

# Expression analysis and tissue localization of IgZ in the grouper *Epinephelus coioides* after *Vibrio alginolyticus* infection and vaccination

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## Short report

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# Abstract

## Background

The orange-spotted grouper (*Epinephelus coioides*) is an important marine farmed fish in China. It is affected by the bacterial pathogen *Vibrio alginolyticus*, which causes high mortality and substantial economic losses.

## Results

We studied the transcriptional changes of the IgZ gene in *E. coioides* following *V. alginolyticus* stimulation and investigated the distribution of IgZ in different tissues. The highest expression level of IgZ occurred in the head kidney. When fish were exposed to live and inactivated *V. alginolyticus*, the expression levels of IgZ in head kidney, spleen, intestine, gill, and blood cell were significantly upregulated. In an *in situ* hybridization study, IgZ mRNA-positive cells were detected in head kidney, spleen, and gill, but positive signals were not detected in liver and intestine. IgZ-labeled cells increased in the head kidney, spleen, and gill post-infection with *V. alginolyticus* for 21 days.

## Conclusions

The present study provided additional evidence that IgZ was involved in mucosal immune responses against pathogens. These results help explain the role of IgZ in *E. coioides* defense against *V. alginolyticus* infection.

## Background

Groupers (*Epinephelus*: Serranidae) are economically important marine fish [1, 2]. Over 159 grouper species are distributed in tropical and subtropical areas throughout the world. The orange-spotted grouper (*Epinephelus coioides*), brown marbled grouper (*Epinephelus fuscoguttatus*), and giant grouper (*Epinephelus lanceolatus*) are the most valuable aquaculture species in Asia and China [3]. The total production of farmed groupers in 2019 was 183127 tons with an increase of 15% over 2018 [4].

Grouper farming suffers from diseases caused by bacteria, viruses, and parasites and this hinders the efficient production, development, and sustainable expansion of this industry [2, 3]. Vibriosis is a common bacterial disease that affects all stages of grouper growth and accounts for about 66.7% of all grouper diseases. Vibriosis can result in up to 50% mortality [5]. Several species, including *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio vulnificus*, and *Vibrio carchariae* are pathogenic to groupers [6].

Innate and adaptive immunities play an important role in protecting hosts against invasive pathogens [7]. Immunoglobulins are a central component of adaptive immunity in vertebrates and they are composed of

two heavy chains (H) and two light (L) chains (H<sub>2</sub>L<sub>2</sub> unit) [8]. In mammals, five immunoglobulin (Ig) isotypes (IgD, IgM, IgA, IgG, and IgE) have been reported, while in teleost fish B lymphocytes express three classes of immunoglobulins IgM, IgD, and IgZ [9]. IgZ, also called IgT, was first identified in 2005 in zebrafish [10] and rainbow trout [11]. Other IgZ/IgT sequences have been identified in various teleost fish since then [12]. Protein characterization and functional localization studies have demonstrated that IgZ is mainly involved in mucosal immunity, which is similar to the function of mammalian IgA [13]. Recent studies have shown that the immune response of IgZ in fish against pathogen infection might not be restricted to the mucosal tissues [14]. The heavy chain genes of IgZ have been cloned in *E. coioides* [15] and another study expressed the recombinant IgZ heavy-chain protein and prepared the anti-IgZ polyclonal antibody [16]. However, the induction of IgZ gene expression in immune organ tissues after infection or immunization with *Vibrio* is unclear.

In the present study, we studied the transcriptional changes of the IgZ gene in *E. coioides* following *V. alginolyticus* infection and stimulation with inactivated bacterial pathogen using quantitative real-time PCR. The localization of IgZ positive cells in different organs were studied using a sensitive in situ hybridization method. The distribution pattern of IgZ in different tissues, in response to *V. alginolyticus* infection, was determined using immunohistochemical techniques.

