Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(5): 3139 – 3152 (2025)
www.ejabf.journals.ekb.eg

Evaluation of Immune-Related Gene Expression in the Common Carp After Immunization with an Autogenous Vaccine Derived from a Local Isolate of *Aeromonas hydrophila*

Reem Zaidan Mukhlif AL-Sugmiany*, Agharid A. AL-Rasheed
Department of Microbiology, College of Veterinary Medicine, University of Tikrit, Tikrit, Iraq
***Corresponding Author: rz230005pve@st.tu.edu.iq**

ARTICLE INFO

Article History:
Received: July 20, 2025
Accepted: Sep. 25, 2025
Online: Oct. 25, 2025

Keywords:
Aeromonas hydrophila, vaccination route, formalin-killed vaccine, immune-related genes, *Cyprinus carpio*

ABSTRACT

In the absence of commercial vaccines, autogenous vaccines derived from local pathogen isolates represent a practical and cost-effective approach to disease prevention in aquaculture. We evaluated anti-inflammatory and humoral responses following vaccination with a formalin-killed *Aeromonas hydrophila* (FKAH) vaccine delivered either by intraperitoneal injection (VI) or spray (VS), followed by an experimental challenge. FKAHCs were prepared by treating a local *A. hydrophila* isolate with 0.5% neutral-buffered formalin. Common carp received doses on days 0, 14, and 28. On day 46, a subset was challenged intramuscularly with LPAH (0.1mL; 2.3×10^6 CFU/mL). Liver, gill, and blood were collected for mRNA expression of IL10, IgT, and IgM genes in affected groups were analyzed using the Kruskal–Wallis test with post-hoc comparisons; temporal and organ patterns were assessed across days 14, 28, and 42. Infection alone strongly suppressed IL-10 and showed route-dependent polarization (P= 0.038): VS drove additional suppression post-challenge, while VI shifted to IL-10 upregulation after challenge, indicating anti-inflammatory recall. IgM increased with both routes (P= 0.037), maximally with VS pre-challenge, but VS responses contracted after challenge; VI sustained or increased IgM post-challenge. IgT amplification was robust and largely route-independent (P= 0.558 overall), with stable post-challenge levels, especially high in the gills, suggesting conserved mucosal protection. These results indicate that the delivery route shapes distinct immune architectures: VS biases toward pro-inflammatory recall with transient IgM, while VI favors IL-10-linked anti-inflammatory control with sustained antibodies. IgT provides a stable, route-independent mucosal response.

INTRODUCTION

The common carp (*Cyprinus carpio*) is one of the most important freshwater species in global aquaculture, contributing substantially to food security. However, its intensive culture is threatened by infectious diseases, particularly those caused by *Aeromonas hydrophila*, which induces motile aeromonad septicemia and leads to high mortality and severe economic losses **(Pridgeon & Klesius, 2013)**. One of the more important methods that has changed the way we investigate fish immune responses is gene expression analysis **(Jaies *et al.*, 2024, Mariam *et al.*,**

2025).The increase in the expression of immune genes is usually considered as a sign immune stimulation or enhanced immune response **(Abo-Al-Ela, 2018)**. The immune response against bacterial antigens involve the activation of innate immunity (e.g., antimicrobial peptides and pro-inflammatory cytokines and alongside regulatory cytokines (IL-10). Furthermore, immunoglobulins IgM and IgT mediate systemic and mucosal adaptive immunity in fish **(Yu *et al.,* 2020; Jeong *et al.,* 2025, Mahmood, *et al.,* 2025)**. In the absence of commercial vaccines, inactivated autogenous homogeneous vaccines (from killed bacterial strains) are a practical and low-cost option. They can be rapidly customized to target problematic infections or respond rapidly to emerging pathogens of particular concern **(Ma *et al.,* 2019)**. Protection can be enhanced by adopting a method that increases the contact time of the vaccine with the fish **(Harrell *et al.,* 1975; Noraini & Sabri, 2013)**. Vaccines are typically administered by injection, immersion / spray shower, or orally **(Gould *et al.,* 1978)**. High-pressure spray vaccination of fish is a modified form of immersion commonly used for fish caught in nets or shallow raceways. It is economical and can process larger numbers than other immersion techniques **(Colwell & Grimes, 1984; Subramani & Michael, 2017)**. In the fish, the liver plays an important role in innate immunity and growth, supporting host defense and promoting metabolic regulation of effective immune function **(Causey *et al.,* 2018; Mehanna *et al*., 2023)**.

