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บทความวิจัย

การตอบสนองทางภูมิคุ้มกันและโลหิตวิทยาในปลาทอง (*Carassius auratus*) ด้วยวัคซีนป้องกันเชื้อ *Aeromonas hydrophila*

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บทคัดย่อ

การแพร่ระบาดของเชื้อ *Aeromonas hydrophila* ในปลาสวยงามมีเพิ่มขึ้น การพัฒนาวิธีการป้องกันจึงมีความสำคัญ โดยการศึกษาการตอบสนองทางภูมิคุ้มกันและโลหิตวิทยาของวัคซีนป้องกันเชื้อ *A. hydrophila* ในปลาทอง (*Carassius auratus*) ในปลาทอง น้ำหนักเฉลี่ย 47.40 ± 1.95 กรัม แบ่งออกเป็น 5 กลุ่มการทดลอง โดยแบ่งความเข้มข้นของวัคซีนออกเป็น กลุ่มควบคุม (0), $1x10^2$, $1x10^4$, $1x10^6$ และ $1x10^8$ เซลล์ต่อมิลลิลิตร ปลาทองทุกกลุ่มได้รับวัคซีนที่เตรียมจากเชื้อ *A. hydrophila* พบว่า ที่ความเข้มข้นของวัคซีน 1×10^6 เซลล์ต่อมิลลิลิตร มีอัตราการเจริญเติบโตต่อวันสูงที่สุด คือ เท่ากับ 0.91 ± 0.04 กรัมต่อวัน และมีค่าปริมาณเม็ดเลือดแดงอัดแน่น (% Hematocrit: Hct) ไม่มีความแตกต่างกันทางสถิติของทุกความเข้มข้น และพบว่าค่าแอนติบอดี้ไตเตอร์ เท่ากับ 3.19 ± 0.06 ไม่แตกต่างกันทางสถิติกับความเข้มข้น 1×10^8 เซลล์ต่อมิลลิลิตร ซึ่งมีแอนติบอดี้ไตเตอร์เท่ากับ 3.22 ± 0.03 ซึ่งเป็นค่าที่สูงที่สุดในการทดลอง โดยระดับความเข้มข้นของวัคซีนที่เหมาะสมคือ 1×10^6 เซลล์ต่อมิลลิลิตร

Introduction

Aquaculture plays a crucial role in global food security and economic development. The swift proliferation of intensive aquaculture systems has heightened the risk of infectious disease outbreaks, especially those induced by bacterial pathogens, which significantly contribute to production losses and economic downturns in the aquaculture sector (FAO, 2020). Goldfish (*Carassius auratus*) is one of the most economically significant ornamental fish, contributing substantially to Thailand's aquaculture industry. It is regarded as a premium export commodity in the ornamental fish industry. Thailand is acknowledged as a leading producer and exporter of ornamental fish globally, especially in Asian, European, and American markets (FAO, 2020; Department of Fisheries, 2021). The ornamental fish industry in Thailand provides revenue for farmers, breeders, and entrepreneurs across the production chain, encompassing breeding, rearing, transportation, and exportation. Goldfish are immensely popular owing to their many strains and appealing appearance.

Intensive goldfish production methods often encounter bacterial infections that adversely impact survival rates and fish quality. *A. hydrophila* is a prominent bacterial pathogen. This bacterium is a Gram-negative organism belonging to the family Aeromonadaceae, typically located in freshwater habitats (Janda & Abbott, 2010). It is an opportunistic disease that can infect various aquatic species, including goldfish (*C. auratus*) (Harikrishnan et al., 2003). Infections caused by *A. hydrophila* frequently correlate with environmental stresses, notably substandard water quality characterized by organic waste accumulation and abrupt temperature variations. These variables compromise the fish immune system, facilitating

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pathogen invasion of host tissues and resulting in acute or chronic infections (Zhang et al., 2017).