## Materials And Methods

### Fish and sample preparation

A total of 200 *E. coioides* were obtained from Dayawan Aquaculture Center of Guangdong province, China. The mean body length of these fish was  $25 \pm 3$  cm, and the mean body weight was  $250 \pm 50$  g. All fish were allowed to adapt for 6 d in 100-L opaque tanks (10 fish per tank) supplied with re-circulated seawater. Water temperature was maintained at  $27 \pm 1^\circ\text{C}$  with an air conditioner. For the tissue-specific expression study, healthy fish were anesthetized with 150 mg/L tricaine methanesulfonate (MS-222, Sigma), and then head kidney, spleen, thymus gland, intestine, gill, blood, heart, muscle, and gonad were collected, immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

### RNA extraction and complementary DNA preparation

Total RNA was extracted from grouper tissues using TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to manufacturer instructions. The RNA was quantified using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the first complementary DNA (cDNA) was synthesized with a M-Mulv Reverse Transcriptase cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer protocol. The first cDNA was stored at  $-20^\circ\text{C}$  prior to use.

### Preparation of *V. alginolyticus*

The *V. alginolyticus* strain was obtained from the Institute of Hydrobiology, Jinan University (Guangzhou, China). The strain was recovered at  $30^\circ\text{C}$  for 18 h on a medium plate, then a single colony was picked

and inoculated into 5 mL broth at 28°C for 18 h in a shaker incubator at 150 rpm. The harvested *V. alginolyticus* cells were centrifuged at 10000×g for 10 min, the supernatant was removed, and *V. alginolyticus* was washed three times with 1% sodium chloride (NaCl). The *V. alginolyticus* was resuspended in 1% sodium chloride with a concentration of  $5.0 \times 10^4$  CFU/mL and stored at 4°C prior to use.

To prepare inactivated *V. alginolyticus*, 10 mL of the bacteria solution was obtained as above. The concentration of *V. alginolyticus* was determined by a plate count method. Then 0.5% formalin was added to inactivate the bacteria. The inactivated *V. alginolyticus* was centrifuged and washed as described above. Finally, *V. alginolyticus* was resuspended in 1% sodium chloride at a concentration of  $2.0 \times 10^5$  CFU/mL.

### ***Vibrio alginolyticus* stimulation and IgZ expression analysis**

To analyze the IgZ expression induced by *V. alginolyticus* infection, 90 fish were randomly transferred into six 100-L tanks (10 fish per group) each containing 80 L seawater. Fish in the infection treatment were injected with 100 µL *V. alginolyticus* solution at a concentration of  $5.0 \times 10^4$  CFU/mL by intraperitoneal injection. Fish in the control treatment were injected with 100 µL 1% NaCl solution. Five fish were randomly sampled at 0 h, 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 5 d, 6 d, 14 d, 21 d, and 28 d post-infection. The head kidney, spleen, thymus gland, intestine, gill, blood, and heart were collected after fish were anesthetized with 150 mg/L MS-222. All samples were immediately frozen in liquid nitrogen and stored at -80°C for total RNA extraction.

To analyze the IgZ expression stimulated by the inactivated *V. alginolyticus*, 90 fish were randomly transferred into seven 100-L tanks (10 fish per group) containing 80 L seawater. Fish in the infection treatment were injected with 100 µL inactivated *V. alginolyticus* solution at a concentration of  $2 \times 10^5$  CFU/mL by intraperitoneal injection. Fish in control treatment were injected with 100 µL 1% NaCl solution. Five fish were randomly sampled at 0 h, 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 5 d, 6 d, 14 d, 21 d, 28 d, and 35 d post-stimulation. The head kidney, spleen, thymus gland, intestine, gill, blood, and heart were collected after fish were anesthetized with 150 mg/L MS-222. All samples were immediately frozen in liquid nitrogen and stored at -80°C for total RNA extraction.

IgZ expression in head kidney, spleen, thymus gland, intestine, gill, blood, and heart on different days after *V. alginolyticus* stimulation was analyzed by real-time quantitative PCR (qPCR). All samples had five replications, and the amplification was carried out in iCycler iQ Real-time PCR Detection System (Bio-Rad, Hercules, California, USA) with an initial denaturing step of 95°C for 3 min, followed by 40 cycles of 94°C for 20 s, 60°C for 20 s, 72°C for 30 s. To ensure only one specific-sized single amplicon was amplified, the specificity of the primer was determined by a dissociation curve. The relative quantification of the IgZ gene was normalized with an internal control (b-actin).