Furthermore, gills, although usually inedible, are among the most important organs of fish due to their crucial physiological and immune functions as the first line of immune defense **(Koppang *et al.,* 2015)**. White blood cells (WBCs) including neutrophils, eosinophils, basophils, lymphocytes, and monocytes were participated in both innate and adaptive immune defenses in vertebrates **(Mohr and Liew, 2007; El Far *et al.,* 2025)**. The WBCs contribute to both innate and acquired immune responses by expressing cell-specific immune-relevant genes **(Abbas *et al.,* 2005; Morera & MacKenzie, 2011; Shen *et al.,* 2018)**.

Accordingly, this study aimed to evaluate the efficacy of autogenous vaccine prepared from formalin-killed local *A. hydrophila* in common carp by both injection and spray administration routes, with a focus on the molecular expression of key immune markers IL-10, IgM, nd IgT in internal organs (liver and gill) and blood tissue. This approach is expected to contribute to the development of practical immunization strategies that enhance fish health and reduce economic losses in aquaculture.

MATERIALS AND METHODS

Fish and aquaria management

Clinically healthy common carp (*Cyprinus carpio*) weighing 20–23 g were purchased from a local supplier (Salah Al-Deen, Iraq). Fish were acclimatized for 14 days in glass aquaria (60 × 40 × 30 cm^3 for experimental groups; 40 × 25 × 23 cm^3 for controls). Aquaria were filled with dechlorinated tap water (20 cm depth) and continuously aerated, with more than 75% of water exchanged daily. Fish were fed commercial pellets every two days. Water parameters were maintained at 24– 26°C, dissolved oxygen at 8– 10ppm, and pH at 7.0–7.5., all parameters were

monitored at the College of Education for Pure Sciences, Tikrit University. Randomly selected fish were confirmed free of *A. hydrophila* **(Ramos-Espinoza *et al.*, 2020)**. Anesthetization for fish was performed using clove oil **(Hajek *et al.*, 2006; Fernandes *et al.*, 2017)** before injection.

Bacterial isolate and activation

Local *A. hydrophila* isolate (LAH) was obtained from Microbiology, College of Veterinary Medicine, Tikrit University. The strain, originally isolated from infected common carp, was identified by biochemical and molecular assays, including detection of toxin genes (hly, aerolysin, act) **(Weli, 2024)**. The isolate was further confirmed as *A. hydrophila* by growth on *Aeromonas* agar base (Dinkelberg Analytics, Germany) followed by positive catalase and oxidase tests **(MacFaddin, 2000)**. The AH isolate was activated in Brain Heart Infusion BHI (Himedia, India), A suspension of LAH was subjected to centrifugation and PBS washing; turbidity was adjusted to McFarland 4 (~1.2×10^9 CFU/mL). The AH was activated *in vivo* according to **Mateos and Paniagua**'s **(1995)** method with semi modification. The common carp were firstly anesthetized and intramuscularly injected with 1.2×10^9 CFU/mL of activated LAH. After passage (3 fish/pass), a local passage AH was cultured in BHI overnight at 37°C and was then maintained with 30% glycerol at −20°C.

Autogenous Vaccine preparation

Formalin-killed *Aeromonas hydrophila* cells (FKAHCs) were prepared according to prior research **(Noraini & Sabri, 2013; Radhakrishnan *et al.*, 2023)** by killing local passage *A.hydrophila* (LPAH) in 0.5% neutral-buffered formalin in PBS, and stored overnight at 4°C. Killing was confirmed by plating on AAB at 37°C for 48h (no growth), succeeded by washing the pellets after centrifugation performed 3× in PBS (centrifuge 3,000 × g, 15min each) to remove residual formalin. The deactivation was confirmed by spreading the culture on *Aeromonas* agar plates, subsequently the cells were resuspended in sterile PBS; turbidity was adjusted by McFarland to 1.2×10^9 CFU/mL; stored at 4°C.

FKAHCs administrations

The FKAHCs suspension at a concentration of 1.2×10^9 CFU/mL was diluted in sterile PBS to a final concentration of 1.2×10^8 CFU/mL. For injection, 0.1mL of inactivated LPAH was administered. According to **Noraini and Sabri (2013)** with modification, the spray vaccine was prepared at a concentration of 1.2×10^8 CFU/mL. Fish were sprayed from head to tail, following the procedure described by **Gould *et al.* (1978)**.

