Expressions of Toll Like Receptor (TLR) Genes in *Paralichthys olivaceus* After Induced by Different Extracts of *Edwardsiella tarda*

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Abstract   Toll like receptors (TLRs) are the main innate immune 'pattern recognition receptors' of animals, which play a central role in host cell recognition and responses to invasive pathogens, particularly common structures of microbial pathogens. In this study, the gene expression profiles of TLRs in the spleen, head kidney, gill, small intestine, liver, muscle, and heart of healthy *Paralichthys olivaceus* were detected by real-time quantitative PCR (qPCR). The TLR family members were widely expressed in different tissues with different basic expression profiles. The highest expressions of TLR1, 5m, 7, 8, 9, 14, and 21 were found in the spleen; the highest expressions of TLR3 and TLR21 were found in the gill; the highest expressions of TLR2 and 5s were found in the small intestine. The second highest expressions of TLR3, 7, and 8 were found in small intestine. The gene expression profiles of TLRs stimulated with *Edwardsiella tarda* DNA, RNA, and lipopolysaccharide (LPS) were also detected in spleen, head kidney and gill. TLR9 and TLR21 were sensitive to *E. tarda* DNA; TLR 8 and TLR21 were sensitive to *E. tarda* RNA; and TLR1 and TLR14 were sensitive to *E. tarda* LPS. The expressions of the other TLR genes showed no significant changes. The results imply that the expressions of these TLR genes in *P. olivaceus* are differently regulated in the whole body and play important roles in the immune response against *E. tarda* infection.

Key words   *Edwardsiella tarda*; *Paralichthys olivaceus*; TLRs; expression; real-time fluorescence quantitative PCR

1 Introduction

Paralichthys olivaceus belongs to the flounder family and is also called flounder or partial mouth in China. It is delicious with high nutritional value. It is mainly distributed in the east and west coasts of South America and North America as well as in Bohai Sea, Yellow Sea, East China Sea, South China Sea and far east coastal areas of Korea, Japan, and Russia in Asia.

Toll-like receptors (TLRs) are a class of important protein molecules involved in pattern recognition receptors (PRRs) and have recognition function (Zhou *et al*., 2018). TLR is a single transmembrane protein that can recognize microorganisms. When they break through the physical barriers of the body, such as skin and mucous membrane, TLRs can recognize such barriers and activate immune cell response. The TLR family includes many members. At present, 17 TLR members have been found in fish, including TLRs 1, 2, 3, 4, 5, 5s, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, and 23. In this study, we mainly investigate the expression and distribution of 10 TLR genes (1, 2, 3, 5m, 5s, 7, 8, 9, 14, and 21) in important immune tissues of *P. olivaceus*.

Although fish is a relatively lower vertebrate, it has innate immune response and acquired immune response (Whyte, 2007). Fish is in a special evolution position between higher vertebrates and lower invertebrates, and its acquired immune mechanism is not perfect (Wilson, 2017). For the two kinds of immune responses, innate immune response plays a very important role.

Edwardsiella tarda is a pathogenic bacterium that is harmful to freshwater and marine fishes. It can cause diseases in more than 20 kinds of fishes, such as flounder, *Trionyx sinensis*, carp, and so on. *E. tarda* is a common pathogen for both human and fish, and can pose a threat to human health (Gao *et al*., 2013; Zhou and Sun, 2016). Therefore, research of *E. tarda* has attracted extensive attention. This bacterium mainly invades fish through the digestive tract, gill, or injured epidermis, causing great losses to aquaculture (Qin *et al*., 2017). A comprehensive and in-depth study on the molecular mechanism of the immune response of *P. olivaceus* to the pathogen will provide a theoretical basis for the establishment of an effective immune control measure to prevent and control *E. tarda* infection.

TLRs are widely distributed in tissues and cells of the body, and the types and numbers of TLRs are different in different tissues or cells. On the other hand, a pathogen often has a variety of pathogen related molecular patterns PAMP that can be recognized by animal bodies. PAMP is recognized by different TLRs members at different time

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periods of infection, How these TLRs family members work together to activate the innate immune system and activate the specific immune response is still unknown. Therefore, it is not enough to study the immunoregulation mechanism of TLRs only from the perspective of single tissue or single TLR member. The aim of this study is to investigate the immune responses of TLRs in various tissues of *P. olivaceus*, and to obtain the temporal and spatial expression profiles of TLRs family members during *E. tarda* infection, so as to reveal the natural law and immunobiological significance of the interaction between TLRs and *E. tarda*.

2 Materials and Methods

2.1 Materials

2.1.1 Experimental fish

Healthy *P. olivaceus* was purchased from a fish farm in Tianjin. Flounders with normal morphology and a length of $10-12\,cm$ were selected. They were raised at $25℃$ in artificial sea water in shaded aquarium for a week to adapt to the environment and fed once a day.

2.1.2 Pathogen

E. tarda was provided by Tianjin Aquaculture Disease Control Center and preserved in our laboratory.

2.2 Methods

2.2.1 Pathogen culture

E. tarda was inoculated into LB medium (pH=7) on the ultra-clean stage, and cultured at $37℃$ with shaking until OD_{600} value was 0.6. The bacteria were fully washed with sterile PBS buffer three times and stored in PBS buffer solution. The concentration was adjusted to $1\times10^7\,cells\,mL^{-1}$.

2.2.2 Extraction of DNA from *E. tarda*

Phenol-chloroform extraction method was used. The bacteria were placed into $500\,\mu L$ of $20\times SSC$ solution and mixed overnight with 1% SDS. The next day, the same volumes of phenol, chloroform, and isoamyl alcohol were added. After mixing, the bacteria were centrifuged at $4℃$ and $9590.4\,g$ for 20 min. The suspension was added with two volumes of anhydrous ethanol, placed at $-20℃$ for 2 h, and centrifuged. The precipitate was placed in double SSC solution, added with RNase, placed at $37℃$ for 1 h, added with protease K, and maintained for 1 h. The DNA solution in the aqueous phase was mixed evenly with sodium acetate (10%, pH=5.2) and 2 volumes of pre-cooled absolute ethanol. DNA was precipitated in an ice bath for 2 h. The supernatant was centrifuged at $4℃$ with $9590.4\,g$ for 10 min. The supernatant was precipitated twice with 70% ethanol, centrifuged again, dissolved in TE buffer solution, and stored at $4℃$ for standby or at $-20℃$ for further analyses.

