Fish and Shellfish Immunology 86 (2019) 449–458

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular characterization, expression analysis, and ontogeny of complement component C9 in southern catfish (*Silurus meridionalis*)

Yao-Wu Fu[a,1], Cheng-Ke Zhu[b,1], Qi-Zhong Zhang[a,*], Ting-Long Hou[a]

[a] Institute of Hydrobiology, Jinan University, Engineering Research Center of Tropical and Subtropical Aquatic Ecological Engineering Ministry of Education, Key Laboratory of Eutrophication and Red Tide Prevention of Guangdong Higher Education Institutes, West 601 Huangpu Avenue, Tianhe District, Guangzhou, 510632, People's Republic of China

[b] College of Animal Science, Southwest University Rongchang Campus, 160 Xueyuan Road, Rongchang District, Chongqing, 402460, People's Republic of China

ARTICLE INFO

Keywords:
Silurus meridionalis
Complement component C9
Expression analysis
Whole mount *in situ* hybridization
Ontogeny
Aeromonas hydrophila

ABSTRACT

The complement system plays an important role in host defense against invading microorganisms. Complement component C9 is the last component that is involved in the formation of the membrane attack complex (MAC) on the surface of target cells. In the present study, the full length C9 cDNA sequence of 1984 bp with an open reading frame (ORF) of 1809 bp was cloned from southern catfish (*Silurus meridionalis*). The deduced amino acid sequence showed similarity with other teleost fish. The mRNA expression of C9 was detected in the liver, spleen, stomach, intestine, and head kidney, with highest levels detected in the liver. The mRNA of C9 was first detected in the yolk syncytial layer at 34 h post fertilization (hpf) with whole mount *in situ* hybridization, followed by the liver at 36 h post hatching (hph). The mRNA expression of C9 was upregulated significantly in the liver, spleen, and intestine following the injection with *Aeromonas hydrophila*, suggesting that C9 played an important role in defense against invading pathogens in southern catfish. Therefore, these results provide important information to understand the functions of C9 during fish early development in fish.

1. Introduction

The complement system consists of more than 35 soluble and membrane-bound proteins [1]. It plays an important role in host defense by interacting with components of both innate and adaptive immunity [2]. The complement system is activated through three pathways: the classical complement activation pathway, the alternative complement pathway, and the lectin complement pathway [3]. All three pathways merge through a common intersection of complement C3, resulting in the subsequent formation of C5 convertase [2,3]. Then C5 convertase cleaves C5 into C5b and C5a. C5a is a smaller fragment and contributes to the inflammation process by attracting phagocytic cells to the site of infection [4]. C5b, the larger fragment, induces the terminal complement activation pathway by binding of C6, C7, C8, and C9 [4]. The polymerization of C9 bound to the C5b-8 complex forms the membrane attack complex (MAC) [1]. Formation of MAC creates a channel or pore on the cell surface of pathogen, thus leading to the destruction of foreign pathogens and the death of infected cells [5].

Fish embryos and larvae are exposed to aquatic pathogens immediately before the maturation of their lymphoid organs [5,6]. Thus, the possession of an effective immune system in fish embryos and larvae is particular important for the protection of early development stages fish against pathogens [7]. Studies have been conducted to investigate the ontogeny of complement in early stages of fish. For example, the transcripts of C3 have been demonstrated to be expressed in the developing embryos or larvae of southern catfish (*Silurus meridionalis*) [8], Atlantic halibut (*Hippoglossus hippoglossus* L.) [9,10], Atlantic cod (*Gadus morhua* L.) [11,12], spotted wolffish (*Anarhichas minor* Olafsen) [13], rainbow trout (*Oncorhynchus mykiss*) [14], Atlantic salmon (*Salmo salar*) [15], carp (*Cyprinus carpio*) [16], zebrafish (*Danio rerio*) [7], Indian major carp (*Labeo rohita*) [17], and olive flounder (*Paralichthys olivaceus*) [18]. The ontogeny of C3 has been investigated with RT-PCR and whole mount *in situ* hybridization in our recently work [8]. The other complement factors, such as bf, C4, C6, MBL, MASP, and complement factor 1 r/s - mannose binding lectin associated serine protease-like molecule, have also been investigated in the fish [7,16]. However, limit knowledge is available on the ontogeny of complement C9 in fish.

Southern catfish is an important economic fish in China with a wide distribution in south of the Yangtze River Basin [19]. However, the

* Corresponding author.
E-mail address: zhangqzdr@126.com (Q.-Z. Zhang).
[1] Yao-Wu Fu and Cheng-Ke Zhu contributed equally to this work.

https://doi.org/10.1016/j.fsi.2018.11.069
Received 20 September 2018; Received in revised form 7 November 2018; Accepted 29 November 2018
Available online 30 November 2018

southern catfish cultured in the fish farm is likely to have reduced disease resistance in recent years, leading to extremely vulnerable to various pathogens [20,21]. *Aeromonas hydrophila*, a common pathogen in southern catfish, could result in high mortality of the embryo, larva, and juvenile [22]. Recent study has suggested that complement factors of dojo loach (*Misgurnus anguillicaudatus*) were involved in the innate response against *A. hydrophila* [23]. While, the information of southern catfish complement component C9 is still unavailable.

The present study aimed to clone full length cDNA sequence of complement C9 of southern catfish, investigate the tissue expression, determine the ontogeny of C9 with whole mount *in situ* hybridization and RT-PCR, and assess the expression level of C9 mRNA in southern catfish after infection with *A. hydrophila*.

2. Materials and methods

2.1. Animal preparation and sample collection

For the cloning, tissue specific expression, and infection with *A. hydrophila* experiments, southern catfish with body length of 18.5 ± 2.3 cm and body weight of 65.5 ± 4.8 g were obtained from the Key Laboratory of Fisheries Science of Chongqing, China. All fish were kept at 25 ± 2 °C in flow through water system, and the fish were fed daily with granule feed at 1% of fish weight. For the tissue specific expression study, tissues from five healthy fish including blood, stomach, liver, kidney, head kidney, gut, muscle, brain, skin, gill, heart, and spleen were collected, immediately frozen in liquid nitrogen and stored at −80 °C.

For the ontogeny and whole mount *in situ* hybridization experiments, parent southern catfish were obtained from the Hechuan reach to the Beibei reach of Jialing River. The body lengths of the female and male were 92.6 and 79.0 cm, respectively. The body weights of the female and male were 9.5 and 5.8 kg, respectively. The parents were cultured in flow through water system at 19.5 ± 1.0 °C for 1 week. Then, chorionic gonadotropin (HCG) at a dose of 1000 U/kg and luteinizing hormone-releasing hormone analogues (LRH-A) at a dose of 25 μg/kg were injected into female fish by intraperitoneal injection. And HCG at a dose of 500 U/kg and LRH-A at a dose of 12.5 μg/kg were injected into male fish. Artificial insemination was conducted at 24 h after the injection of HCG and LRH-A. Eggs, embryos, and larvae were collected at 0, 1, 2, 5, 7, 11, 13.5, 15, 17, 20.5, 22.5, 25.5, 30, 32, 34, 36, 39, 42, 47.5, 50, 52, 54, 58, and 63 hpf (hour post fertilization), as well as 8, 16, 24, 32, 44, 56, 80, 104, 136, 172, and 212 hph (hour post hatching) (Table 2). The temperature during the development showed in Table 2. All samples were washed with 1 × PBS (phosphate buffered saline). Then the samples were frozen in liquid nitrogen and stored at −80 °C.

Table 1
Primer sequences of complement C9 used in the present study.

Primer	Sequence	Use
C9-F1	GGGTATTGAGGTCTTTGGGCAGTT	Cloning
C9-R1	TCTGCCACGGCTGACACTTGC	
C9-F2A	TGTTACAGAGCACAGCACGACTTCAGA	3′ RACE
C9-F2B	GAAATCAAAGACCCAAAGAAGACGGA	3′ RACE
C9-R2A	TGTGGATGGGTCCTTGACACGCTC	5′ RACE
C9-R2B	ATTAGAGGAGCAGACAGGTGGTGGA	5′ RACE
C9-F3	GGGCATCGGGGAAGGTCAG	Expression analysis
C9-R3	GAAATCAAAGACCCAAAGAAGACG	
18S-F	CCTGAGAAACGGCTACCACATCC	Expression analysis
18S-R	AGCAACTTTAATATACGCTATTGGAG	
C9-F4	CCCACAGATGACAAGAACAATA	For *in situ* hybridization probe
C9-R5	GTCCCTGGTGAGCACATACA	

2.2. RNA isolation and cDNA library construction

The liver of southern catfish was extracted using a TRIzol Reagent (Invitrogen, USA) to obtain the total RNA following the manufacturer's instructions. The obtained total RNA was quantified using a Nanodrop 2000 (Thermo Scientific, USA), and cDNA synthesis was performed with a M-Mulv Reverse Transcriptase cDNA Synthesis Kit (TaKaRa, Japan) following manufacturer's protocol.