Experimental groups & schedule

1. Control groups: (n = 10/group): No vaccine, no infection (C). Used for negative and infected group(I).
2. Vaccinated groups (n = 20/group); vaccination timeline: Days 0, 14, and 28.

a. Injection (Intraperitoneal) subgroup (VI).
b. Spray subgroup (VS).

3. Challenge groups (TVI,TVS): On day 46, fish were challenged intramuscularly with *A. hydrophila* (LPAH) at 2.3×10^6 CFU/mL.
4. Sampling for gene expression: Days 0, 14, 28 and 14 days post-challenge.

Sampling and molecular procedures

Fish in the experimental groups (C, I, VI, VS, TVI, and TVS) were anesthetized and blood was collected by caudal transection, stabilized immediately in TRIzol; liver and gills were snap-frozen in liquid nitrogen, ground, and homogenized in TRIzol.

Analysis of the expression of immune genes

Total RNA was extracted from liver, gills, blood tissues using Trizol reagent (Invitrogen, USA), and residual genomic DNA was removed with RNase-free DNase I (Life Technologies, USA). cDNA was synthesized using a Reverse Transcription Kit (Proma, USA) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed with Master SYBR Green Mix (PCM08, Proma, USA) on a Real-Time PCR system using gene-specific primers (Table 1).The sequences were designed by Dr. Ahmed Abdul-Jabbar Suleiman in the molecular biology laboratory at the College of Science, University of Anbar using the Primer3 program. β-actin was employed as the internal reference gene, and relative expression levels were calculated using the 2^−ΔΔCt method.

Table 1. Primer sequences used for gene expression analysis

Gene	Sequence	Annealing temp (c)	Product size(bp)
IL10	F:AACGAGATCCTGCGCTTTTA R:TATCCCGTTGAGATCCTGA	58	121
IgM	F:AGTGGCCTAGTGTCCTCCAC R:GCTGGCTCATGCTGTTTGTA	58	161
Igt	TATGCAGCATTCGGGAGTGG AGGAACCAAGCTCAGGGTTG	60	171
B_Actin	F:CTCTTCCAGGCCTTCCTTCC R:CTTCTGCATACGGTCAGCAA	58	165

Statistical analysis

Analyses were performed using Python. Gene expression was logarithmically transformed ($\log_2$), and non-parametric methods were used to account for non-normal distribution. The Kruskal-Wallis equation was used to test for multiple group differences, and Mann-Whitney U pairwise comparisons were performed when statistically significant. Data were presented as median with interquartile range (IQR). For pre-/post-challenge comparisons, pairwise tests were used whenever possible. Statistical significance was set at $P < 0.05$, with values reported with three-digit precision. The temporal dynamics analysis examined expression changes across days 14, 28, and 42 post-vaccination as well as after challenge, with percentage changes calculated relative to baseline measurements to assess kinetic response patterns across different organs (liver organ A, gill organ B, blood organ C) and vaccination routes.

RESULTS

Molecular diagnosis of immune-related genes in infected group

IL10 (Anti-inflammatory Cytokine)

Overall treatment effects (Fig. 1-A)

IL10 expression differed significantly among groups (Kruskal–Wallis, *P*= 0.038). Controls showed baseline suppression (median –0.916 $\log_2$), and infection further reduced IL10 (median –2.053, *P*= 0.126). Vaccination also yielded suppression: VS median –1.557 (*P*= 0.526) and VI median –1.847 (*P*= 0.920). Post-hoc tests identified VS differing from control (*P*= 0.003) and VI (*P*= 0.009), consistent with a distinct VS regulatory signature. Notably, the challenged injection group (TVI) upregulated IL10 (median +0.270, *P*= 0.005), differing from controls (*P*= 0.002), suggesting anti-inflammatory activation after VI + challenge.

Organ-specific patterns (Fig. 1-B)

Liver (Organ A) showed highly significant effects (*P*= 0.005): Controls remained suppressed (–2.25 $\log_2$), VI was further suppressed (–4.09), while TVI reversed to upregulation (+0.28). In gill (Organ B), differences were significant (*P*= 0.037): VI was enhanced (+1.21), and TVI maintained mild enhancement (+0.14). Blood showed no significant effects (*P*= 0.483), yet TVI again showed enhancement (+0.29).