2.2.3 Extraction of RNA from *E. tarda*

The frozen tissue (fresh or preserved at $-70℃$ or in liquid nitrogen) was placed in a centrifuge tube, added with 1 mL of Trizol, homogenized, and allowed to stand at room temperature for 5 min. The solution was then added with 0.2 mL of chloroform, shaken for 15 s, and allowed to stand for 2 min. The supernatant was centrifuged at $4℃$ with $9590.4\,g$ for 15 min, added with 0.5 mL of isopropanol, mixed gently, and allowed to stand at room temperature for 10 min. The supernatant was centrifuged at $4℃$ with $9590.4\,g$ for 10 min and added with 1 mL of 75% ethanol. The precipitate was washed gently. The supernatant was discarded after the pellet was centrifuged at $4℃$ with $3746.25\,g$ for 5 min. The supernatant was dried in the air and added with appropriate amount of DEPC H_2O to dissolve (promote the dissolution at $65℃$ for $10-15\,min$). The extracted RNA was placed in a refrigerator at $-80℃$ prior to use.

2.2.4 Extraction of Lipopolysaccharides (LPS) from the cell wall of *E. tarda*

About 10 mL of the bacterial suspension ($10^7\,piece\,mL^{-1}$) and 90% phenol solution were preheated for 10 min in a water bath at $66℃$. The suspension was added with the same volume of phenol (preheated), stirred for 25 min, cooled at room temperature, and refrigerated overnight at $4℃$. The next day, centrifugation was performed with $2775\,g$ for 15 min, and the supernatant was removed. The residue and phenol layer were added with 10 mL of water, stirred in a water bath at $66℃$ for 30 min, cooled, and centrifuged to obtain the supernatant. The supernatant extracted twice was dialyzed in 0.85% normal saline for 48 h, during which the normal saline was changed, and the crude lipopolysaccharide was obtained by measuring phenol free (purple free) with ferric chloride. The crude LPS was concentrated with polyethylene glycol 6000 and added with $50\,\mu g\,mL^{-1}$ DNase and RNase. Enzymolysis was carried out at $37℃$ for 4 h. The solution was heated in $66℃$ water bath for 10 min, cooled, and centrifuged at $4℃$ with $249.75\,g$ for 30 min. The supernatant was collected and added with two volumes of acetone to obtain the purified LPS.

2.2.5 Immunity of *P. olivaceus*

Each tail of *P. olivaceus* was intraperitoneally injected with $100\,\mu L$ of pathogenic stimulants (*E. tarda* DNA, RNA, or LPS). The control group was injected with the same volume of PBS. At each time point after injection, three fish were randomly chosen to be executed for analyses. The tails were taken, and the other tissues were dissected under sterile conditions and then were frozen in liquid nitrogen. The next day, the tissues were transferred to $-80℃$ for storage.

2.2.6 Quantitative analysis of gene expression

The cDNA kit purchased from Sangon Biotech® Inc. was used to synthesize the first strand of TLR genes in this experiment according to its recommended method.

Transcriptome sequencing kit was provided by Beijing Nuohe Zhiyuan Bioinformation Technology Co., Ltd. The sequencing platform was Illumina hiseqTM2500.

The relative quantitative expression data of 10 genes in this experiment were obtained using ABI 7500 fluorescent

PCR instrument through the method recommended by Promega's GoTaq® qPCR Master Mix kit with reverse transcriptional cDNA as template. The primer sequences are listed in Table 1. The reaction system (24 μL) consisted of 4 μL of the cDNA template, 0.4 μL of upstream primer (10 μmol L^{-1}), 0.4 μL of downstream primer (10 μmol L^{-1}), 10 μL of Promega's GoTaq® qPCR master mix, and 9.2 μL of nuclear free water. The relative quantitative analysis was performed by 2$^{-\Delta\Delta CT}$ method, and histogram analysis was performed by Origin 8 software.

Table 1 Gene specific primers used in real time RT-PCR in this project

Primers	Nucleotide sequences (5'−3')	GenBank number
TLR1.q-F	TCCGCACTTCTCATCTTTAT	NG_016228.1
TLR1.q-R	ATTTCACCACAGCCCTTC	
TLR2.q-F	ACTTCCTTCCTGGACACTG	NG_016229.1
TLR2.q-R	TCGTAGCGGCACCAATCA	
TLR3.q-F	GCGGTGCTGCTCAGTATGT	NG_007278.1
TLR3.q-R	TGGTTTGTGACGTGGTTG	
TLR5m.q-F	AACTGTGTGAACTTACGGAG	AB562153.1
TLR5m.q-R	GCAGCAGCTGTTTTTGGATCA	
TLR5s.q-F	AGGCGTTTCAGCCGAGTC	AB562155.1
TLR5s.q-R	TCTGCGGGAGTTAGGTTT	
TLR7.q-F	ATCGAGGACAGTTACCAC	NG_012569.1
TLR7.q-R	TACCCACAAGTCTGAAAG	
TLR8.q-F	AGTTTCACCCGCAGATTG	NG_012882.2
TLR8.q-R	CTGGGATGCCTCCTATGT	
TLR9. q-F	CGTGCTGGTTCTGTTGGATGAG	NG_033933.1
TLR9. q-R	GCTGAGCCTTTGGGTTTTTTGG	
TLR14.q-F	TACTGAAGCGTCAATCGT	AB576806.1
TLR14.q-R	TTGCGTGACATAGCCAAC	
TLR21.q-F	TAAACTTTGCCTACATCACA	NM_001199335.1
TLR21.q-R	AACACGAGCAGAAGAACAT	
β-actin-F	AGGTTCCGTTGTCCCG	EF026002.1
β-actin-R	TGGTTCCTCCAGATAGCAC	