2.3. Full length C9 cDNA cloning

The conserved regions of complement C9 sequences of *O. mykiss*, *D. rerio*, *Ctenopharyngodon idella*, *Xenopus laevis*, and *Paralichthys olivaceus* were used to design the primers of C9. The PCR was conducted with a Takara PCR Amplification Kit (Takara, Japan). The PCR program was as follows: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min, with final extension at 72 °C for 10 min. The target products were purified using a TIANgel Midi Purification Kit (TIANGEN, China) and then cloned into the pMD-19T vector (Takara, Japan) for sequence determination. The obtained sequence was used to design the primers of 5′-RACE and 3′-RACE. Then, 5′-RACE and 3′-RACE were performed using a BD SMART RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer's protocol. The PCR products were cloned into the pMD-19T vector and subsequently sequenced at the Beijing Genomics Institute (China). All primers used in the study were listed in Table 1.

2.4. Sequence analysis

The open reading frame (ORF) of the southern catfish complement C9 cDNA sequence was predicted using the ORFfinder of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The C9 cDNA sequence was analyzed at both nucleotide and amino acid levels using the Nucleic Translation of the EMBOSS explorer (http://emboss.bioinformatics.nl/). Prediction of domains was carried out using SMART (http://smart.embl-heidelberg.de/) [24]. Amino acid sequences of complement C9 of related species were obtained from NCBI GenBank. Multiple sequence alignment was performed using BioEdit [25].

2.5. Semi-quantitative RT-PCR analysis of C9 tissue expression pattern

To assess pattern of gene expression level of C9, semi-quantitative RT-PCR was performed for transcript from various tissues of the southern catfish. The total RNA of each fish was extracted as described above. Next, the total RNA of five samples with same quantity was pooled to synthesize the first chain cDNA. PCR amplifications were performed with a PCR program as follow: 94 °C for 3 min, followed by 33 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s, with final extension at 72 °C for 10 min. Reactions of each sample were performed in triplicate. The internal control, the negative control, and the positive control were conducted with 18S rRNA, reaction with no cDNA template, and reaction with C9 template, respectively. PCR products were electrophoresed on a 1.2% agarose gel, and then examined with a Gel DocTM XR documentation system (BioRad, USA).

2.6. In situ hybridization

Dioxigenin (DIG)-labeled RNA probe and control probe for C9 were prepared with DIG RNA Labeling Kit (Roche Molecular Biochemicals, Switzerland) according to manufacturer's instructions.

Liver samples were washed two times with PBS (pH 7.4, RNase-free) and then fixed with 4% paraformaldehyde (PFA) for at least 24 h at 4 °C. Tissues were immersed with a graded ethanol series and stored in 100% ethanol for 24 h at −20 °C. The tissues were then dehydrated sequentially in ethanol and xylene and embedded in paraffin. Ultrathin sections (8 μm in thickness) were prepared and mounted onto slides.

Table 2
Development stages/characters of eggs, fertilized eggs, embryos, and larvae used for the ontogeny study.

Time	WT (°C)	Development stage	Time	WT (°C)	Development stage/character
	19.0	Egg	42 hpf	19.8	Cardiac formation stage
0 h	19.0	Fertilized egg	47.5 hpf	20.5	Anal primordia appearance stage
1 hpf	19.0	Blastoderm stage	50 hpf	19.8	Otolith appearance stage
2 hpf	18.8	4 cell stage	52 hpf	19.8	Blood circulation stage
5 hpf	18.8	Multicellular stage	54 hpf	19.8	Pectoral fin primordia formation stage
7 hpf	18.8	Early blastula stage	58 hpf	19.8	Prehatching stage
11 phf	18.8	Middle blastula stage	63 hpf	19.8	Hatching stage
13.5 hpf	18.6	Late blastocyst stage	8 hph	21.0	Eye pigment formation
15 hpf	18.6	Early gastrula stage	16 hph	20.0	Eye pigment increase
17 hpf	18.6	Middle gastrula stage	24 hph	19.6	The second barbel primordia lower jaw appearance
20.5 hpf	18.6	Late gastrula stage	32 hph	18.8	Mandibular movement
22.5 hpf	19.0	Neurula stage	44 hph	20.0	Swim bladder appearance
25.5 hpf	19.2	Blastopore closure stage	56 hph	21.0	Stomach differentiation
30 hpf	19.6	Eye primordia formation stage	80 hph	20.5	Start feeding
32 hpf	20.0	Eye vesicle stage	104 hph	20.5	Larva stage
34 hpf	20.0	Early tail bud stage	136 hph	21.0	Dorsal fin differentiation
36 hpf	19.6	Middle tail bud stage	172 hph	20.5	Pelvic fin formation
39 hpf	19.6	Late tail bud stage	212 hph	21.0	Dorsal fin separation

WT, water temperature; hpf, hour post fertilization; hph, hour post hatching.

Tissue sections were deparaffinized and rehydrated with xylene and ethanol. The slides were washed twice in PBS buffer. Sections were permeabilized by incubating in 20 μg/mL proteinase K at 37 °C for 30 min. Prehybridization was done by fixing the sections in prehybridization solution for 2 h at 60 °C. Hybridization was performed using DIG-labeled oligonucleotide anti-sense and sense probes in hybridization solution (50 μg/mL heparin, 50% formamide, 0.5 mg/mL torula yeast tRNA, 0.1% Tween-20, 5 × SSC (NaCl + Na_3citrate)) for 18 h at 60 °C in a humidified chamber. The cover slips were carefully removed by washing in 2 × SSC, followed by washing twice in 50% deionized formamide and 50% 2 × SSC for 15 min at 60 °C, twice in 2 × SSC for 15 min, and twice in 1 × SSC for 15 min, respectively. The RNA in the sections was removed with 20 μg/mL RNAase at 37 °C in 1 × SSC for 30 min, followed by washing three times in 0.1 × SSC for 15 min at 37 °C, and then washed twice in washing buffer (0.1 M Tris +0.15 M NaCl, pH 7.4) for 10 min. The sections were then incubated with blocking solution for 1 h. Anti-DIG Fab fragments at a dilution (in blocking solution) of 1:1000 were applied and allowed to react for 4 h at room temperature. The color reaction was developed over 3 h at 37 °C using Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. The reaction was visualized with a microscope (Nikon 80i, Japan).

2.7. Ontogeny of C9 in southern catfish

2.7.1. Expression of C9 in egg, embryo, and larva

The ontogeny expression of C9 in egg, embryo, and larva was analyzed by semi-quantitative RT-PCR. All samples were performed in triplicates, and the conditions used for RT-PCR were described in section of 2.5. PCR products were electrophoresed on a 1.2% agarose gel, and then examined with a Gel DocTM XR documentation system (BioRad, USA).

2.7.2. Whole mount in situ hybridization

DIG-labeled RNA probe and control probe for C3 were prepared as section 2.6 described. Embryos were fixed in 4% PFA in phosphate saline solution (pH 7.4) for 24 h, followed by washing with 1 × PBT (10 × PBS 100 mL, DEPC water 890 mL, 10% Tween 20 10 mL) three times (5 min each). The embryos were dechorionated with 100% methanol five times (5 min each) and then stored at −20 °C at least 24 h.