Pre- vs post-challenge (Fig. 1-C)

VS decreased further post-challenge (–1.557 → –1.909, *P*= 0.009; 556% reduction), consistent with pro-inflammatory bias. VI shifted from suppression to mild enhancement (–1.847 → +0.270, *P*= 0.714; 115% increase).

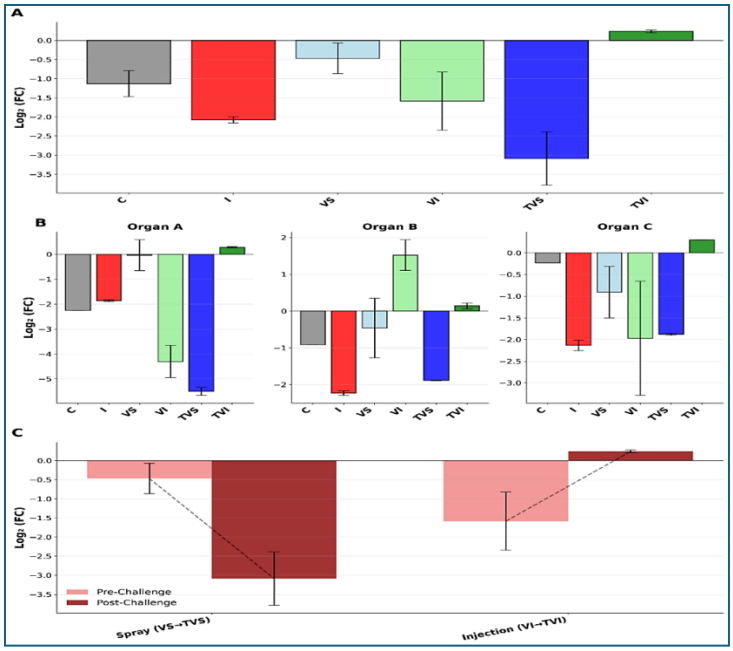

Fig. 1. *IL10* expression analysis showing: A) Gene expression overview of overall intervention effects across all groups, B) Organ-specific expression patterns of tissue-dependent immunization efficacy, and C) Pre- vs post-challenge comparison of immunization response dynamics. All plots show $\log_2$ fold change values with error bars representing SEM. Liver organ A),gill (organ B) and blood (C).

Temporal dynamics (Fig. 2)

In liver, VS showed early transient activation (+0.90 at day 14) followed by suppression (–1.40 at day 42), while VI showed sustained inhibition (–4.09 → –2.51 → –6.33). Gill fluctuated (VI +2.89 at day 14 → +0.46 at day 28 → +1.21 at day 42), and blood oscillated across routes.

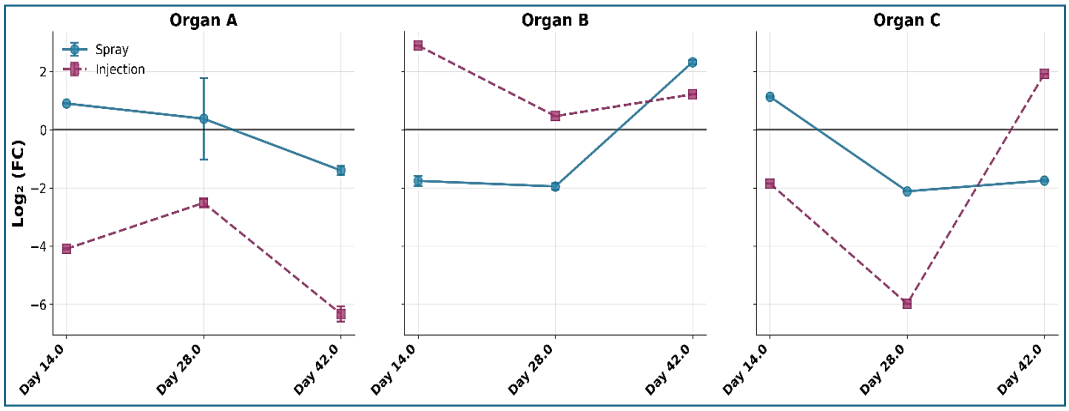

Fig. 2. Temporal dynamics of *IL10* expression during immunization showing the response kinetics over time for each organ separately. Each panel represents one organ (A liver, B gill, C blood)) with measurements at Days 14, 28, and 42 post-vaccination. Solid blue lines represent spray vaccination (VS), dashed purple lines represent injection vaccination (VI). Error bars indicate SEM. Circles mark spray route measurements, squares mark injection route measurements.