3 Results

3.1 Expression Profile of TLRs Genes in Normal Tissues of Healthy *P. olivaceus*

All members of the TLRs family of *P. olivaceus* were widely expressed in various tissues, but their basic expression profiles were different (Fig.1). Many TLR members were expressed with a high level in the spleen, gill, and small intestine. The highest expression levels of TLR1, 5m, 7, 8, 9, 14, and 21 were found in the spleen; and the highest expression of TLR3 was found in the gill tissue. TLR21 was also highly expressed in the gill tissue. The highest expressions of TLR2 and 5S were found in the small intestine, where TLR 3, 7, and 8 were also expressed with a high level. The spleen and gill of fish are important immune organs, and TLRs have a high basic expression level in immune organs, consistent with their defense function (Rebl *et al.*, 2010). The small intestine is an organ that comes in contact with foreign food and is exposed to a large number of microorganisms for a long time. TLR members exhibited basic expression distribution in this organ, which is also related to the environment of small intestine (Jault *et al.*, 2004; Meijer *et al.*, 2004). In contrast to expectations, as another important immune organ, the head kidney showed low expression of TLR genes, though TLR1, 2, 3, and 21 could be detected in head kidney. Under the health status, the spleen functions as a main preventive immune organ, and the head kidney can keep the lowest energy consumption of the body. Their function are different. However, the real reason remains to be further investigated. Our subsequent immune stimulation experiments showed that the gene expression of TLRs in the head kidney of *P. olivaceus* was significantly up-regulated in response to pathogen invasion upon stimulation by pathogenic microorganisms. This expression characteristic may be used as an indicator for health monitoring of *P. olivaceus*.

To better understand the difference in the basal expression levels of TLR genes in the same healthy tissue, we rearranged the data and presented the basal expression profile of TLRs in the same tissue (Fig.2). TLR2 and TLR9 genes generally had higher basal expression levels in each tissue than the other TLR members. The overall basal expression levels of TLR family genes were relatively higher in the spleen than in the other tissues.

TLRs were widely expressed in all the tissues of healthy *P. olivaceus*, but the basic expression pattern of TLRs varied among different tissues. There are many TLRs in the spleen of healthy *P. olivaceus*, and their basic expression values are high, which indicates that the spleen plays an important role in the prevention of infection. However, the expression of TLRs in the head kidney was low, and the expression of some TLRs increased after immune stimulation, suggesting that the head kidney was more focused on the immune response during acute infection. This may suggest that the spleen is the main preventive immune organ in healthy *Paralichthys olivaceus*. Various TLR genes were also expressed in the heart, liver, and muscle that are non-immune organs, but the expression level was low, which may be beneficial to maintain the overall health of the body. Among the 10 TLR genes, TLR2 and TLR9 had the highest basal expression levels.

3.2 TLR Gene Expression Profile of *P. olivaceus* Immunized with *E. tarda* DNA Extract

The gene expression profiles of TLR 1, 2, 3, 5m, and 5s after immunization with *E. tarda* DNA are shown in Fig.3. The expressions of TLR1 and TLR2 were up-regulated. The TLR3 gene expression in the gill and spleen was down-regulated at less than 1 h and was also down-regulated and up-regulated in the head kidney and reached the peak at 6 h. The expressions of TLR5m and TLR5s were up-regulated

and reached the highest levels in the gill and spleen at 6 h and in the head kidney at 3 h. The expression profiles of TLR7, 8, 9, 14, and 21 genes are shown in Fig.4. Only TLR9 and TLR21 genes were significantly up-regulated, among which TLR9 was the most up-regulated in the spleen and presented two peaks at 3−6 h and at 1 d. TLR21 showed the largest up-regulation in the head kidney and reached the peak in the three immune organs at 6 h. Few significant changes were observed in other genes. Hence, TLR9 and TLR21 were sensitive to the DNA of *E. tarda*.
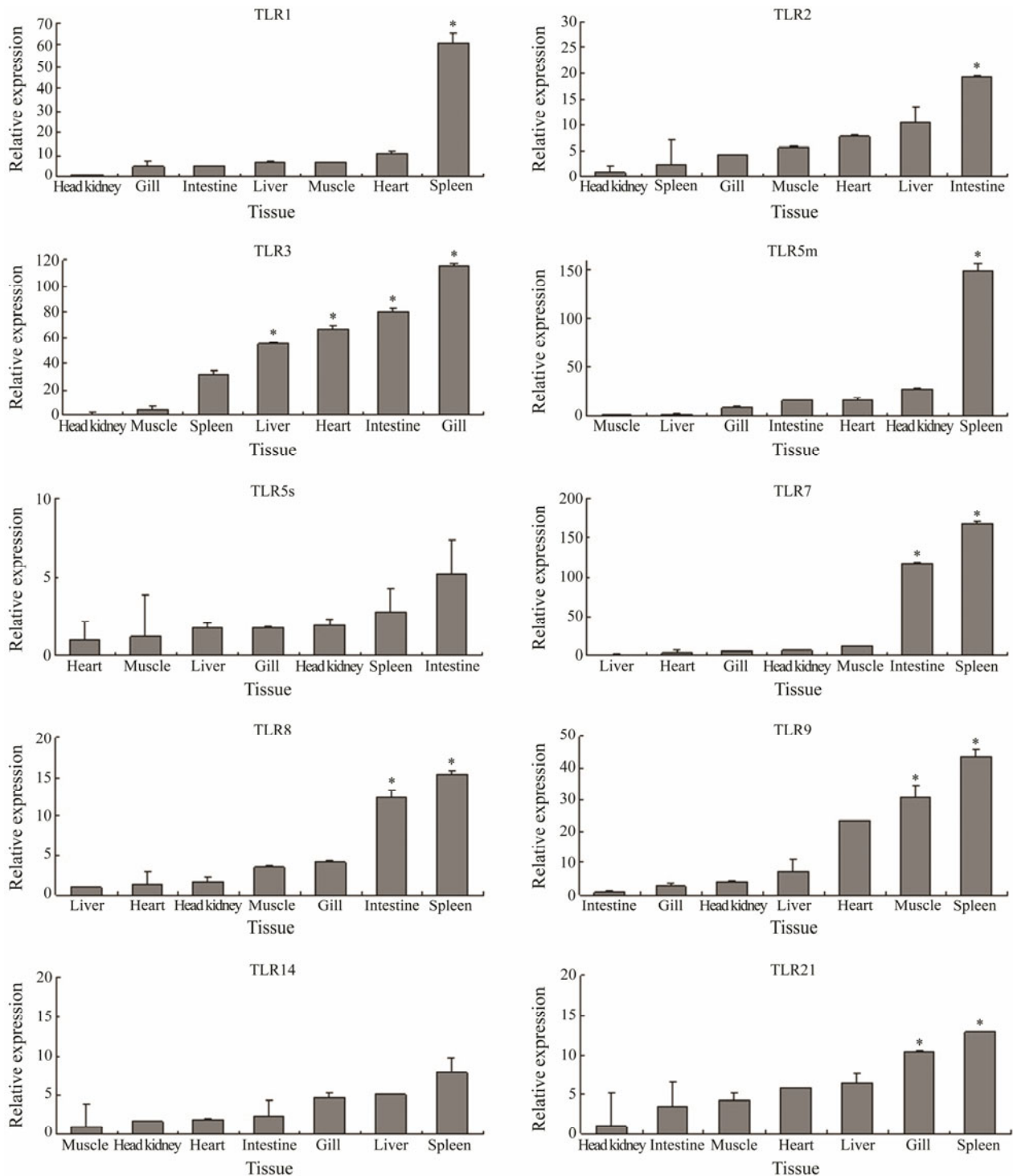