Aeromonas hydrophila is the etiological agent of motile Aeromonas septicemia (MAS), a serious and highly transmissible bacterial disease in aquaculture systems. Clinical manifestations encompass hemorrhagic lesions on the integument, abdominal distension (ascites), inflammation of fins and gills, septicemia, and anemia (Semwal et al., 2023). The pathogen generates many virulence factors, including as hemolysin, aerolysin, and protease, which lead to host tissue destruction and systemic physiological dysfunction (Akmal et al., 2020; Semwal et al., 2023). This disease has been documented in various fish species, including catfish, common carp, goldfish, and koi carp (Thangaviji et al., 2012). *A. hydrophila* infection poses a significant risk to aquatic animal health and the viability of aquaculture output (Nhinh et al., 2021).

Despite the prevalent use of antibiotics for disease management, their misuse can result in antimicrobial resistance and environmental pollution, imposing considerable constraints on contemporary aquaculture systems (Nhinh et al., 2021). As a result, vaccination has garnered heightened focus as an efficacious and sustainable approach to disease prevention. Vaccines can activate the fish immune system, namely the humoral immunological response, which can be assessed by measuring antibody titers (Semwal et al., 2023). Vaccination has become an important approach for controlling bacterial diseases caused by *A. hydrophila* in aquaculture. Previous studies demonstrated that vaccines could enhance immune responses and improve survival rates in several fish species, including common carp, Nile tilapia, and zebrafish (Alishahi et al., 2024; Qosimah et al., 2023; Rivas et al., 2023). In ornamental fish, protective immunity against *A. hydrophila* has also been reported in goldfish following vaccination with bacterial antigens (Viji et al., 2013; Rahman et al., 2000). However, information regarding hematological responses and vaccine efficacy in goldfish (*C. auratus*) remains limited. Therefore, further investigation is necessary to support disease prevention and improve health management in ornamental fish culture. This study aims to assess the impact of varying dosages of *A. hydrophila* vaccination on immunological responses (antibody titer), hematological alterations (hematocrit), and growth performance in goldfish. The results aim to determine an ideal vaccination concentration for efficient and sustainable use in aquaculture systems.

Materials and Methods

Fish Samples and Rearing Conditions

Goldfish (*C. auratus*) were obtained from a local ornamental fish supplier in Mueang District, Maha Sarakham Province, Thailand. The fish exhibited an average body weight of 47.4 ± 1.95 g. Before the experiment, the fish were acclimatized in glass aquaria for one week to facilitate adaptation to laboratory settings. Throughout the acclimatization and experimental phases, the fish were administered a commercial feed (35% crude protein) twice daily (morning and evening) at 3% of their body weight. The experiment was performed in triplicate, and the fish were raised for two weeks under regulated conditions. Water quality measurements, such as pH and temperature, were consistently observed over the trial period.

Vaccine and Antigen Preparation

The vaccination utilized in this investigation was an inactivated (killed) vaccine derived from *A. hydrophila*. The bacterial strain was isolated and cultivated on Brain Heart Infusion Agar (BHIA) and incubated at 25 °C for 24 to 48 hr. (Akmal et al., 2020; Austin & Austin, 2016). A single colony was subsequently injected into 100 ml of Brain Heart Infusion Broth (BHIB) and cultured at 25 °C for 24 hr. to augment bacterial cell density. The bacterial suspension was then collected using centrifugation at 2,500 rpm for 15 min (Harikrishnan et al., 2003). The pellet underwent three washes with sterile 0.85% sodium chloride (NaCl) solution to eliminate contaminants. The bacterial concentration was further modified to 1×10^2, 1×10^4, 1×10^6, and 1×10^8 cells/ml (Thangaviji et al., 2012). The bacteria were rendered inactive using heat treatment at 70 °C for 30 min a conventional technique employed to maintain antigenic structures while eradicating pathogenicity (Semwal et al., 2023; Nayak, 2010). The efficacy of inactivation was validated by re-culturing the treated bacteria on BHIA plates to confirm

the lack of bacterial growth before its application as a vaccination in experimental fish (Akmal et al., 2020).