IgM (Systemic Antibody)

4.x.1 Overall treatment effects (Fig. 3-A)

IgM differed significantly (Kruskal–Wallis, *P*= 0.037). Controls were near baseline (median –0.315), while infection elevated IgM (median +1.555, *P*= 1.000), contrasting with IL10 suppression. Both vaccinations increased IgM: VS +4.332 (*P*= 0.012) and VI +2.080 (*P*= 0.368). Post-hoc: VS vs VI (p = 0.016) and VS vs TVS (p = 0.012) were significant, identifying VS as the strongest inducer.

Organ-specific patterns (Fig. 3-B)

Liver showed moderate effects (*P*= 0.064): infection +7.52; VS +3.92; challenged VS switched to –1.59. Gill showed the strongest effect (*P*= 0.023), with VS +7.27 (the highest overall). Blood was non-significant (*P*= 0.769), though TVI maintained high IgM (+4.33).

Pre- vs post-challenge (Fig. 3-C)

VS declined significantly +4.332 → –1.591 (*P*= 0.012; 108% reduction), indicating contraction after recall. VI rose +2.080 → +3.180 (*P*= 0.617; 69% increase), suggesting maintained antibody memory.

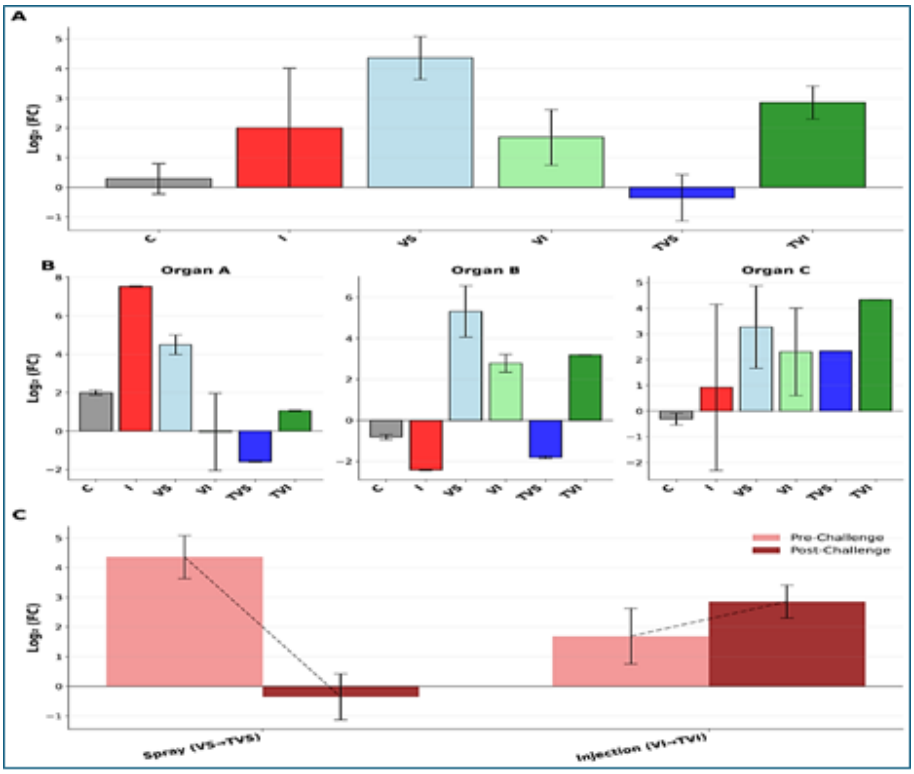

Fig. 3. Comprehensive analysis of *IgM* expression in immunization study: A) Gene expression overview of overall intervention effects across all groups, B) Organ-specific expression patterns of tissue-dependent immunization efficacy, and C) Pre- vs post-challenge comparison of immunization response dynamics. All plots show $\log_2$ fold change values with error bars representing SEM. Liver (organ A), gill(organ B) and blood (organ C).