Fig.1 Distribution of TLR gene expression in different tissues of healthy *P. olivaceus*. Data are expressed as mean±SD ($n =$ 3) ($P < 0.05$).
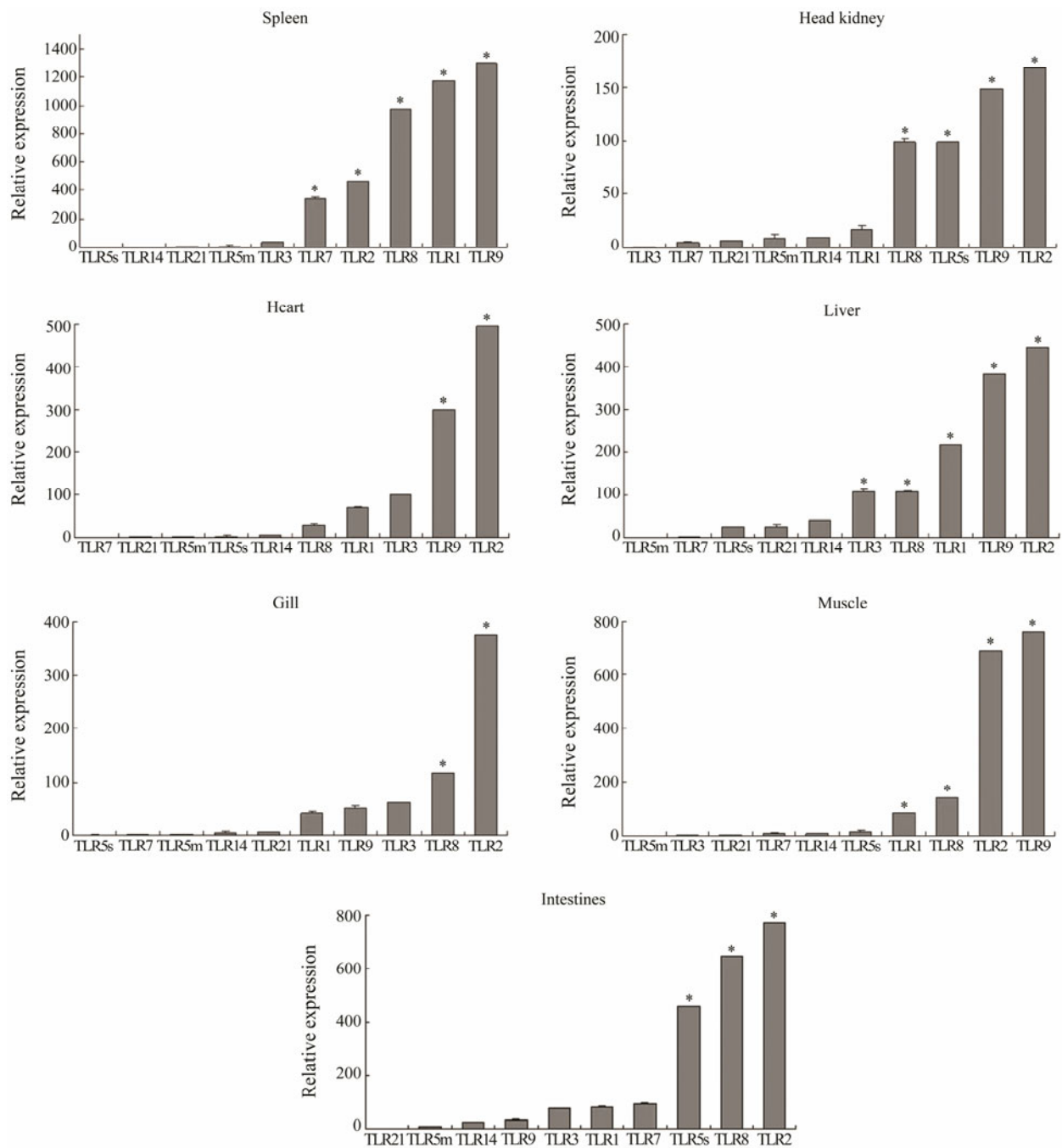
Fig.2 Basic expression profile of TLR genes in the same tissues of healthy *P. olivaceus*. Data are expressed as mean±SD (*n* =3) (*P*<0.05).