### ***In situ* hybridization**

A digoxigenin (DIG)-labeled RNA probe and control probe for IgZ were prepared according to the method described by Sato et al. [17]. The head kidney, spleen, intestine, gill, and liver samples were directly placed into 4% paraformaldehyde (PFA) to remove the residual blood. The samples were transferred into 4% PFA with 10 times volume and fixed for 24 h at 4°C. Then, the samples were treated with four processes: (1) dehydration and embedding, (2) tissue sectioning, (3) prehybridization, and (4) hybridization. The more detailed process was similar to a previous study [18].

### **Immunohistochemistry on paraffin sections**

The anti-IgZ polyclonal antibody used in immunohistochemical procedures was obtained as in a previous study [16].

The head kidney, spleen, intestine, gill, and heart samples were directly placed into 4% PFA to remove the residual blood. The fixation, dehydration, embedding, sectioning, and deparaffinization of tissues were conducted as described above. Antigen retrieval was performed by boiling slides in citrate antigen retrieval solution (sodium citrate 21.01 g, citric acid 26.41 g, H<sub>2</sub>O 1000 mL, and pH 6.0) in a pressure cooker for 6 min. Endogenous peroxidase activity was quenched by incubating sections in 1.5% H<sub>2</sub>O<sub>2</sub> for 10 min. The sections were incubated in blocking solution for 15 min at room temperature to block non-specific binding of antibodies. Antibodies at a dilution (in blocking solution) of 1:1000 were added to react for 2 h at 37°C. The sections were washed three times in 1×PBS for 3 min. Thereafter the slides were incubated for 15 min at 37°C in secondary antibody (goat anti-rabbit antibody) and then washed times in 1×PBS for 3 min. Bound antibody was visualized by incubating slides in DAB for 3 min at room temperature. All sections were counterstained with Ehrlich hematoxylin for 30 s and subsequently washed with dilute ammonia water, followed by washing with water. All sections were dehydrated in graded ethanol series, followed by treating in xylene. The sections were mounted with neutral balsam. All sections were examined using a Nikon 80i microscope (Japan).

### **Statistical analysis**

Relative gene expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method [19]. The assumptions of normality and homogeneity of variances were confirmed using the Shapiro-Wilk test and Levene's test, respectively. One-way ANOVA was used to analyze the data in SPSS 19.0 software. The results were considered statistically significant at  $p < 0.05$  and  $p < 0.01$ .

## **Results**

### **Distribution analysis of IgZ**

The tissue distribution analysis indicated that head kidney had the highest level of IgZ, followed by blood cells, spleen, gill, and thymus gland. Muscle, gonad, heart showed the lowest expression levels.

### **IgZ expression changes**

When *E. coioides* were injected with *V. alginolyticus*, expression levels of IgZ in head kidney, spleen, thymus gland, intestine, gill, and blood cell were significantly upregulated, with no significant changes in heart. The significant up-regulation of IgZ was first detected in the thymus gland at 3 d, followed by head kidney at 4 d, spleen at 4 d, blood cell at 5 d, gill at 5 d, and intestine at 21 d. IgZ expression peaked at 6 d in gill with a 3.9-fold higher expression compared to the control, followed by spleen at 14 d (5.6-fold), head kidney at 21d (5.5-fold), thymus gland at 21 d (2.5-fold), intestine at 21 d (2-fold), and blood cell at 28 d (6.2-fold). IgZ expression showed no significant changes in gill from 21 d to 28 d post-injection, while it was significantly upregulated in head kidney, spleen, thymus gland, intestine, and blood until 28 d post-injection.

When fish were stimulated with inactivated *V. alginolyticus*, expression levels of IgZ were significantly increased in head kidney, spleen, intestine, gill, and blood cell, with no significant changes in heart and thymus gland. The significant upregulation of IgZ was first detected in gill at 6 d followed by the spleen at 14 d, head kidney at 21 d, intestine at 21 d, and blood cell at 28 d. The highest expression levels of IgZ in gill were detected at 6 d post-stimulation with a 2.6-fold upregulation, followed by spleen at 14 d (3.7-fold), head kidney at 21 d (4.2-fold), intestine at 21 d (1.7-fold), and blood cell at 28 d (5.1-fold).

### **Distribution of IgZ-producing cells**

IgZ-producing cell expression was detected in head kidney, spleen, and gill. The positive cells in head kidney and spleen were mainly distributed around the blood vessels. While in gill, positive cells were located along gill filaments and occasionally in gill lamellae. No positive signals were detected in liver and intestine (data not shown).