Immunological Analysis (Antibody Titer Evaluation)

Antibody titers were determined using the direct agglutination method. Serum samples were serially diluted using a two-fold dilution series in 0.85% saline solution. The final volume in each well was adjusted to 50 µl. Subsequently, 50 µl of the prepared antigen was added to each well, resulting in a series of serum dilutions up to 1:2,048. The plates were gently mixed and incubated at 25 ℃ for 24 hours. Agglutination results were recorded by comparison with a negative control containing only saline solution and antigen. The antibody titer was defined as the highest serum dilution showing visible agglutination. All experimental fish were screened for baseline antibody titers prior to vaccination (Thangaviji et al., 2012; Nayak, 2010).

Vaccination Procedure and Experimental Design

The experimental goldfish were randomly assigned to five treatment groups, each with three replicates and 15 fish per replicate, following a completely randomized design (CRD). All treatment groups received an inactivated *A. hydrophila* vaccine prepared by heat inactivation. Vaccination was administered via intraperitoneal injection at a dose of 1 ml/kg body weight. All fish were maintained under identical environmental and feeding conditions throughout the experimental period. The experimental groups were as follows:

Treatment 1 (Control): non-vaccinated

Treatment 2: vaccinated with 1×10^2 cells/ml

Treatment 3: vaccinated with 1×10^4 cells/ml

Treatment 4: vaccinated with 1×10^6 cells/ml

Treatment 5: vaccinated with 1×10^8 cells/ml

All experimental fish were randomly selected for blood collection to ascertain baseline levels following two weeks of feeding. The initial vaccination (primary vaccination) was subsequently provided. Blood samples were obtained again two weeks after the main vaccination to assess the initial immune response. A booster immunization was delivered three weeks following the original vaccination, or one week after the second blood sampling. Blood samples were taken two weeks post-booster vaccination to evaluate the immune response subsequent to immunological stimulation.

Throughout the experimental duration, growth performance was assessed by recording body weight at both the commencement and conclusion of the trial. Weight gain was assessed to determine growth performance. The complete trial duration spanned 8 weeks (2 months) under regulated rearing settings.

Hematological Analysis

Hematological parameters of goldfish (*C. auratus*) were evaluated before and after the 2-month experimental period. Ten fish were randomly selected from each treatment group for blood collection. Blood samples were obtained with heparinized syringes to inhibit coagulation during collection (Hrubec et al., 2000). The obtained blood was utilized for hematological analysis, specifically for the estimation of hematocrit (Hct) (Harikrishnan et al., 2003). The microhematocrit method was employed to measure hematocrit. Blood samples were placed into capillary tubes (two tubes per sample) and spun in a hematocrit centrifuge at 11,000 rpm for 5 minutes to segregate red blood cells from plasma (Hrubec et al., 2000). The percentage of packed cell volume (hematocrit) was subsequently measured and documented.

Statistical Analysis

Differences in antibody titers between the first and second sampling points, as well as growth performance among experimental groups, were analyzed using one-way analysis of variance (ANOVA) based on a completely randomized design (CRD). Mean comparisons were performed using Duncan's New Multiple Range Test (DMRT) at a 95% confidence level ($p<0.05$). All statistical analyses were conducted using SPSS software (version 17.0).

Ethical Approval

All experimental procedures were conducted in accordance with ethical standards for scientific research and the use of animals in research. The study was approved by the relevant institutional authority, and the researcher held a valid Animal Use License for Scientific Purposes (License No. U1045012559, ThaiACUC).