3.3 TLR Gene Expression Profile of *P. olivaceus* Immunized with *E. tarda* RNA Extract

The expression profiles of TLR1, 2, 3, 5m, and 5s in *E. tarda* RNA-immunized *P. olivaceus* are shown in Fig.5. The expression of all five genes changed, but the amplitude was not very significant. The gene expression profiles of TLR7, 8, 9, 14, and 21 are shown in Fig.6. The expression of TLR8 and 21 genes was significantly up-regulated. TLR8 showed two peaks in the head kidney and was up-regulated in different degrees in the gill and spleen. The TLR21 expression increased at a lower rate and showed the largest increase in the gills. TLR7 was only weakly up-regulated in the gills. The expression of the other genes had no signi-

ficant change in the three immune organs. Hence, TLR8 and TLR21 were sensitive to *E. tarda* RNA.

3.4 TLR Gene Expression Profile in *P. olivaceus* Immunized with LPS Extracted from *E. tarda* Cell Wall

The expression profiles of TLR1, 2, 3, 5m, and 5s genes in *P. olivaceus* immunized with LPS of *E. tarda* are shown in Fig.7, and those of TLR7, 8, 9, 14, and 21 genes are shown in Fig.8. The expressions of TLR2 and 14 genes were significantly up-regulated in all the three immune organs. The TLR1 gene was up-regulated in the head kidney only at 3 h, whereas the other genes did not change significantly. Hence, TLR2 and 14 were sensitive to the LPS of *E. tarda*.
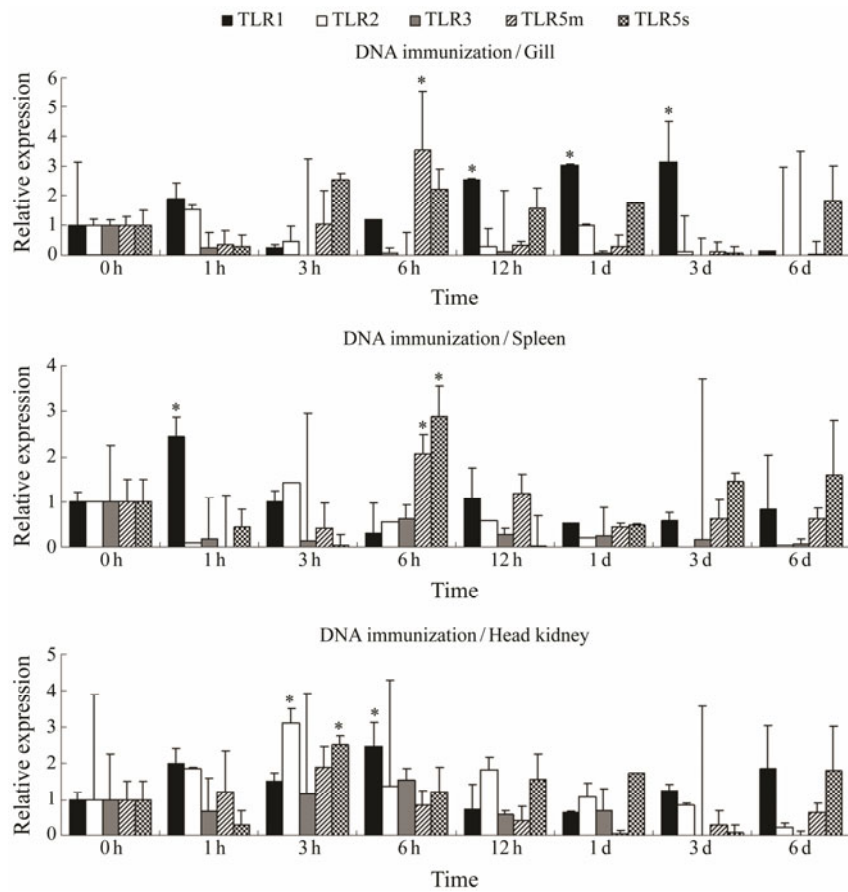
Fig.3 Gene expression profiles of TLR 1, 2, 3, 5m, and 5s in *P. olivaceus* immunized with *E. tarda* DNA. Data are expressed as mean±SD ($n=3$) ($P<0.05$).
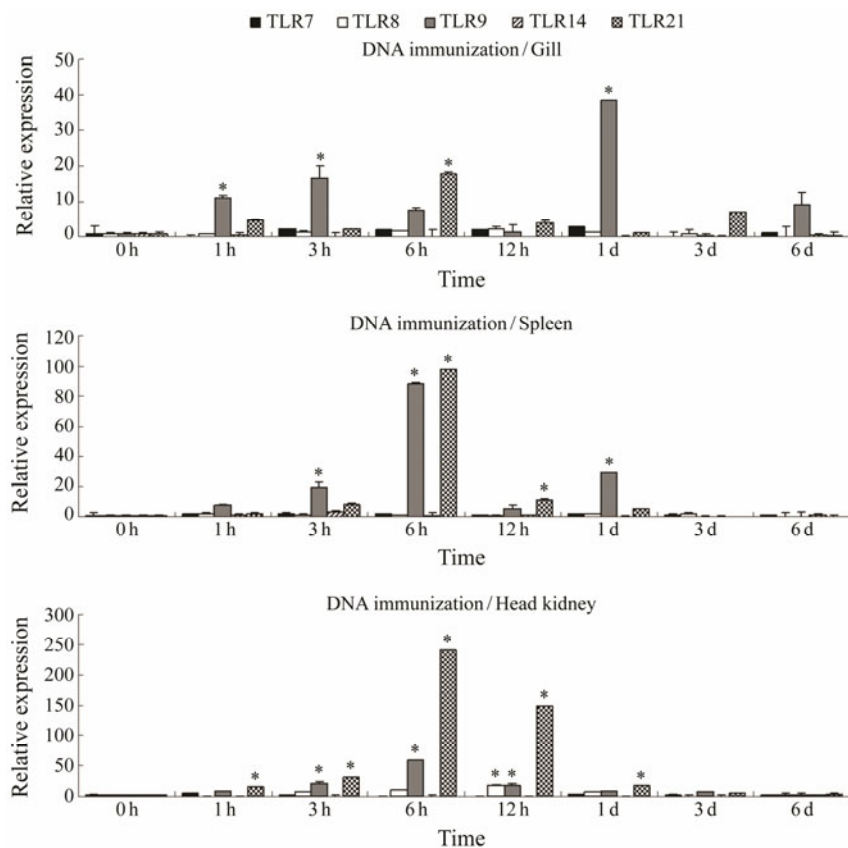

Fig.4 Gene expression profiles of TLR 7, 8, 9, 14, and 21 in *P. olivaceus* immunized with *E. tarda* DNA. Data are expressed as mean±SD ($n=3$) ($P<0.05$).