### **Immunohistochemistry**

In healthy *E. coioides*, a small number of the IgZ labelled cells were found mainly around the blood vessels in the head kidney and spleen. Positive signals were also detected in gill with a position at the junction of gill filaments and gill lamellae, as well as in the mucous layer of the intestine. At 21 d after groupers were injected with *V. alginolyticus*, the positive signals increased in the head kidney, spleen, and gill, showing an aggregation around blood vessels in head kidney and spleen. No positive signals were detected in the heart.

## **Discussion**

Immunoglobulins play an important immunological role of the adaptive immune system of teleost fish against aquatic pathogens. The nucleotide and protein sequences of IgM, IgZ, and IgD heavy chains in *E. coioides* have been characterized [15] and four isotypes of immunoglobulin light chain genes were described in a previous study [20]. Our previous study examined the expression of IgM gene stimulated by thermal stress, infection, and immunization of *V. alginolyticus*. But there is still limited information on the biological functions of IgZ in response to pathogenic bacteria in *E. coioides*. This study examined the

immune response pattern of IgZ in the process of infection and immunization by *V. alginolyticus* and investigated the localization of IgZ in tissues using *in situ* hybridization and immunohistochemistry.

In healthy *E. coioides*, a high level of IgZ expression was detected in head kidney, blood cells, and spleen, followed by the thymus gland. The expression and distribution pattern of IgT in *E. coioides* was consistent with results in previous studies. For example, in rainbow trout (*Oncorhynchus mykiss*) [21], zebrafish *Danio rerio* [22], flounder *Paralichthys olivaceus* [23], and sea bass *Dicentrarchus labrax* [12], IgZ was also mainly found in the primary and secondary lymphoid tissues, such as head kidney and spleen, respectively. The head kidney of teleost fish represents the primary systemic immune organ involved in production of antibodies and other immune cells. It is functionally and morphologically considered to be equivalent to the bone marrow in higher vertebrates [24]. *In situ* hybridization results showed that IgZ mRNA-positive cells were present in head kidney and spleen, and the immunohistochemistry results demonstrated a strong positive signal of IgZ in head kidney and spleen. IgZ<sup>+</sup> cells were present either in clusters or isolated in the vicinity of melanomacrophage centers (MMCs) and small blood vessels. After *Vibrio* infection, the levels of IgZ increased significantly in the head kidney and spleen, and IgZ tended to be distributed surrounding the MMCs and the blood vessels. This association between IgZ, MMCs, and blood vessels indicated that IgZ might cooperate with MMCs during specific immune responses [25]. Thus, the present results indicate that IgZ might play an important role in the systemic immunity of *E. coioides*.

Gills, the specialized gas-exchange structures, are a mucosal tissue of fish with a similar function to those of the mammalian respiratory mucosa [26]. The gill epithelium contains a gill-associated lymphoid tissue with macrophages/granulocytes, B cells, and T cells, which protect the fish from pathogen infections [27]. IgZ B<sup>+</sup> cell and pathogen-specific IgZ can be induced locally in gill tissue, indicating that the IgZ system plays a key role in mucosal immune responses to pathogens in the gills of teleost fish [26]. The present study was consistent with previous findings. The qPCR, *in situ* hybridization, and immunohistochemistry results demonstrated that IgZ-producing cells were present, and gave strong positive signals, in the gills of *E. coioides*. In healthy *E. coioides*, IgZ-producing cells were mainly detected in gill filament and the base of gill lamella. The staining signals of IgZ in gill filament, especially in gill lamella, increased significantly after *V. alginolyticus* infection. The distribution of IgT-producing cells in the gills of *E. coioides* was similar to that of IgZ in the gills of mandarin fish [28] and sea bass [12]. The increased IgZ positive signal in gills post-pathogen infection might due to the local proliferative of IgZ-producing cells and production of pathogen-specific IgT. Thus, distribution of IgZ<sup>+</sup> cells in gills implied that the mucus-associated immune system plays an important role in protection against pathogens.