4 Temporal dynamics (Fig. 4)

VS in liver: early elevation (+3.80 day 14), peak (+5.75 day 28), stabilization (+3.92 day 42). VI in liver: suppression (–3.91 day 14) → sharp peak (+6.91 day 28) → decline (–3.11 day 42). In gill, VS stayed high on days 14–28 (+7.27 → +7.66) then fell (+1.01 day 42); VI fluctuated within a narrower range (+4.30 → +2.01 → +2.05). Blood varied across time.

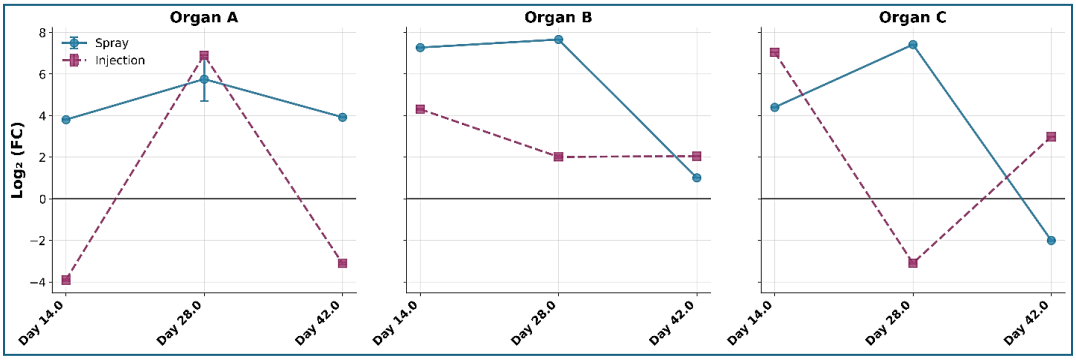

Fig. 4. *IgM* expression analysis during immunization showing the response kinetics over time for each organ separately. Each panel represents one organ (A, B, C) with measurements at Days 14, 28, and 42 post-vaccination. Solid blue lines represent spray vaccination (VS), dashed purple lines represent injection vaccination (VI). Error bars indicate SEM. Circles mark spray route measurements, squares mark injection route measurements.

IgT (Mucosal Antibody)

4.1 Overall treatment effects (Fig. 5-A)

IgT showed moderate, biologically meaningful differences (Kruskal–Wallis, *P*= 0.558). Controls were variably elevated (median +2.639), while infection reduced IgT (median –1.588, *P* = 0.818). Both routes amplified IgT: VS +4.822 (*P*= 0.102) and VI +4.632 (*P*= 0.194), with no route difference (*P*= 0.714). Challenged groups retained amplification (TVS +3.712, *P*= 0.394; TVI +3.509, *P*= 0.247), consistent with stable IgT memory.

4.2 Organ-specific patterns (Fig. 5-B)

Liver showed strong effects (*P*= 0.041): infection +7.91; VS variable (median –1.16), recovering after challenge (+3.71). Gill exhibited consistent amplification (*P*= 0.105), with VS +7.64 (highest among tissues). Blood was non-significant (*P*= 0.197), though both challenged groups remained elevated (TVS +1.16; TVI +2.35).

4.3 Pre- vs post-challenge (Fig. 5-C)

IgT remained route-independently stable. VS: +4.822 → +3.712 (p = 0.526; 9.2% increase). VI: +4.632 → +3.509 (p = 0.434; 71% relative increase).