Results and Discussion

Effects of Aeromonas hydrophila Vaccination on Growth Performance in Goldfish

As shown in Table 1, vaccination with *A. hydrophila* significantly influenced the growth performance of *C. auratus*. Fish in all vaccinated groups exhibited higher average daily gain (ADG) compared to the control group ($p<0.05$). The highest ADG was observed in fish vaccinated with 1×10^6 cells/ml (0.91 ± 0.04 g/day), although this value was not significantly different from those in the 1×10^4 and 1×10^8 cells/ml groups ($p>0.05$). In contrast, the control group showed the lowest ADG (0.46 ± 0.07 g/day), indicating that vaccination contributed positively to growth performance.

The enhanced growth observed in vaccinated fish may be attributed to improved health status and reduced subclinical infections, which likely facilitated better feed utilization and metabolic efficiency (Uribe et al., 2011). These findings are consistent with previous studies reporting that vaccination can indirectly improve growth by minimizing disease-related stress and enhancing physiological condition. However, increasing the vaccine concentration beyond 1×10^6 cells/ml did not result in further significant improvements in growth. This suggests that excessively high antigen doses may not provide additional benefits and could potentially impose physiological stress. Therefore, 1×10^6 cells/ml appears to be the optimal vaccine concentration for maximizing growth performance in this study.

Table 1 Average Daily Gain (ADG) of *C. auratus* after vaccination against *A. hydrophila*

Concentration	Average Daily Growth (g/day) (ADG)
	Means ± SD
control (0)	0.46 ± 0.07^c
1×10^2	0.61 ± 0.06^b
1×10^4	0.81 ± 0.04^a
1×10^6	0.91 ± 0.04^a
1×10^8	0.83 ± 0.04^a

* Means in each column followed by different letters are significantly different at $p< 0.05$

Hematological Parameters

Hematological parameters, particularly hematocrit (Hct), were assessed prior to and during the experimental period to evaluate the physiological status of goldfish post-vaccination against *A. hydrophila*. Blood samples were obtained from 10 fish per treatment group for a duration of 2 months. The findings indicated that hematocrit levels were not substantially different among treatment groups at both sample intervals (week 3 and week 6) ($p>0.05$). Nonetheless, minor discrepancies were noted among the therapies. During week 3, the peak hematocrit (Hct) value was noted in fish immunized with 1×10^6 cells/ml (25.60 ± 0.52%), whereas in week 6, the highest value was obtained in the 1×10^8 cells/ml cohort (27.00 ± 1.00%) (Table 2). Despite the absence of statistically significant differences, hematocrit values exhibited a tendency to rise from the first to the second sample across all treatment groups. This tendency may signify an enhancement in physiological condition over time. The elevation in hematocrit aligns with the superior growth performance noted in vaccinated fish, indicating higher oxygen transport capacity and overall metabolic efficiency. Vaccination is an effective strategy for controlling bacterial diseases caused by *A. hydrophila* in aquaculture. Following vaccination, fish immune systems are stimulated through both innate and adaptive immune responses, leading to increased phagocytic activity, cytokine production, and antibody synthesis, particularly immunoglobulin M (IgM) (Ellis, 2001; Uribe et al., 2011). Injectable vaccines have been reported to enhance immune responses, improve hematological parameters such as hematocrit levels, and increase survival rates following bacterial challenge tests (Plant & LaPatra, 2011). Hematocrit serves as a crucial measure of piscine health and signifies

the oxygen transport capability of blood (Clauss et al., 2008). The lack of notable differences among treatments indicates that the immunization did not provoke hematological stress or detrimental physiological effects. Previous research has shown analogous findings, suggesting that vaccination does not substantially modify hematological parameters but may enhance physiological state and immunological competence (Hrubec et al., 2000; Harikrishnan et al., 2003; Fazio, 2019).