The intestine, a mucus-associated immune organ in both teleost fish and mammals, is populated by high densities of bacteria which live in symbiosis with the host's mucosal environment [9]. The intestine is rich in bacterial products derived from commensal bacteria (such as lipopolysaccharide and DNA) and food antigens. Thus, immunoglobulins play a key role in the maintenance of mucosal homeostasis in mammals and teleost fish [9, 13, 29]. Parasite-specific IgZ was detected in the intestine mucus of rainbow trout that survived infection of the intestinal parasite *Ceratomyxa shasta*. IgZ-producing cells penetrated

the intestinal epithelium of the fish, indicating that IgZ was responsible for inducing protection against the parasite infection[30]. In the present study, qPCR, *in situ* hybridization, and immunohistochemistry were conducted to confirm whether IgZ and its transcripts were present in the *E. coioides* intestine. The qPCR result demonstrated the IgZ transcripts were present in the intestine, with a distribution of positive signals in the lamina propria identified by immunohistochemistry. However, no IgZ-producing cells were present in the intestine when examined using *in situ* hybridization. This might be because the level of IgZ mRNA in healthy *E. coioides* is too low to be detected by *in situ* hybridization. While the IgZ-positive signals detected by anti-IgZ antibodies might be that the majority of IgZ in the lamina propria were transported via blood from other organs.

The role of IgZ in coping with parasitic, bacterial, and viral challenges has been studied in several fish species, such as mandarin fish [28], common carp [31], sea bass [12], rainbow trout [13], gilthead sea bream [32], Nile tilapia [33], flounder [23], turbot [34], and rohu [35]. In flounder, the fish were infected and vaccinated with *Edwardsiella tarda* via intraperitoneal injection and immersion, and the IgZ mRNA levels were significantly upregulated in all tested tissues [23]. In sea bass, IgZ expression in the gills and spleen increased significantly after infection with nodavirus [12]. In rohu, IgZ showed early upregulation at 12 h post-*Argulus siamensis* infection in the head kidney, skin, and mucus [36]. These studies revealed that IgZ responses could be simultaneously induced in both mucosal and systemic tissues after pathogen infection and that IgZ might play a more important role in mucosal immunity than in systemic immunity [13, 26]. A previous study investigated the immune gene expressions in grouper larvae induced by bath and oral vaccinations with inactivated nervous necrosis virus. The results showed that significant gene expression of IgZ could be detected in viscera, skin, and gill of bath immunized fish, while IgZ gene expression significantly increased in viscera, skin, and gut of orally immunized fish [37]. In the present study, the response of IgZ was studied in mucosal and systemic immunity and the results demonstrated that the expression of IgZ was significantly increased after *E. coioides* were infected and vaccinated with live and inactivated *V. alginolyticus* by intraperitoneal injection. These results revealed that IgZ is involved in the immune response of *E. coioides* against bacterial pathogens, and both infection and vaccination routes can simultaneously induce systemic and mucosal immune responses. In addition, the upregulation of the IgZ mRNA level in gill was significantly faster than that in the other tissues, suggesting that IgZ could play a predominant role in mucosal immunity in *E. coioides*, which was consistent with results reported in other teleost fishes.

## Conclusions

The results demonstrated expression changes of IgZ following bacterial infection and vaccination. The *in situ* hybridization and immunohistochemistry data strongly suggested that IgZ was involved in mucosal immune responses against pathogens. These results have important implications for the design of *E. coioides* vaccines to control diseases.

## Abbreviations

Ig: Immunoglobulin

MS-222: methanesulfonate

PFA: paraformaldehyde

MMCs: melanomacrophage centers

## Declarations

### Acknowledgments

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**Contributions:** **QZ** designed the work; **ZJ, M** organised and performed the samplings, **ZJ, YW** analysed and interpreted the data; **YW, QZ** drafted and revised the work.

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### Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

All study procedures were approved by the Jinan University Laboratory Animal Ethics Committee (20200707-02). The Guide for the Care and Use of Laboratory Animals and the National Standards of Laboratory Animals of the People's Republic of China were followed by the authors.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