Embryos/eggs/larvae (EEL) were rehydrated with successive dilutions of methanol in PBT (75% methanol, 50% methanol, and 25% methanol; vol/vol), each for 5 min. Then, the EEL were washed four times with 1 × PBT for 5 min. The rehydrated embryos were digested with proteinase K (5 μg/mL) at room temperature for 30 min. The digested EEL were washed three times with PBT: two quick washes and one 5-min wash. The EEL were subsequently incubated in 4% PFA for 20 min and washed five times with 1 × PBT (for 5 min each). Prehybridization was conducted by incubating EEL with 200 μL prehybridization solution for 2–5 h in a 67 °C water bath. Next, the prehybridization solution was removed, and 200 μL hybridization solution containing 3% of probe stock solution (vol/vol) was added to incubate with the EEL overnight in a 67 °C water bath.

The next day, the hybridization reaction mixture containing the probe was removed, and 1 mL of prewarmed hybridization solution was added to wash the EEL for 20 min. Then, the EEL were washed with prewarmed solutions of 2 × SSCT (20 × SSC 100 mL, DEPC water 890 mL, 10% Tween 20 10 mL) in formamide (50% 2 × SSCT three times, 75% 2 × SSCT one time, 100% 2 × SSCT two times) for 20 min at 67 °C. The EEL were subsequently washed with prewarmed 1 × PBT for 5 min at 67 °C, followed by washing with 1 × PBT for 5 min at 37 °C. The embryos were then incubated with 1 mL blocking solution for 5 h at 37 °C. Lastly, the EEL were incubated with a solution of anti-DIG-AP antibody (1:2000) in blocking solution overnight at 4 °C.

On the third day, the EEL were washed three times with 1 × PBT for 5 min each, followed by washing six times with 1 × PBT for 30 min each. Then, the EEL were washed three times with NTMT (2 mL 100 mM pH 9.5 Tris HCl, 1 mL 50 mM $MgCl_2$, 2 mL 100 mM NaCl, 500 μL 0.1% Tween-20, and 14.5 mL water) solution for 5 min each. The EEL were subsequently transferred to a 24-well plate. The color reaction was developed at 37 °C using Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. The staining reaction was monitored every 15 min using a microscope. When the desired staining intensity was reached, the EEL were immediately washed three times with 1 × PBT. The reaction was subsequently stopped by the stop solution. Lastly, the EEL were stored in 0.5 mL 80% glycerol stock solution (vol/vol) at 4 °C. The reaction was visualized with a microscope (Nikon 80i, Japan).

2.8. A. hydrophila infection and the C9 expression analysis

The bacteria *A. hydrophila* strain was obtained from the Laboratory of Molecular Biology of Institute of Hydrobiology at Jinan University (Guangzhou, China). Single colonies of *A. hydrophila* were picked after the strain being incubated with Luria-Bertani Medium (LB) plate. Then, the single colonies were inoculated into 20 mL LB at 28 °C for 24 h. The harvested *A. hydrophila* was centrifuged at 4500 × *g* for 10 min, and then it was resuspended in PBS and adjusted at a concentration of

1.0×10^6 CFU/mL. Two groups with each containing 40 southern catfish were prepared for the challenge trial. A total of 200 μL A. *hydrophila* solution and 0.65% sodium chloride solution were injected into the trial fish group and control fish group by intraperitoneal injection, respectively. The fish were randomly sampled on days 1, 3, 7, 14, 21, and 35 with each group in triplicate. The liver, intestine, and spleen were collected after fish were anesthetized with 150 mg/L tricaine methanesulfonate (MS-222, Sigma). All samples were immediately frozen in liquid nitrogen and stored at −80 °C for total RNA extraction.

Semi-quantitative RT-PCR was used to detect the C9 mRNA expression in the liver, intestine, and spleen on different days after *A. hydrophila* infection. The program used for RT-PCR was described in section 2.5 with each sample in triplicate. PCR products were electrophoresed on a 1.2% agarose gel, and then examined with a Gel DocTM XR documentation system (BioRad, USA).

2.9. Statistics

All experimental data are expressed as the mean ± SD (standard deviation). Assumptions of normality and homogeneity of variances were confirmed with the Shapiro-Wilk test and Levene's test, respectively using a statistical analysis system software (SPSS 19.0). Significance differences between the control and infected groups were determined using independent samples *t*-test. Results were considered to be statistically significant when $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Isolation and sequence analysis of full length C9 from southern catfish

Full length C9 gene was isolated from southern catfish cDNA and were analyzed through sequencing. The full length C9 sequence was 1984 bp with a 5′ UTR of 50 bp, an ORF of 1809 bp, and a 3′ UTR of 125 bp (Fig. 1). The 3′ UTR of C9 had the characteristic polyadenylation signals (AATAAA) at position of 1926 bp. The ORF of C9 sequence encoded a predicted protein of 602 amino acids with a molecular formula $C_{2917}H_{4609}N_{813}O_{914}S_{36}$, and a predicted isoelectric point (pI) of 6.82.

C9 of southern catfish comprised of two thrombospondin type 1 repeats (TSP1), a low-density lipoprotein receptor class A domain (LDLa), a MAC/Perforin domain (MACPF) (Fig. 2). Two TSP1 domains were at position 44–97 and 559–601 amino acids, the LDLa domain was at 103–139 amino acids, and the MACPF domain was at 141–511 amino acids with a structure characteristic of YFGFLEDFGTHY at position of 337–348 amino acids (Figs. 1 and 2). C9 had a signal peptide cleavage site at residues 26–27 (Gly-Lys).

3.2. Tissue expression analysis of C9 and in situ hybridization

The mRNA expression levels of C9 were detected in the blood, liver, spleen, kidney, head kidney, stomach, intestine, skin, gill, brain, and heart using semi-quantitative RT-PCR with 18S rRNA as the internal reference gene. The results showed that C9 mRNA was highly expressed in the liver, weekly expressed in the spleen, stomach, intestine, and head kidney (Fig. 3). There was no mRNA expression of C9 in the blood, skin, kidney, muscle, gill, brain, and heart (Fig. 3). *In situ* hybridization result revealed that the mRNA of C9 was detected in all liver cells (Fig. 4). While in the endothelial cells of the liver blood vessel, no signal of C9 mRNA was presented (Fig. 4).

3.3. Ontogeny of C9 in southern catfish

The results showed that no C9 transcript was detected in the unfertilized eggs, the fertilized eggs, and the embryos at 1 to 25.5 hpf (Fig. 5). A weak mRNA expression of C9 was first detected at 30 hpf (Fig. 5). The mRNA expression of C9 was upregulated at 34 hpf and 36 hpf. It further increased significantly to the highest level at 39 hpf and was maintained until 63 hpf. After hatching, the mRNA expression of C9 was still maintained at a high level until 212 hph (Fig. 5).

Whole mount *in situ* hybridization results revealed that C9 mRNA expression was not detected at 25.5 and 30 hpf (Fig. 6A–D). A low signal of C9 mRNA was first detected at 34 hpf and distributed in the yolk syncytial layer (Fig. 6 E, F). The transcript of C9 was increased significantly from 36 hpf to 46 hpf (Fig. 6G–Q), and it was presented locating all over the yolk sac at 52 hpf (Fig. 6 R, S) with embryo development. At 0, 12, and 24 h post hatching, the mRNA signal of C9 was enhanced in the abdomen and postabdomen of the yolk sac (Fig. 7A–L). At 36 and 48 hph, C9 transcript was presented in the liver and the yolk sac (Fig. 7M–U). The yolk sac was gradually absorbed with larva growth. At 104 hph, the yolk sac was completely absorbed, and the mRNA of C9 was presented only in the liver (Fig. 7V–Y).

3.4. Expression of C9 in response to A. hydrophila infection

The transcript of C9 in the liver tissue was significantly upregulated on day 1 post injection of *A. hydrophila* ($p < 0.01$) (Fig. 8 A). The C9 mRNA expression was downregulated on day 3, and it showed no significant difference between infection group and control group from day 7 to day 35 (Fig. 8 A). While in the spleen tissue, the mRNA of C9 was downregulated significantly on day 1 post injection of *A. hydrophila* ($p < 0.05$) (Fig. 8 B). Then, it was upregulated on day 3 and peaked on day 7. In the intestine, C9 mRNA expression showed significant upregulation on day 14 ($p < 0.05$), and no significant change was detected in the others sample day post infection (Fig. 8 C).