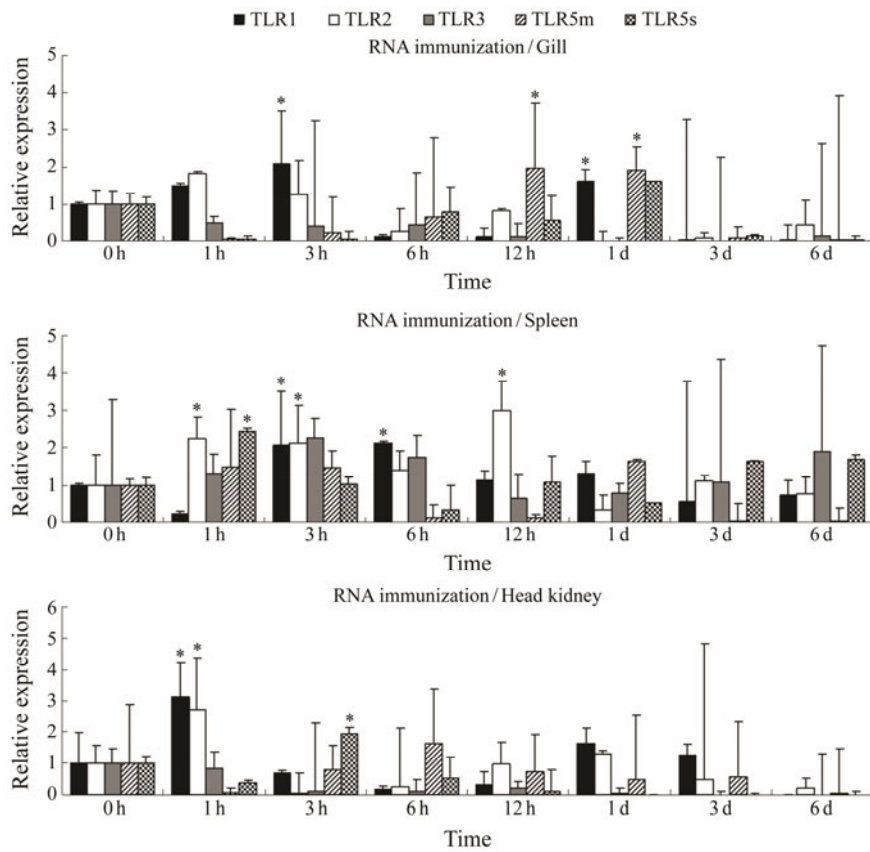Fig.5 Gene expression profiles of TLR1, 2, 3, 5m, and 5s in *P. olivaceus* immunized with *E. tarda* RNA. Data are expressed as mean±SD (*n*=3) (*P*<0.05).
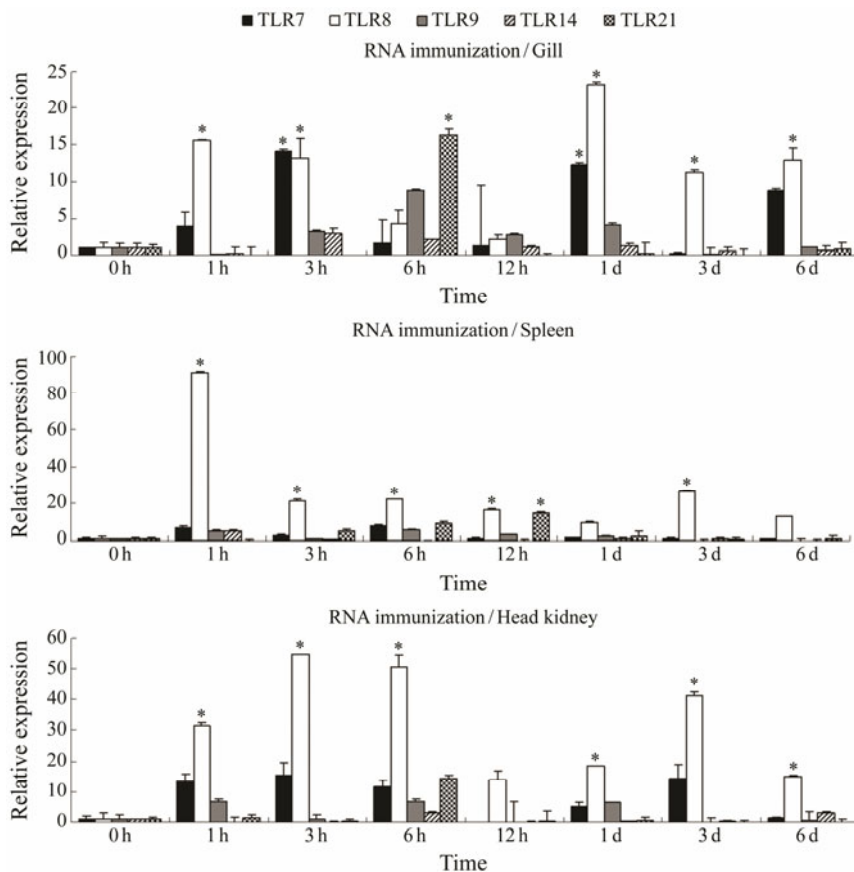