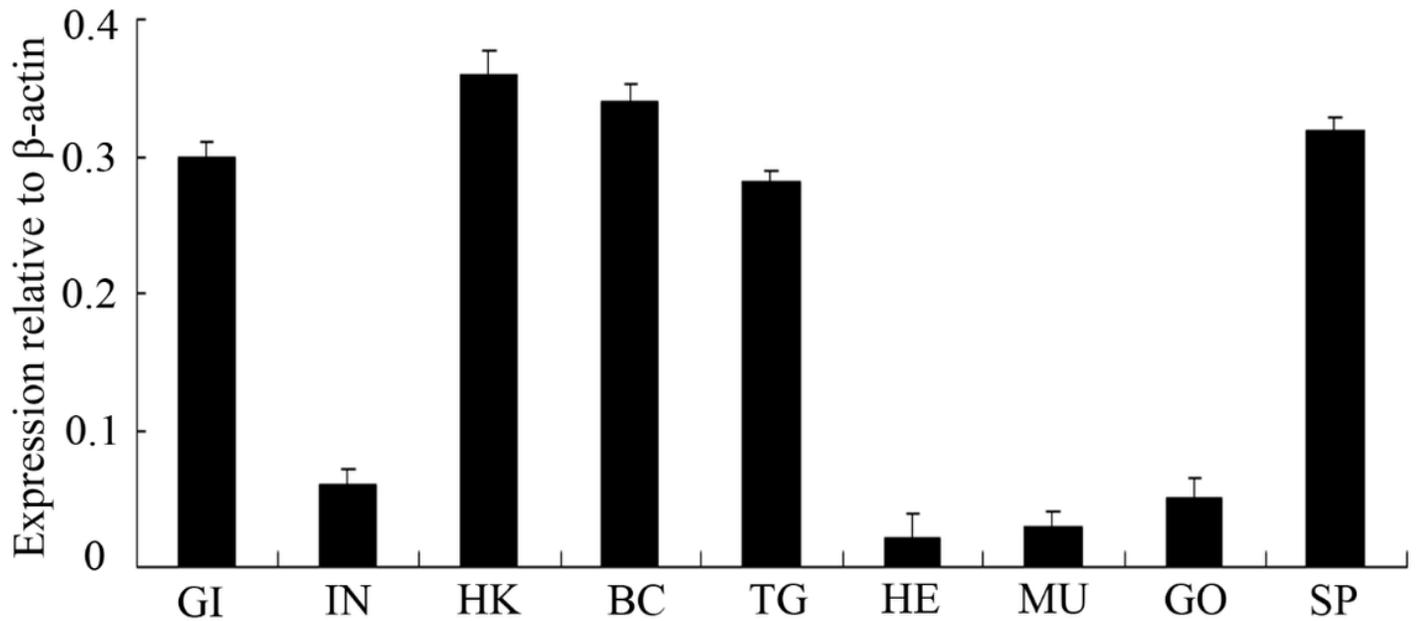
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## Figures