4. Discussion

In this study, the full length cDNA sequence encoding C9 was cloned from the liver of the southern catfish. Until date no C9 cDNA sequence for southern catfish was available. The alignment demonstrated that the overall structure was highly similar to other vertebrates. The SMART domain research revealed the existence of 4 terminal complement complex (TCC) specific domains in southern catfish. The first domain was the thrombospondin type 1 repeats (TSP-1) domain that was involved in cell-cell interactions, inhibition of angiogenesis, and apoptosis [26]. The second domain was the low-density lipoprotein receptor domain class A (LDLa) domain. The third and fourth domains were the membrane attack complex/perforin (MACPF) domain and epidermal growth factor-like (EGF) domain, respectively. In the C-terminal region, the C9 cDNA had a second TSP 1 domain. In mammals and birds, there are no the second TSP 1 domain [27]. In all reported C9 cDNA in fish, i.e., grass carp [28], Japanese flounder [29], rainbow trout [30], redlip mullet [26], rock bream [31], nile tilapia [32], and yellow catfish [33], have the second TSP 1 domain in the C-terminal region. Both the first and the second TSP1 domains were rich in cysteine, and they contained the C-mannosylation motifs WXXW and WXXWXXW, respectively. Those sequence data suggested that southern catfish C9 functions were similar to the C9 of other animals.

In mammals, the liver is the main production site of the complement components, which suggests that liver is an important organ for innate immunity and plays a key role in complement activation and in immune defense mechanisms against bacterial agents [18,34]. The liver is also the main source complement component proteins in teleost [27]. For example, in rainbow trout, dojo loach, and large yellow croaker, the expressions of C3 in the liver were higher than the other tissues [14,23,35]; in redlip mullet, yellow catfish, and grass carp, the transcripts of C9 were highly expressed in the liver [26,28,33]. In the present work, the highest mRNA expression of C9 was detected in the liver with RT-PCR and *in situ* hybridization. This indicated that liver was indeed the main supplier of C9 in southern catfish.

The mRNA expression of C9 produced in the other sites of southern catfish were found in the spleen, stomach, intestine, and head kidney.

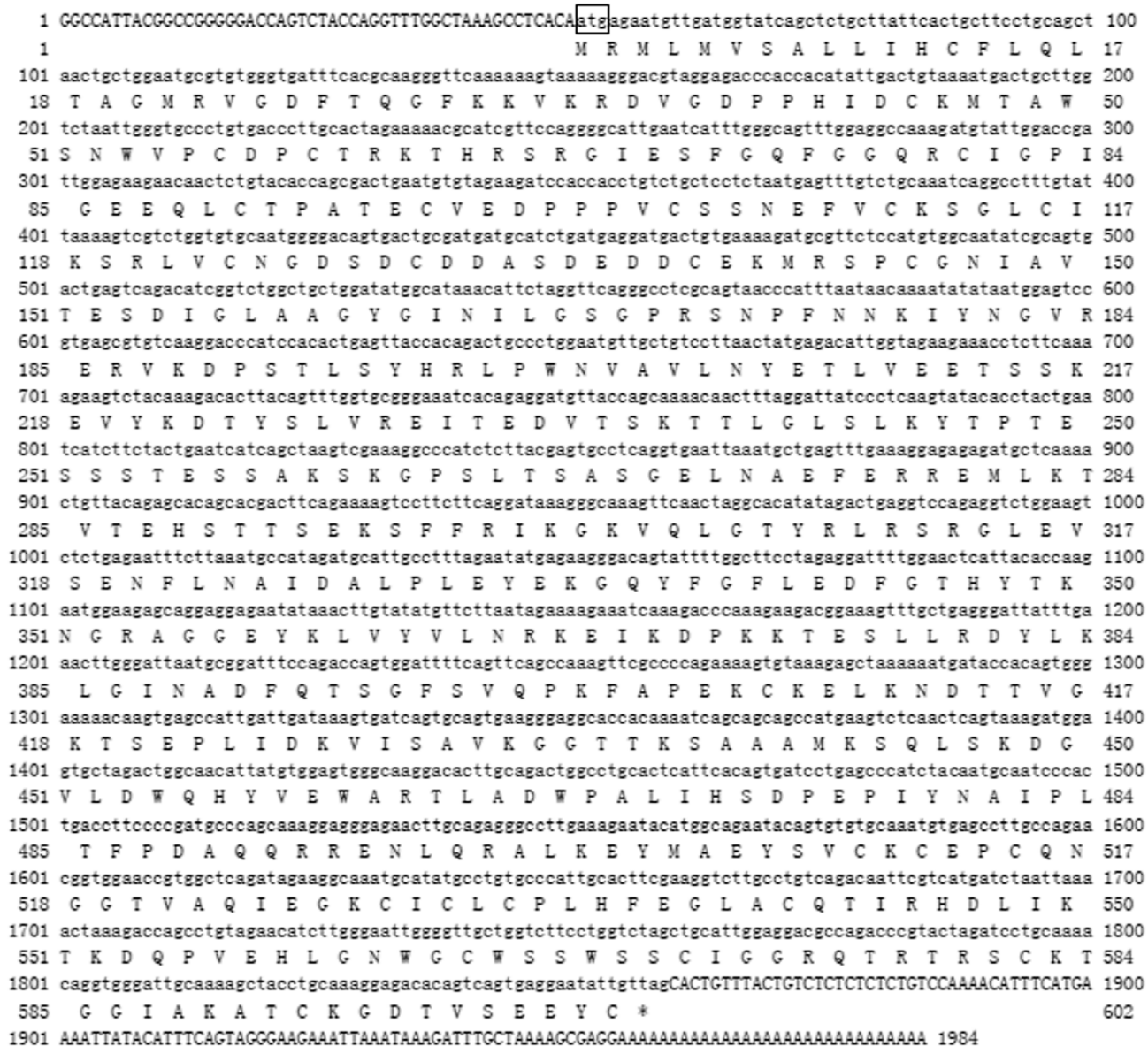

Fig. 1. Nucleotide and deduced amino acid sequence of the southern catfish. Initiation codon (box) and stop codon (*).

Recently studies also showed the extrahepatic expression of C9 in other fish. For example, in grass carp, the transcription of C9 was detected in the spleen, skin, head kidney, renal kidney, brain, muscle, thymus, gill, blood, heart, and intestine [28]; in rock bream, the mRNA of C9 was presented in the brain, gills, skin, heart and spleen, as compared to muscle [31]. These results also demonstrated that extrahepatic expression of C9 changed with different fish. Extrahepatic expression of complement components plays an important role in regulation of local immune responses including crosstalk with the adaptive immune system, as well as damage and repair of tissues [5]. Southern catfish, belonging to the carnivore family, would ingest many pathogens during feeding. So the mRNA expression C9 in the stomach and intestine were most likely acting in defense against invading pathogens ingested through the mouth. Thus, the extrahepatic expression of C9 might make the immune system more effective.

Egg, fertilized egg, embryo, and larva are immediately exposed to aquatic pathogens. It has been demonstrated that transferred maternal complement components were involved in defense against microbial attacks in the aquatic environment during early embryonic development [13,14,33]. However, the maternal supplies are low and did not persist [14]. Thus, the mRNA of complement components need to be expressed in the early development stages. A few studies reported the mRNA expression of C3 in Indian major carp [17], rainbow trout [14], Atlantic salmon [7], Atlantic cod [6], and Atlantic halibut [9], and the C3 transcripts were detected at 6 hpf, 7 dpf, 14 dpf, 1 dph, and 15 dph, respectively. In addition, the mRNA expression of C3 in carp was detected as early as 0 hpf [16]. These results suggested that the complement components were presented at early stage development and ready to react against bacterial infection. However, there are limit studies on the expression of C9 in egg, fertilized egg, embryo, and larva. In yellow catfish, transcripts of the C9 gene were low in the newly fertilized egg, it was upregulated to a moderate level at the gastrula stage and to a high level at the blood circulation stage, and then it reached the highest value at the newly hatched larval stage [33]. In large yellow croaker, the mRNA expression of C9 were detected at the two-cells stage [27]. In the present study, there were no C9 transcripts in the eggs, fertilized eggs, and embryos at 1 to 25.5 hpf. While a weak mRNA expression of C9 was first detected at 30 hpf. The mRNA expression of C9 was upregulated significantly at 34 hpf and reached to the highest level at 39 hpf. After hatching, the mRNA expression of C9 was still maintained at