Fig.6 Gene expression profiles of TLR7, 8, 9, 14, and 21 in *P. olivaceus* immunized with *E. tarda* RNA. Data are expressed as mean±SD (*n*=3) (*P*<0.05).
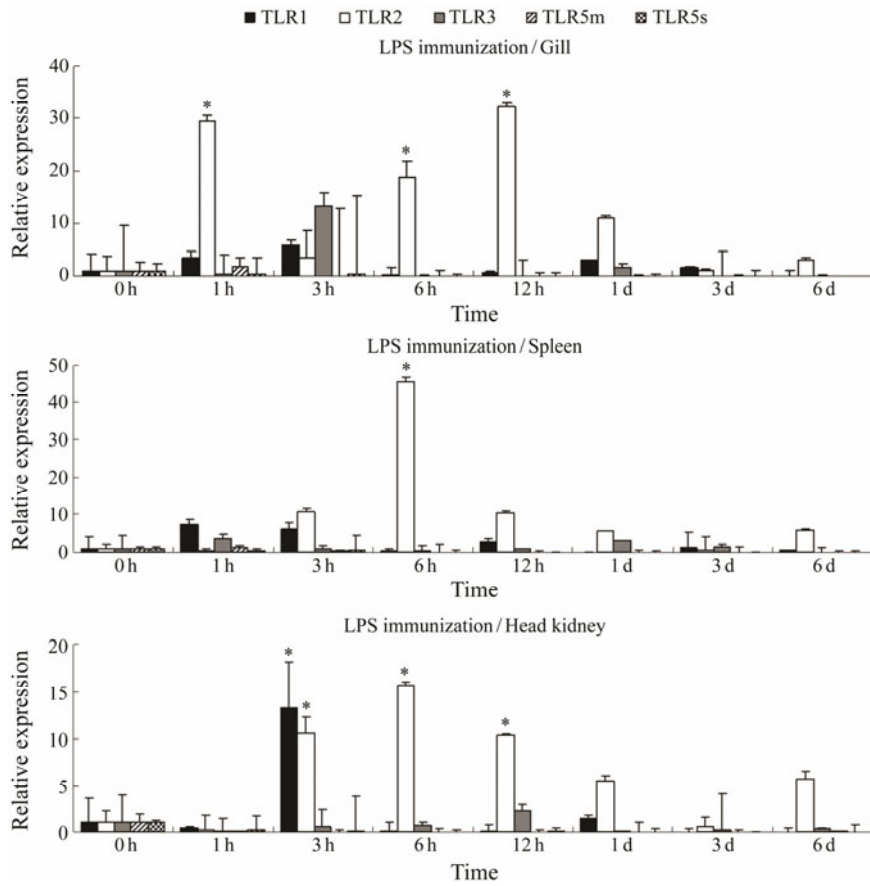
Fig.7 Gene expression profiles of TLR1, 2, 3, 5m, and 5s in *P. olivaceus* immunized with *E. tarda* LPS. Data are expressed as mean±SD (*n*=3) (*P*<0.05).
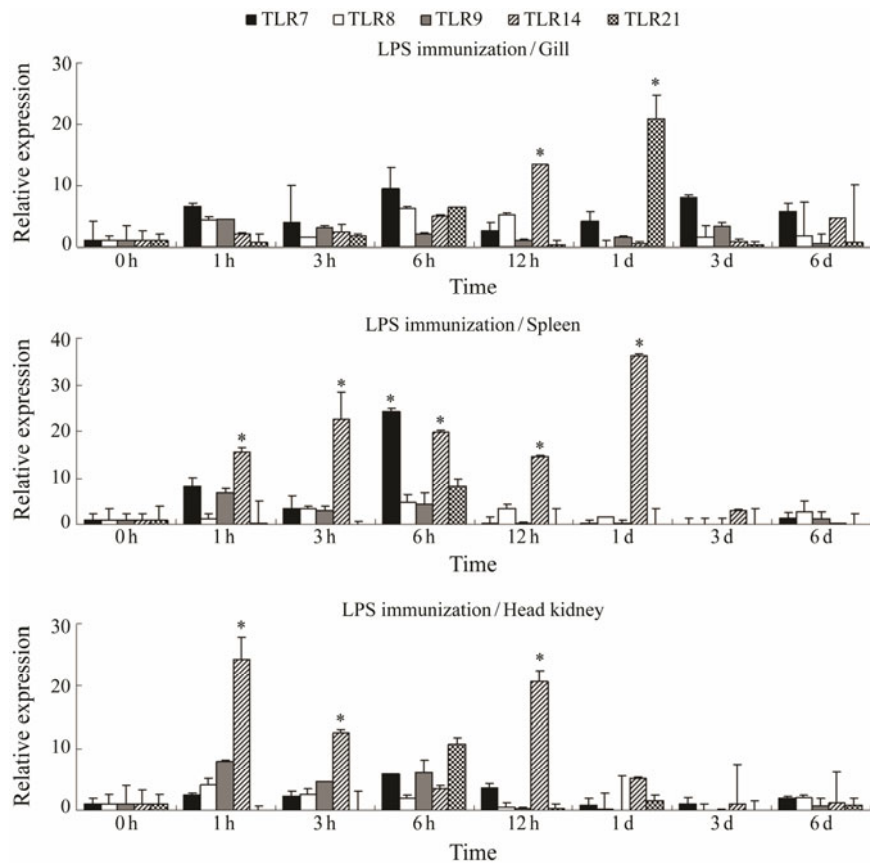

Fig.8 Gene expression profiles of TLR7, 8, 9, 14, and 21 in *P. olivaceus* immunized with *E. tarda* LPS. Data are expressed as mean±SD (*n*=3) (*P*<0.05).