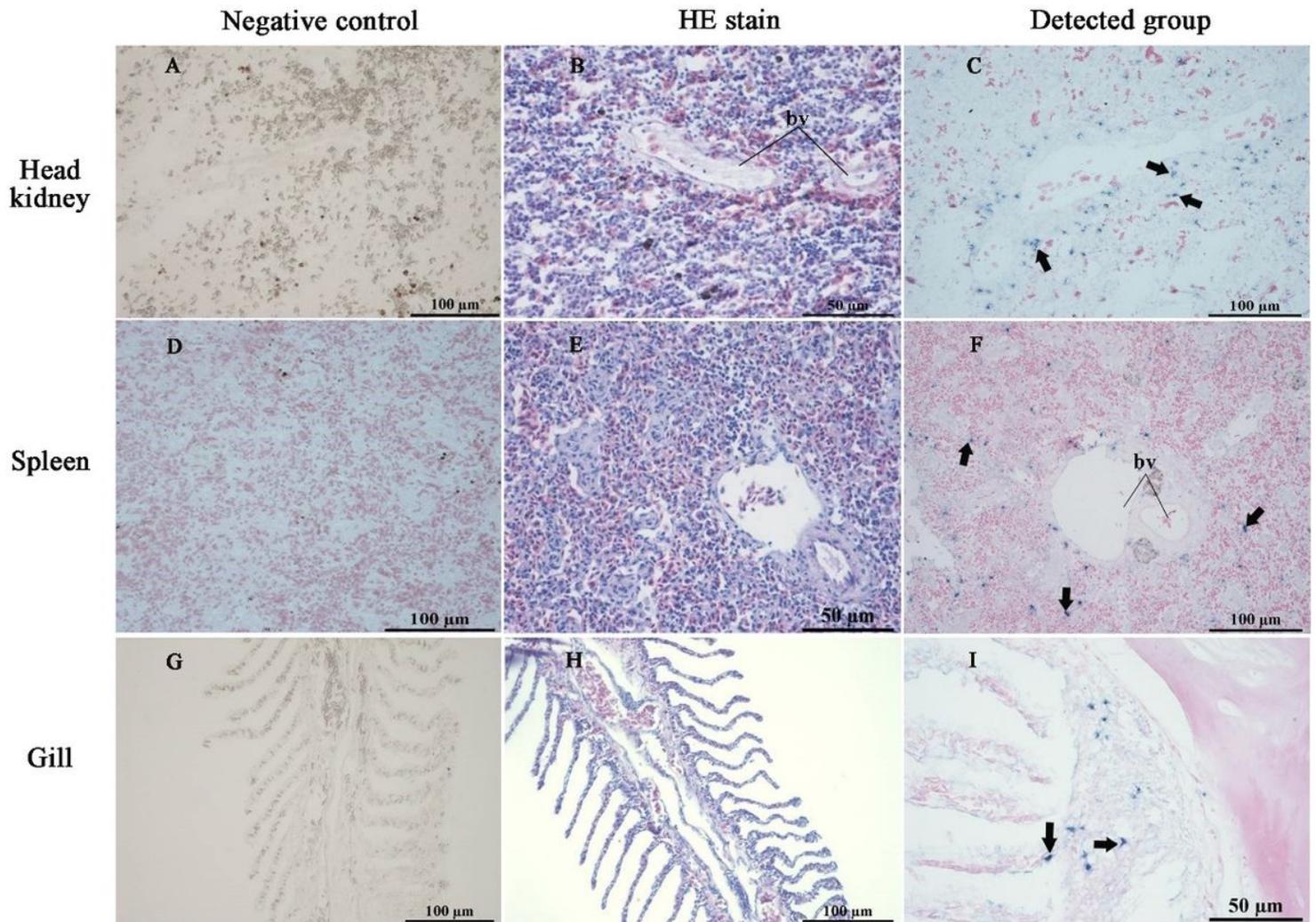


**Figure 1**

Expression profiles of IgZ in different tissues of *E. coioides*. GI: gill; IN: intestine; Hk: head kidney; BC: blood cell; TG: thymus gland; HE: heart; MU: muscle; GO: gonad; SP: spleen.