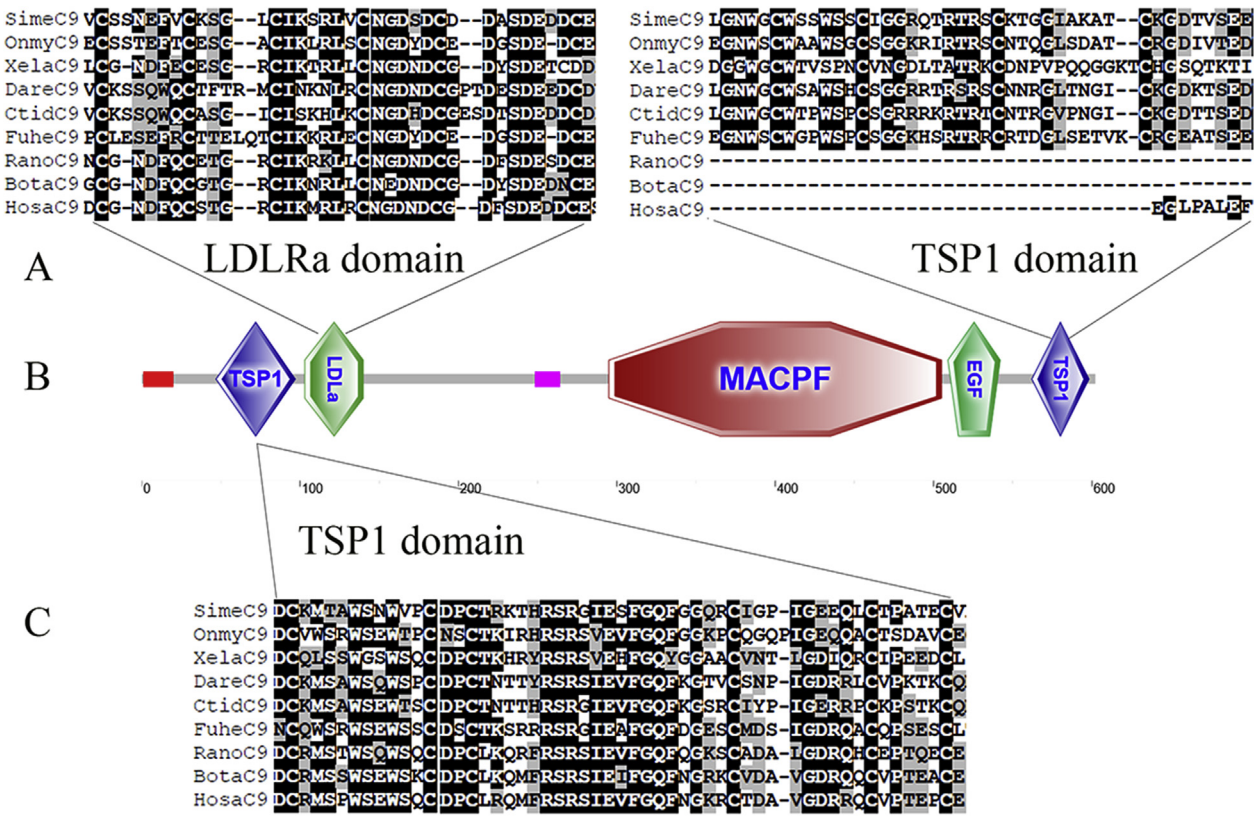

Fig. 2. The predicted protein domain characteristics of complement component C9 (B). Thrombospondin type 1 repeats (TSP 1) domain, low-density lipoprotein receptor domain class A (LDLa) domain, membrane attack complex/perforin (MACPF) domain, and epidermal growth factor-like (EGF) domain. Multiple alignments of the southern catfish C9 LDLa domain and the second TSP 1 domain (A), the first TSP 1 domain (C). The GenBank accession numbers of the sequences used in the alignment are shown as follows: OnmyC9 (*Oncorhynchus mykiss*) NP_001117898; XelaC9 (*Xenopus laevis*) NP_001079814; DareC9 (*Danio rerio*) CAX15434; CtidC9 (*Ctenopharyngodon idella*) ABN49522; FuheC9 (*Fundulus heteroclitus*) AAR87007; RanoC9 (*Rattus norvegicus*) AAH91127; HosaC9 (*Homo sapiens*) CAA69849; SimeC9 (*Silurus meridionalis*), MH763625.

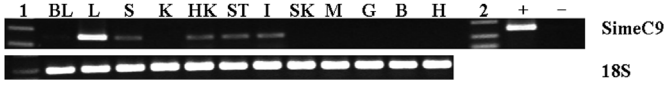

Fig. 3. Tissue distribution of the C9 mRNA in southern catfish. 18S rRNA was used as an internal control. BL, blood; L, liver; S, spleen; K, kidney; HK, head kidney; ST, stomach; I, intestine; SK, skin; M, muscle; G, gill; B, brain; H, heart; 1 and 2, DL 2000 maker; +, positive control; -, negative control; SimeC9, *Silurus meridionalis*.

a high level until 212 hph. The mRNA of C9 that was not expressed in the eggs, fertilized eggs, and embryos at 1 to 25.5 hpf might due to the transferred maternal C9 presented in these stages. In addition, the time of appearance of C9 mRNA which varied in early embryonic development stages of southern catfish, yellow catfish, and large yellow croaker might be related to the different fish species. These results also indicated that the complement component C9 was indeed expressed at early stage development.

Whole mount *in situ* hybridization demonstrated that the mRNA of C9 was first detected at 34 hpf and distributed in the yolk syncytial layer. The signal of C9 mRNA increased significantly in the following time and located all over the yolk sac at 52 hpf. The result suggested that yolk syncytial layer was the primary site for the expression of C9. This phenomenon was in accordance with the C3 which was first detected in the yolk syncytial layer of fish [8,13,16]. With embryo development, the yolk sac was gradually absorbed. An increased mRNA expression of C9 was presented in hepatic cells and a decreased mRNA

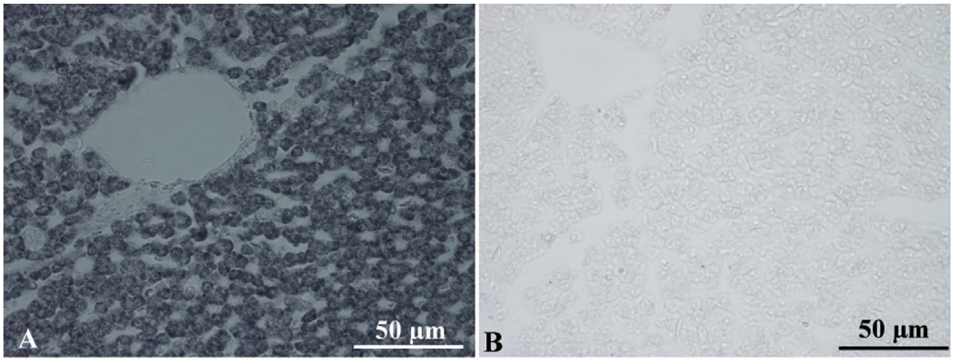

Fig. 4. Expression of complement C9 mRNA in the liver of southern catfish detected by *in situ* hybridization. B, negative control by sense probe. Scale bar = 50 μm.