4 Discussion

Up to now, there are many studies on the identification and expression of immunity-related genes in fish. As an important immunity-related gene, the immune regulatory mechanism of TLR is still unclear in teleost fish. Some studies have shown that stimulation with bacterial pathogens can up-regulate TLR gene expression in fish. For example, TLR20 and TLR21 have been identified in several teleost fishes, such as *P. olivaceus*, *Epinephelus coioides*, large yellow croaker, zebrafish *etc.* (Li *et al.*, 2012; Gao *et al.*, 2013; Yeh *et al.*, 2013; Sun *et al.*, 2016). TLR21, TLR22 and TLR25 are also expressed in *Acipenser dabryanus* (Qi *et al.*, 2018). Fan isolated and identified full-length cDNA of TLR2 in giant yellow crocker and determined gene expression after immune stimulation (Fan *et al.*, 2015). In catfish, the changes of TLR genes spectrum were detected after parasite ciliate infection at different time points, which confirmed that some TLRs may play an important role in protecting the fish from infection (Zhao *et al.*, 2013). High expressions of TLR3, TLR4, TLR9 and TLR22 genes can be induced when yellow catfish is infected with *Aeromonas hydrophila* (Zhang *et al.*, 2017). The expression of TLR5s and 5m was up-regulated in *P. olivaceus* infected by *E. coli* and *E. tarda* (Hwang *et al.*, 2010). In addition to the original innate immunity, Rauta *et al.* (2014) investigated the additional role of acquired immunity. By supplementing TLR activator to stimulate dendritic cells, they can prepare effective vaccines against major diseases in many aquatic animals.

Although many TLRs have been cloned and identified in different fishes, the function and mechanism of TLRs are still unclear. Previous studies mainly analyzed the immune expression regulation of single TLR from a specific tissue of fish, and then determine their biological function and immune role. However, TLRs are widely distributed in various tissuesof the body, and the types and numbers of TLRs are different in different tissues. On the other hand, a pathogen often has a variety of molecular patterns that can be recognized by immune system. In different periods of infection, PAMP is recognized by different members of TLRs. How these family members work accurately in various tissues to activate the innate immune system and activate the specific immune response is still unknown. Therefore, it is not enough to study the immune regulation mechanism of TLRs only from the perspective of single tissue or single TLR gene. It is necessary to investigate the full range of immune responses of various TLR members in a variety of important tissues of fish, and to analyze the interaction and relationship between TLR members and pathogenic microorganisms, so as to obtain a more systematic and in-depth understanding.

In this study, ten TLR (1, 2, 3, 5m, 5s, 7, 8, 9, 14, 21) genes were detected by real-time quantitative PCR in different tissues of *P. olivaceus* infected with *E. tarda* to find the expression patterns of ten TLR genes after pathogen stimulation.

The results of real-time PCR detection in healthy *P. oli-vaceus* showed that all members of the TLRs family were widely expressed in the tested tissues, but the basic expression profiles of each member varied among different tissues. As immune organs, the spleen and gill had variety members of TLR with the highest or high expression. The intestine also had variety members of TLR with the highest or higher expression. In the head kidney, the expression levels of TLR genes were very low, and some expression levels were nearly zero. The result may suggest the spleen is the main preventive immune organ in healthy *P. olivaceus*. Ten TLRs were also detected in non-immune organs such as heart, liver, and muscle, but their expressions were very low. The low expression may be helpful to maintain the overall health of the body. Among the ten TLRs, TLR2 and TLR9 showed the highest basal expression, while TLR1 showed the highest expression in spleen. Previous studies also showed that the relative expression of TLR1 in spleen was the highest in grouper tissues (Wei *et al.*, 2011).

According to the ligands and the subcellular location, TLRs can be divided into two subgroups. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are located primarily on the cell surface and recognize mainly microbial membrane components such as lipids, lipoproteins, and lipopolysaccharide (LPS). On the other hand, TLR3, TLR7, TLR8, and TLR9 reside on the membranes of intracellular compartments, such as endosomes, lysosomes, endolysosomes and endoplasmic reticulum, and are responsible for the recognition of microbial nucleic acids (Palti, 2011). Eight members of TLRs (TLR5S, 14, 18, 19, 20, 21, 22, 23) were specific in teleost, which have not been identified in mammals. A unique feature of teleost TLRs is the presence of a soluble TLR5 molecule (TLR5S). TLR5S ligands have not been identified in fish, and mammalian TLR5 are thought to recognize flagellin. Teleost TLR14 shares sequence and structural similarity with TLR1 and 2 (Palti, 2011), which was divided into subgroups of TLR1 in fish and recognize gram-negative bacteria, gram-positive bacteria, polyI:C and LPS (Fan *et al.*, 2015).

In the present study, most of the immune stimulation studies of fish are based on the complete pathogen. In order to explore which component of the pathogen can effectively activate the TLRs response, and further understand the interaction between *E. tarda* and TLRs family of *P. olivaceus*, we examined the effects of LPS, DNA and RNA extracted from *E. tarda* on TLRs family genes expression in immune organs (spleen, head kidney, and gill) of *P. olivaceus*.

The results showed that only TLR9 and TLR21 gene expression were significantly up-regulated after *E. tarda* DNA immunization in *P. olivaceus*. This implied that both TLR9 and TLR21 were sensitive to *E. tarda* DNA. The expressions of TLR8 and TLR21 genes were significantly up-regulated after *E. tarda* RNA immunization. The results implied that TLR8 and TLR21 were sensitive to *E. tarda* RNA. The expressions of TLR2 and TLR14 genes were significantly up-regulated after *E. tarda* LPS immunization, and no significant changes were found in other genes. The results implied that TLR2 and TLR14 were sensitive to *E. tarda* LPS.

In our experiments, TLR1 and TLR5s were up-regulated

unexpectedly after the immunization with *E. tarda* DNA, which may be caused by the stimulation of inflammatory factors produced by *P. olivaceus* immune system in response to *E. tarda* DNA. Another interesting phenomenon is that there were two peaks of up regulation in TLR8 after immunization with *E. tarda* RNA and in TLR14 after immunization with *E. tarda* LPS. The maximum expression peak values of TLR8 and TLR14 were much higher than those of other TLR genes, which implied that TLR8 and TLR14 were more sensitive to the stimulation overexpression. However, over-expression may be suppressed by negative feedback of immune system. The expressions of TLR8 and TLR14 were restored and reached the second peak after the negative regulation stopped.

From all of the gene expression profiling in this study, we can get lots of information about the dynamic changes in the gene expression of the immune organs and non-immune organs under different stresses or stimulations. The results will be helpful to clarify the interaction between *E. tarda* infection and host recognition, and provide a theoretical basis for *E. tarda* vaccine development for *P. olivaceus*.

Acknowledgements

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