compared to their unvaccinated counterparts, reinforcing the role of vaccination as a preventive strategy against bacterial infections **(Sombe *et al*., 2024)**. Various vaccine formulations, including whole-cell and subunit vaccines targeting *A. hydrophila*, have demonstrated significant protective effects in different fish species **(Monir *et al*., 2020)**. For instance, studies have reported that fish vaccinated with inactivated bacteria showed improved survival rates and enhanced immune responses compared to unvaccinated fish **(Mulia *et al*., 2022)**.

The water quality parameters measured during the study were stable and consistently within the ideal ranges for tilapia (temperature = 25 to 30°C, dissolved oxygen = ≥5 mg/L, pH = 6.5 to 8.5). The stability of these water quality parameters indicates effective management practices that support a healthy environment for juvenile tilapia culture **(Hargreaves & Tucker, 2004; Regal Springs, 2023)**. Therefore, the recorded mortality in Treatment 2 was not attributed to water quality but it was due to bacterial infection.

CONCLUSION

The findings affirm that the developed vaccine via pH manipulation is a viable method for enhancing disease resistance and providing a significant advancement in strategies to combat bacterial infections in fish farming. Specifically, the recorded 100% RPS in Treatment 4 (vaccinated and challenged) is an indication of effective protection against mortality when exposed to *A. hydrophila*.

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