Table 2 Hematocrit of *C. auratus* after vaccination against *A. hydrophila*

Concentration	Hematocrit, Hct % (Means ± SD)	
	First (weeks 3)	Second (weeks 6)
control (0)	24.76 ± 0.40^{a}	25.33 ± 0.76^{a}
1 x 10^{2}	25.00 ± 0.86^{a}	26.00 ± 1.00^{a}
1 x 10^{4}	25.00 ± 1.00^{a}	25.83 ± 1.04^{a}
1 x 10^{6}	25.60 ± 0.52^{a}	26.00 ± 1.00^{a}
1 x 10^{8}	25.33 ± 0.57^{a}	27.00 ± 1.00^{a}

* Means followed by the same letters within rows are not significantly different at $p>0.05$

Table 3 Antibody titer of *C. auratus* after vaccination against *A. hydrophila*

Concentration	Antibody titer (log_{10}) (Means ± SD)	
	First (weeks 3)	Second (weeks 6)
control (0)	0.06 ± 0.00^{Ad}	0.09 ± 0.03^{Ad}
1 x 10^{2}	0.63 ± 0.03^{Bc}	2.31 ± 0.03^{Ac}
1 x 10^{4}	1.14 ± 0.06^{Bb}	2.69 ± 0.01^{Ab}
1 x 10^{6}	1.23 ± 0.03^{Ba}	3.19 ± 0.06^{Aa}
1 x 10^{8}	1.29 ± 0.03^{Ba}	3.22 ± 0.03^{Aa}

* Means followed by different lowercase letters within columns and different uppercase letters within rows indicate significant differences at $p<0.05$.

Antibody Titer Response

The antibody titers of goldfish (*C. auratus*) were assessed utilizing the direct agglutination technique. Baseline antibody levels were evaluated before immunization. Table 3 demonstrates that antibody titers significantly increased in all vaccination groups relative to the control group ($p<0.05$).

Subsequent to the initial vaccination (week 3), antibody titers exhibited a steady increase correlating with elevated vaccine concentrations, varying from 0.63 ± 0.03 to 1.29 ± 0.03 (log_{10}). Following the booster vaccination at week 6, a significant elevation in antibody titers was noted across all vaccinated cohorts, with values spanning from 2.31 ± 0.03 to 3.22 ± 0.03. The peak antibody titers were seen in the 1 × 10^{6} and 1 × 10^{8} cells/ml groups, with no statistically significant difference between these treatments ($p > 0.05$) (Figure 1).

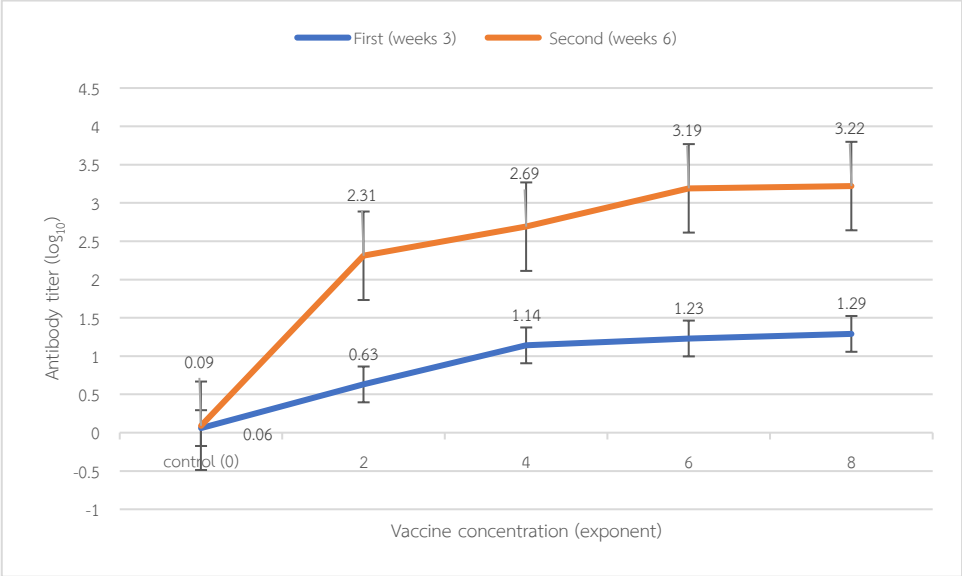


Figure 1 Antibody titer of *C. auratus* after vaccination against *A. hydrophila*