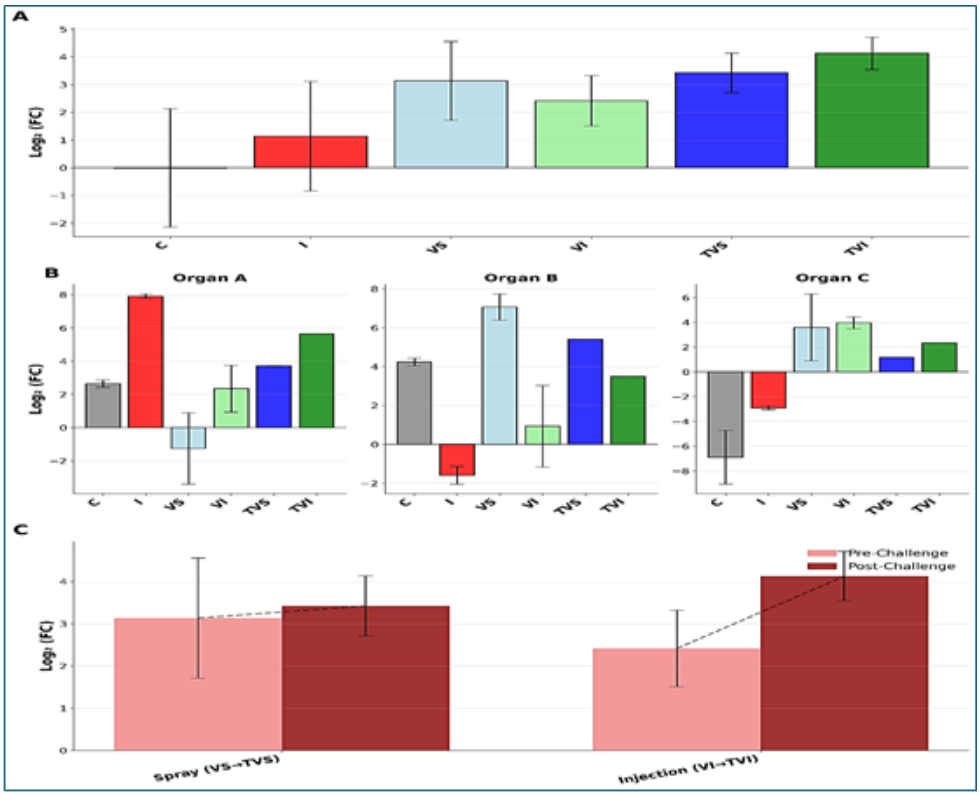

Fig. 5. *IgT* expression analysis showing: A) Gene expression overview of overall intervention effects across all groups, B) Organ-specific expression patterns of tissue-dependent immunization efficacy, and C) Pre- vs post-challenge comparison of immunization response dynamics. All plots show log_2 fold change values with error bars representing SEM. Liver organ A),gill(organ B)and blood (organ C).

Temporal dynamics (Fig. 6)

In liver, VS transitioned from suppression (–6.00 day 14) to recovery (–1.33 day 28) and strong increase (+3.57 day 42). VI showed earlier amplification: –2.54 (day 14) → +4.95 (day 28) → +4.63 (day 42). In gill, VS remained high (+4.82 → +8.72 by day 42), while VI fluctuated (+7.90 → –4.31 → –0.81).

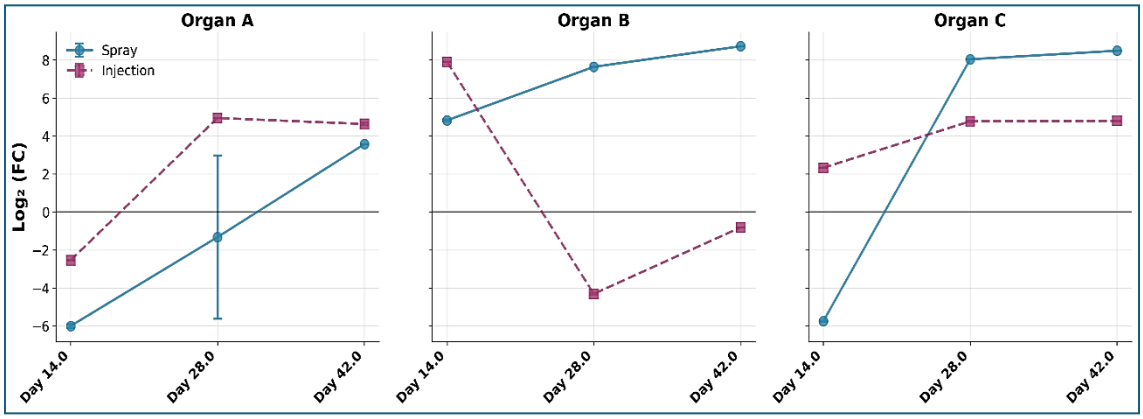

Fig. 6. *IgT* expression during immunization period showing the response kinetics over time for each organ separately. Each panel represents one organ (A liver, B gill, C blood) , with measurements at Days 14, 28, and 42 post-vaccination. Solid blue lines represent spray vaccination (VS), dashed purple lines represent injection vaccination (VI). Error bars indicate SEM. Circles mark spray route measurements, squares mark injection route measurements.