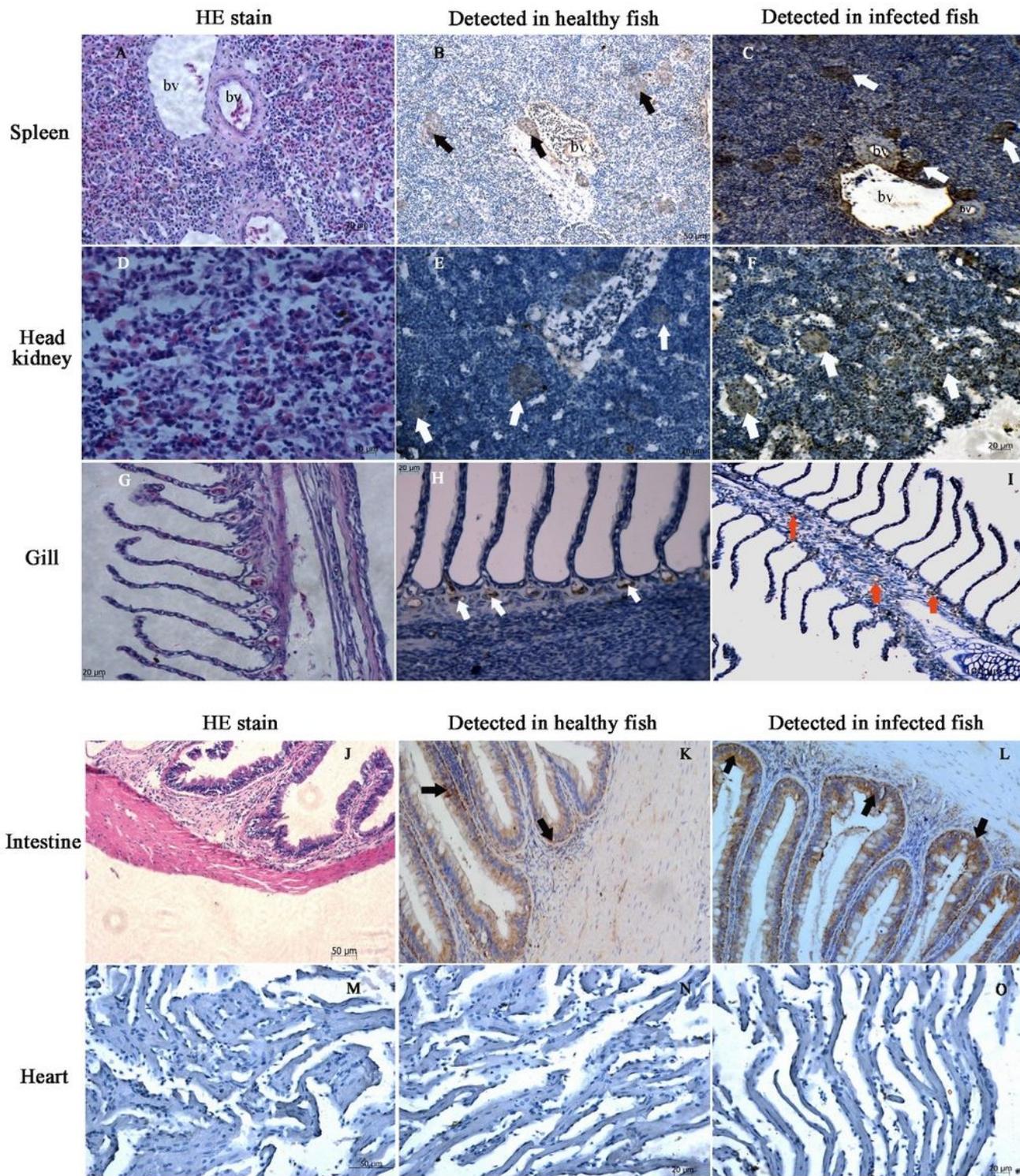
**Figure 2**

Transcriptional changes of IgZ in different tissues of grouper post-immunization with *Vibrio alginolyticus*. A, C, E, and G: immunized by intraperitoneal injection with live *V. alginolyticus*; B, D, F, and H: immunized by intraperitoneal injection with inactivated *V. alginolyticus*. Values are presented as means  $\pm$  standard deviation (n=5). Asterisks indicate a significant difference among the different sample times (\*p < 0.05, \*\*p < 0.01). Transcriptional changes of IgZ in different tissues of grouper post-immunization with *Vibrio alginolyticus*. I, K, and M: immunized by intraperitoneal injection with lived *V. alginolyticus*; J, L, and N: immunized by intraperitoneal injection with inactivated *V. alginolyticus*. Values are presented as means  $\pm$  standard deviation (n=5). Asterisks show significant difference among different sample times (\*p < 0.05, \*\* p < 0.01).



**Figure 3**

In situ hybridization of IgZ-expressing cells in *E. coioides* head kidney (A, B, and C), spleen (D, E, and F), and gill (G, H, and I). A, D, and G: negative control of posterior intestine with sense probe; B, E, and H: section stained with hematoxylin-eosin (HE); C, F, and I: arrows indicate the IgZ mRNA-positive cells. Scale bars: A, C, D, F, G, H, 100 µm; B, E, I, 50 µm.



**Figure 4**

Immunohistochemical detection of IgZ in spleen (A, B, and C), head kidney (D, E, and F), and gill (G, H, and I) of groupers. A, D, and G: section stained with HE; B, E, and H: detected in healthy groupers; C, F, and I: detected in groupers at week 3 post-*Vibrio alginolyticus* infection. Arrows indicate the IgZ positive signals. Scale bars: A, E, F, G, H, 20  $\mu$ m; B, 50  $\mu$ m; D, 10  $\mu$ m; I, 100  $\mu$ m. Immunohistochemical detection of IgZ in intestine (J, K, and L), and heart (M, N, and F) of groupers. A, D, and G: section stained with HE; B, E, and

H: detected in healthy groupers; C, F, and I: detected in groupers at week 3 post-*Vibrio alginolyticus* infection. Arrows indicate the IgZ positive signals. Scale bars: J, M, 50  $\mu\text{m}$ ; N, O, 20  $\mu\text{m}$ .