The significant increase in antibody titers from week 3 to week 6 indicates a strong secondary immune response, indicating the formation of immunological memory. This reaction is chiefly facilitated by B lymphocytes, which develop into plasma cells and generate specific antibodies targeting *A. hydrophila*. In teleost fish, immunoglobulin M (IgM) is the primary antibody responsible for humoral immunity, essential for pathogen neutralization and elimination (Magnadóttir, 2006; Uribe et al., 2011).

The increased antibody production noted in this study illustrates the efficacy of the inactivated vaccination in eliciting adaptive immunological responses. The results align with other research indicating that vaccination against *A. hydrophila* markedly elevates antibody levels and enhances illness resistance in goldfish (Semwal et al., 2023; Han et al., 2019; Akmal et al., 2020; Thangaviji et al., 2012). Although the antibody titer in the 1×10^8 cells/ml group was the highest, it was not significantly different from that of the 1×10^6 cells/ml group. The vaccine concentration of 1×10^6 cells/ml seems to be the most suitable for practical application in aquaculture systems, taking into account both immune response and growth performance.

Conclusion

This study demonstrated that vaccination with an inactivated *A. hydrophila* vaccine effectively enhanced the immune response of goldfish (*C. auratus*) without causing adverse effects on hematological parameters. Among the tested concentrations, 1×10^6 cells/ml resulted in the highest growth performance (0.91 ± 0.04 g/day) and induced a strong antibody response comparable to that of the highest concentration (1×10^8 cells/ml). Hematocrit values did not differ significantly among treatments, indicating that the vaccine was physiologically safe for the fish. The stable hematocrit levels may indicate normal blood regulation and the absence of severe stress or adverse effects following vaccination. In contrast, antibody titers increased significantly following booster vaccination, reflecting enhanced adaptive immune response and immunological memory. The results indicate that a vaccination concentration of 1×10^6 cells/ml is ideal for optimizing immunological stimulation and growth performance in goldfish. This concentration is thus advised for practical use in ornamental aquaculture. Although vaccination is an effective strategy for disease prevention, its application in the ornamental fish industry remains limited due to economic constraints

and labor-intensive administration methods. Future studies should focus on improving vaccine delivery strategies, such as oral or immersion methods, and evaluating the use of adjuvants to enhance vaccine efficacy and practicality.

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Research article

Immunological and Hematological Responses of Goldfish (*Carassius auratus*) Following Vaccination Against *Aeromonas hydrophila*

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ABSTRACT

Due to the increasing prevalence of *Aeromonas hydrophila* infections in ornamental fish culture, the development of effective preventive strategies has become increasingly important. In this study, a vaccine against *A. hydrophila* was developed and evaluated in goldfish (*Carassius auratus*) with an average body weight of 47.40 ± 1.95 g. The fish were randomly assigned to five experimental groups based on vaccine concentration: control (0), 1×10^2, 1×10^4, 1×10^6, and 1×10^8 cells/ml. All groups, except the control group, received vaccinations derived from *A. hydrophila*. The results indicated that the group inoculated with 1×10^6 cells/ml exhibited the highest specific growth rate, at 0.91 ± 0.04 g/day. Hematocrit (%Hct) values showed no significant differences among the treatment groups ($p > 0.05$). The antibody titer in the 1×10^6 cells/ml group was 3.19 ± 0.06, which did not differ significantly from that in the 1×10^8 cells/ml group (3.22 ± 0.03), the highest value recorded in this study. Therefore, the optimal vaccine concentration was determined to be 1×10^6 cells/ml.

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