DISCUSSION

In this study, inactivated vaccine was prepared from a local isolate of *A. hydrophila*, isolated from infected fish and then activated within the fish by passage. This enables a faster and more appropriate response than commercial vaccines designed for geographically or temporally distant reference strains. At the level of gene expression, our indicators showed pathway- and organ-dependent variation. For the regulatory cytokine IL-10, the spray (VS) approach trended toward a post-recall downregulation, whereas the injection (VI) approach showed a delayed enhancement. These patterns indicate a pathway-dependent immune polarization, with VI toward anti-inflammatory responses, and VS skewed toward pro-inflammatory responses, highlighting the influence of exposure route on immune outcome.IL-10 levels were affected by both the pathway and the organ, with VI showing an increase after challenge, while VS showed inhibition. These differences suggest a pathway-dependent regulation of IL-10. These results are consistent with patterns of cytokine regulation in mucosal and parenteral immunization across multiple tissues **(Korytář *et al.*, 2019; Yang *et al.*, 2021; Yuan *et al.*, 2022; Zhang *et al.*, 2023)**. For IgM, spray (VS) appeared to stimulate the gills early and clearly, then declined after the challenge dose, whereas injection (VI) tended to maintain the response for a longer period. In general, the VS pathway induces a rapid but short-lived activation, while VI supports a more sustained response, suggesting different motor patterns. This aligns with the concept that mucosal immunization pathways: A strong local release but not sustained systemically unless reinforced by mechanisms that enhance antigen capture and presentation **(Huising *et al.*, 2003; Najeeb *et al.*, 2025)**. It is also consistent with the mid-trial peak timing of humoral response maturation in fish, and with the fact that IgM is the major systemic isotype **(Zhang *et al.*, 2010; Parra *et al.*, 2015; Jiang *et al.*, 2016)**. In contrast, the liver signal was less conclusive, and the blood signal remained largely insignificant, suggesting a difference in signaling pathways between mucosal and systemic pathways, while acknowledging the role of the immune liver and the presence of IgM in the liver and bile **(Løvoll *et al.*, 2010; González-silvera & Cuesta, 2021)**. IgM-producing B cell memory formation and plasmacytosis within a time frame corresponding to the follow-up period explains the tendency for a relatively longer-lasting response with post-recall injections **(Parra *et al.*, 2015; Chan *et al.*, 2024)**.

On the other hand, IgT exhibited a more stable behavior across both pathways and persisted after challenge. This stability contrasts with the decrease in IgM and suppression of IL-10 observed in VS, suggesting that mucosal IgT responses are maintained independently of the route. It reflecting the integration of mucosal and systemic immunity and the broad contribution of IgT upon repeated stimulation, with its normal localization in epithelial barriers such as gills and skin **(Zhang *et al.*, 2010; Sitjà-bobadilla, 2016; Yu *et al.*, 2020; Salinas *et al.*, 2021)**. Following the challenge dose, the results demonstrate a practical trade-off between the two pathways: VS provides a rapid mucosal burst followed by contraction, while VI supports longer systemic maintenance, with IgT remaining stable across both pathways as a complementary mucosal substrate. From an applied perspective, this picture suggests the feasibility of a heterogeneous

prime–boost (VS vers VI) design to balance rapid onset and durability of protection, or to adopt VI when systemic persistence is an operational priority.

From an applied perspective, a heterologous prime–boost (VS→VI) may balance rapid onset and durability, whereas VI alone is suitable where sustained systemic protection is prioritized. Limitations include non-significant contrasts in liver and blood, statistical power, and the local-isolate focus which may narrow applicability; future work should link gene-expression endpoints to functional protection.

CONCLUSION

There is lack of information regarding the *Aeromonas hydrophila* linked to the diseases affecting farmed carp in the Salah Al-Din Governorate Iraq, which may lead to *A. hydrophila* growing in ponds, resulting in a high risk of fish infection and substantial financial losses in the aquaculture sector. Therefore, locally produced vaccines from local isolates are needed to match field-circulating strains and curb disease spread and its economic impact. Monitoring gene expression (IL-10/IgM/IgT) provides a realistic representation of tissue-specific immune dynamics and helps evaluate vaccine efficacy in aquaculture.

ACKNOWLEDGEMENT

The authors would like to acknowledge the staff of Molecular Biology Laboratory at the College of Science, Tikrit University for providing the facilities and technical support for the gene expression analyses .

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