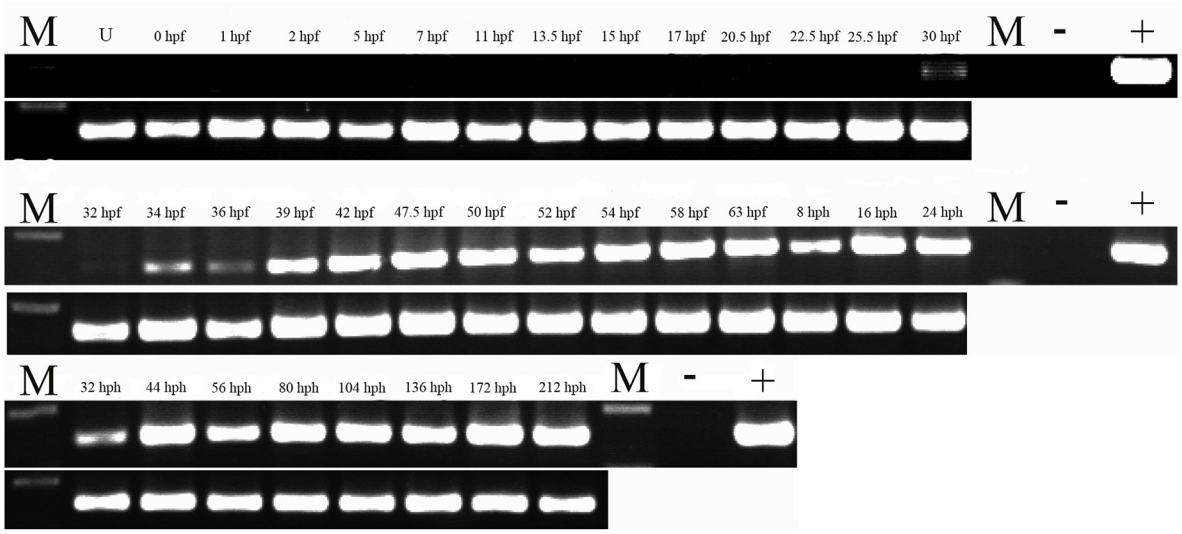

Fig. 5. Expression of C9 mRNA during the embryonic and early larval developmental stages. Eggs, embryos, and hatchlings were sampled at different time points. The RNA was extracted and analyzed by RT-PCR. M, Marker DL2000; U, unfertilization; hpf, hour post fertilization; hph, hour post hatching; +, positive control; -: negative control; 18S rRNA was used as a positive control to normalize the samples.

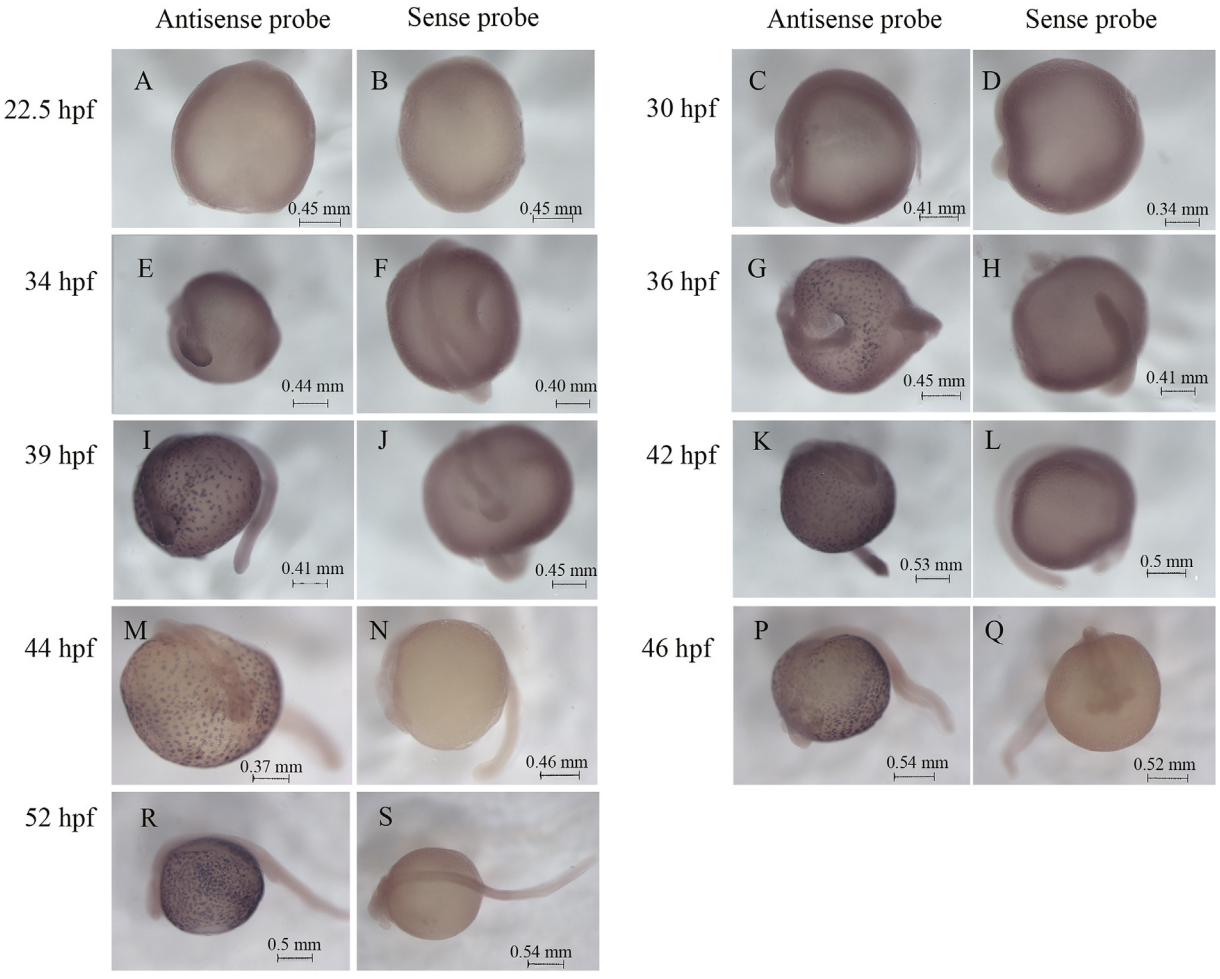

Fig. 6. Whole mount *in situ* hybridization analysis of C9 mRNA in embryos post-fertilization. The transcript of C9 was not expressed at 22.5 (A, B) and 30 (C, D). The mRNA expression of C9 was presented on the yolk sac at 34 (E), 36 (G), 39 (I), 42 (K), 44 (M), 46 (P), and 52 (R) hpf using the antisense probe. The sense probe served as a negative control. hpf, hour post fertilization. Scale bars of A to S were 0.45, 0.45, 0.41, 0.34, 0.44, 0.40, 0.45, 0.41, 0.41, 0.45, 0.53, 0.50, 0.37, 0.46, 0.54, 0.52, 0.50, and 0.54 mm, respectively.

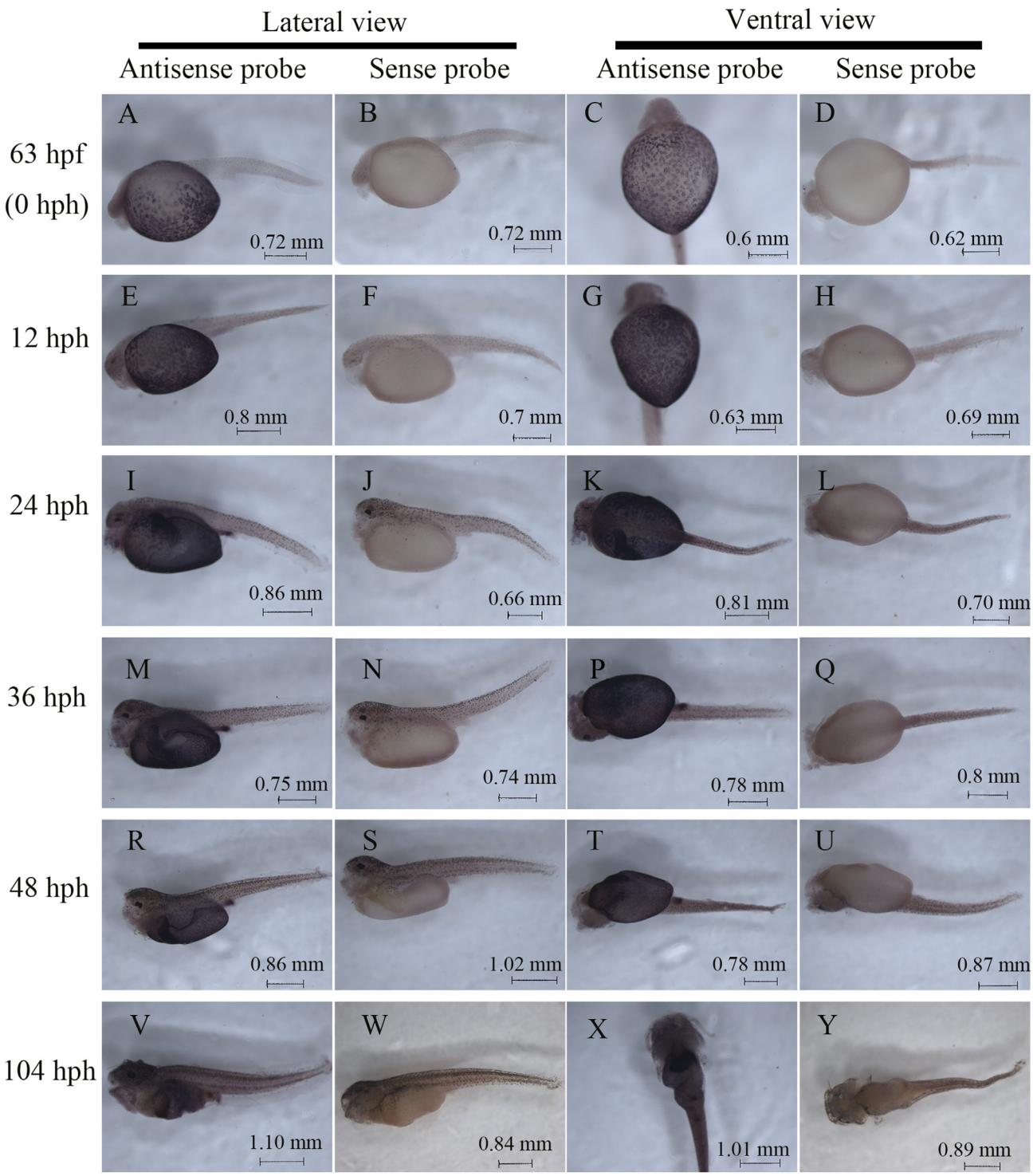

Fig. 7. Whole mount *in situ* hybridization analysis of C9 mRNA in larvae post hatching. The mRNA expression of C9 in liver was detected at 36 hph using the antisense probe. All yolk was absorbed at 104 hph and the mRNA expression of C9 was presented only in the liver of southern catfish. The sense probe served as a negative control. hph, hour post hatching. Scale bars of A to Y were 0.72, 0.72, 0.60, 0.62, 0.80, 0.70, 0.63, 0.69, 0.86, 0.66, 0.81, 0.70, 0.75, 0.74, 0.78, 0.80, 0.86, 1.02, 0.78, 0.87, 1.10, 0.84, 1.01, and 0.89 mm, respectively.

expression of C9 was detected in the yolk syncytial layer. This indicated that liver was the second site for the expression of C9 and played an important role in the expression of C9 at early stages of southern catfish.

The MAC complex has been microscopically observed and contains small pores on cell surface of the pathogen, thus leading to the destruction of foreign pathogens and the death of infected cells [5,36]. A previous study demonstrated that the molar ratios of complement components C5b, C6, C7, C8, and C9 in carp serum were 1:1:1:1:4, respectively [37]. Therefore, C9 behaves as crucial protein among the TCC proteins, and it plays a key role in the innate resistance to pathogens [4]. In rock bream, the mRNA expression of C9 was upregulated significantly post the challenged with *Edwardsiella tarda*, *Streptococcus iniae*, lipopolysaccharide, or rock bream iridovirus [31]. In large yellow croaker, a qPCR study indicated that C9 gene expression was effectively triggered by *Vibrio alginolyticus* at 24 h post-infection [27].

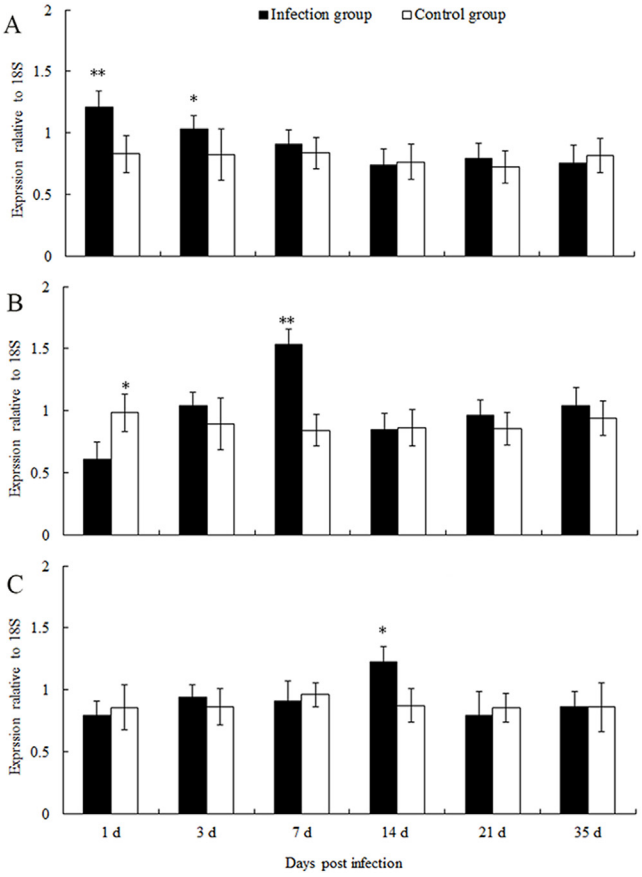

Fig. 8. Expression levels of C9 mRNA on 1, 3, 7, 14, 21, and 35 days in the liver (A), spleen (B), and intestine (C) following infection with *Aeromonas hydrophila* evaluated by real-time PCR. Expression levels are normalized against 18S rRNA. Bars represented the mean ± SD (n = 3) for each time. $*p < 0.05$, $**p < 0.01$.

The present work was consistent with the previous study. The mRNA of southern catfish C9 was upregulated significantly on day 1 post injection with *A. hydrophila*. The mRNA expression of C9 in extrahepatic tissues also showed an upregulation post infection with *A. hydrophila*. In the spleen of southern catfish, the transcription level of C9 reach peaked on day 7; in intestine, the highest level of C9 mRNA expression was detected on day 14. The other fish was in consistent with the phenomenon. C9 transcripts were upregulated significantly in the head kidney, spleen, and hepatopancreas of grass carp after infection with bacterial pathogen *Flavobacterium columnare* [28]. Injection of *A. hydrophila* led to upregulation of the C9 gene in the spleen, head kidney, kidney, and blood tissues of yellow catfish [33]. These results suggested that the southern catfish C9 was sensitive to the bacterial challenge and might contribute to the host's early defense against invading pathogenic.

In conclusion, the present study identified and characterized the full length cDNA of C9 from southern catfish, and deduced the amino acid sequence of C9. The mRNA expression of C9 was detected in the liver, spleen, stomach, intestine, and head kidney. The primary site of embryonic C9 mRNA expression was the yolk syncytial layer, followed by the liver. The transcript of C9 was upregulated significantly in the liver, spleen, and intestine after infection with *A. hydrophila*. These results suggest that C9 plays an important role in defending against invading pathogens in southern catfish.

Conflicts of interest

The authors have no competing interests to declare.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 41476100, 31702382), the Science and Technology Project of Guangdong Province (2016B090918112, 2016B090918029), the Guangzhou Science and Technology Project (201604020040), the Marine and Fishery Special Project of Fish Diseases Treatment and Prevention in Guangdong Province (YueCaiNong [2016] No.11), as well as the China Postdoctoral Science Foundation (2017M612847).

References

[1] H. Boshra, J. Li, J.O. Sunyer, Recent advances on the complement system of teleost fish, Fish Shellfish Immunol. 20 (2006) 239–262.
[2] J.O. Sunyer, H. Boshra, G. Lorenzo, D. Parra, B. Freedman, N. Bosch, Evolution of complement as an effector system in innate and adaptive immunity, Immunol. Res. 27 (2003) 549–564.
[3] M. Nonaka, S.L. Smith, Complement system of bony and cartilaginous fish, Fish Shellfish Immunol. 10 (2000) 215–228.
[4] M.C.H. Holland, J.D. Lambris, The complement system in teleosts, Fish Shellfish Immunol. 12 (2002) 399–420.
[5] M. Nakao, M. Tsujikura, S. Ichiki, T.K. Vo, T. Somamoto, The complement system in teleost fish: progress of post-homolog-hunting researches, Dev. Comp. Immunol. 35 (2011) 1296–1308.
[6] S. Lange, S. Bambir, A.W. Dodds, B. Magnadottira, The ontogeny of complement component C3 in Atlantic cod (*Gadus morhua* L.)-an immunohistochemical study, Fish Shellfish Immunol. 16 (2004) 359–367.
[7] Z. Wang, S. Zhang, G. Wang, Response of complement expression to challenge with lipopolysaccharide in embryos/larvae of zebrafish *Danio rerio*: acquisition of immunocompetent complement, Fish Shellfish Immunol. 25 (2008) 264–270.
[8] Y.W. Fu, C.K. Zhu, Q.Z. Zhang, Molecular characterization and expression analysis of complement component C3 in southern catfish (*Silurus meridionalis*) and a whole mount *in situ* hybridization study on its ontogeny, Fish Shellfish Immunol. 84 (2019) 865–875.
[9] S. Lange, S. Bambir, A.W. Dodds, B. Magnadottir, An immunohistochemical study on complement component C3 in juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.), Dev. Comp. Immunol. 28 (2004) 593–601.
[10] S. Lange, S.H. Bambir, A.W. Dodds, T. Bowden, I. Bricknell, S. Espelid, B. Magnadottir, Complement component C3 transcription in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae, Fish Shellfish Immunol. 20 (2006) 285–294.
[11] B. Magnadottir, S. Lange, A. Steinarsson, S. Gudmundsdottir, The ontogenic development of innate immune parameters of cod (*Gadus morhua* L.), Comparative biochemistry and physiology Part B, Biochemistry & molecular biology 139 (2004) 217–224.
[12] S. Lange, A.W. Dodds, S. Gudmundsdottir, S.H. Bambir, B. Magnadottir, The ontogenic transcription of complement component C3 and Apolipoprotein A-I tRNA in Atlantic cod (*Gadus morhua* L.)-a role in development and homeostasis? Dev. Comp. Immunol. 29 (2005) 1065–1077.
[13] T. Ellingsen, C. Strand, E. Monsen, J. Bøgwald, R.A. Dalmo, The ontogeny of complement component C3 in the spotted wolffish (*Anarhichas minor* Olafsen), Fish Shellfish Immunol. 18 (2005) 351–358.
[14] M. Løvoll, T. Kilvik, H. Boshra, J. Bøgwald, J.O. Sunyer, R.A. Dalmo, Maternal transfer of complement components C3-1, C3-3, C3-4, C4, C5, C7, Bf, and Df to offspring in rainbow trout (*Oncorhynchus mykiss*), Immunogenetics 58 (2006) 168–179.
[15] M. Løvoll, H. Johnsen, H. Boshra, J. Bøgwald, J.O. Sunyer, R.A. Dalmo, The ontogeny and extrahepatic expression of complement factor C3 in Atlantic salmon (*Salmo salar*), Fish Shellfish Immunol. 23 (2007) 542–552.
[16] H.B. Huttenhuis, C.P. Grou, A.J. Taverne-Thiele, N. Taverne, J.H. Rombout, Carp (*Cyprinus carpio* L.) innate immune factors are present before hatching, Fish Shellfish Immunol. 20 (2006) 586–596.
[17] J. Mishra, P.K. Sahoo, B.R. Mohanty, A. Das, Sequence information, ontogeny and tissue-specific expression of complement component C3 in Indian major carp, *Labeo rohita* (Hamilton), Indian J. Exp. Biol. 47 (2009) 672–678.
[18] J.W. Lee, Y.M. Lee, J.H. Lee, J.K. Noh, H.C. Kim, C.J. Park, J.W. Park, I.J. Hwang, S.Y. Kim, The expression analysis of complement component C3 during early developmental stages in olive flounder (*Paralichthys olivaceus*), Dev Reprod 17 (2013) 311–319.
[19] Z.H. Liu, Y.G. Zhang, D.S. Wang, Studies on feminization, sex determination, and differentiation of the southern catfish, *Silurus meridionalis*-a review, Fish Physiol. Biochem. 36 (2010) 223–235.
[20] Q.Z. Zhang, Z.J. Wang, *Dermocystidium* sp. infection in cultured juvenile southern catfish *Silurus meridionalis* in China, Dis. Aquat. Org. 65 (2005) 245–250.
[21] Y. Geng, K.Y. Wang, Isolation and characterization of *Edwardsiella ictaluri* from southern catfish, *Silurus soldatovi meridionalis*, (Chen) cultured in China, J. World Aquacult. Soc. 44 (2013) 273–281.
[22] Q.Z. Zhang, D.K. Jiang, Parasitic diseases and their prevention and treatment of the southern catfish (*Silurus meridionalis* Chen), J Southwest Chin Norm Univ (Nat Sci) 21 (1996) 622–628 (in Chinese with English abstract).
[23] Y. Xu, Y. Yu, X. Zhang, Z. Huang, H. Li, S. Dong, Y. Liu, F. Dong, Z. Xu, Molecular characterization and expression analysis of complement component 3 in dojo loach

(*Misgurnus anguillicaudatus*), Fish Shellfish Immunol. 72 (2018) 484–493.
[24] I. Letunic, P. Bork, 20 years of the SMART protein domain annotation resource, Nucleic Acids Res. 46 (2018) D493–D496.
[25] T.A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucleic Acids Symp. Ser. 4 (1999) 95–98.
[26] D.S. Liyanage, W.K.M. Omeka, G.I. Godahewa, S. Lee, B.H. Nam, J. Lee, Membrane attack complex-associated molecules from redlip mullet (*Liza haematocheila*): molecular characterization and transcriptional evidence of C6, C7, C8β, and C9 in innate immunity, Fish Shellfish Immunol. 81 (2018) 1–9.
[27] B. Guo, C. Wu, Z. Lv, C. Liu, Characterisation and expression analysis of two terminal complement components: C7 and C9 from large yellow croaker, *Larimichthys crocea*, Fish Shellfish Immunol. 51 (2016) 211–219.
[28] L. Li, M.X. Chang, P. Nie, Molecular cloning, promoter analysis and induced expression of the complement component C9 gene in the grass carp *Ctenopharyngodon idella*, Vet. Immunol. Immunopathol. 118 (2007) 270–282.
[29] T. Katagiri, I. Hirono, T. Aoki, Molecular analysis of complement component C8 and C9 cDNAs of Japanese flounder, *Paralichthys olivaceus*, Immunogenetics 50 (1999) 43–48.
[30] M.P. Chondrou, A.V. Londou, I.K. Zarkadis, Expression and phylogenetic analysis of the ninth complement component (C9) in rainbow trout, Fish Shellfish Immunol. 21 (2006) 572–576.
[31] W.D. Wickramaarachchi, Q. Wan, Y. Lee, B.S. Lim, M. De Zoysa, M.J. Oh, S.J. Jung, H.C. Kim, I. Whang, J. Lee, Genomic characterization and expression analysis of complement component 9 in rock bream (*Oplegnathus fasciatus*), Fish Shellfish Immunol. 33 (2012) 707–717.
[32] X.-x. Hu, J.-m. Cao, M.-x. Lu, Q. Chen, K.-p. Chen, Cloning and expression analysis of complement 9 gene in *Oreochromis niloticus*, Freshw. Fish. 47 (2017) 3–11.
[33] H. Zheng, W. Ji, G.R. Zhang, X.T. Zhang, Z.C. Shi, K.J. Wei, R.B. Yang, J.P. Gardner, Molecular characterization and expression analyses of the complement component C8α, C8β and C9 genes in yellow catfish (*Pelteobagrus fulvidraco*) after the *Aeromonas hydrophila* challenge, Int. J. Mol. Sci. 17 (2016) e345.
[34] B.P. Morgan, P. Gasque, Expression of complement in the brain: role in heath and disease, Immunol. Today 17 (1996) 461–466.
[35] H. Wang, P. Qi, B. Guo, J. Li, J. He, C. Wu, Y. Gul, Molecular characterization and expression analysis of a complement component C3 in large yellow croaker (*Larimichthys crocea*), Fish Shellfish Immunol. 42 (2015) 272–279.
[36] J.A. Jenkins, R. Rosell, D. Ourth, L.B. Coons, Electron microscopy of bactericidal effects produced by the alternative complement pathway of channel catfish, J. Aquat. Anim. Health 3 (1991) 16–22.
[37] M. Nakao, T. Uemura, T. Yano, Terminal components of carp complement constituting a membrane attack complex, Mol. Immunol. 33 (1996) 933–937.