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Identification, expression profile and analysis of the antimicrobial activity of *collectin 11* (*CL-11, CL-K1*), a novel complement-associated pattern recognition molecule, in half-smooth tongue sole (*Cynoglossus semilaevis*)

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ABSTRACT

Collectin 11 (CL-11, also known as collectin kidney-1, CL-K1), a new member of the vertebrate C-type lectin superfamily, plays an important role in innate immunity as a pattern recognition molecule of the lectin complement pathway. However, little is known about CL-11 in teleosts. In the present study, a CL-11 homolog was identified and characterized from half-smooth tongue sole (*Cynoglossus semilaevis*) (designated as *CsCL*-11). The full-length cDNA of *CsCL*-11 is 1220 bp long and includes a 5′untranslated region (5′-UTR) of 180 bp, a 3′-UTR of 218 bp and an open reading frame (ORF) of 819 bp encoding 273 amino acids. Multiple sequence alignment revealed that the deduced CsCL-11 protein has the typical modular architecture (EPN and WTD) conserved throughout vertebrates, suggesting a conserved function of CsCL-11. Tissue expression profile analysis by quantitative real-time PCR (qRT-PCR) showed *CsCL*-11 to be ubiquitously distributed in tissues and highly expressed in the ovary and liver. A pattern of significant upregulation of *CsCL*-11 expression was observed in the blood, spleen, head kidney and gill at 6 h, 12 h and 24 h after infection with *Vibrio anguillarum*, and western blotting showed that natural CsCL-11 protein levels in the blood were significantly increased after *V. anguillarum* infection. Moreover, by binding to various bacteria, recombinant CsCL-11 (rCsCL-11) expressed in HEK-293 T cells displayed strong antibacterial activity. Taken together, these results suggest that *CsCL*-11 is a unique C-type lectin that is likely involved in host defense against bacterial infection. To our knowledge, this is the first study on CL-11 in marine fish.

1. Introduction

Collectins are vertebrate C-type lectin (CTL) molecules containing a collagen-like region and a calcium-dependent carbohydrate recognition domain (CRD) [1–5] that are conserved from birds to mammals. Lectins are large oligomeric pattern recognition molecules divided into several groups, including mannose-binding lectin (MBL), collectin liver-1 (CL-L1, CL-10), scavenger receptor, collectin placenta-1 (CL-P1), collectin kidney-1 (CL-K1, CL-11), surfactant protein A and D (SP-A and SP-D) and conglutinin [6–8]. Despite the presence of a highly conserved domain, collectins have diverse functions and are involved in various processes, such as cell adhesion, tissues integration, platelet activation, complement activation, pathogen recognition, endocytosis, and phagocytosis [9–11]. CL-10, CL-11 and CL-P1 are the most recently discovered members of the collectin family [7,12,13]. CL-11 is found in the circulation as part of a heteromeric complex with CL-10 in the lectin complement pathway associated with mannose-binding lectin-associated serine proteases (MASPs) [14,15]. The genomic organization and protein structure of CL-10 is relatively similar to that of CL-11 [16]. Identified after CL-10, CL-11 was initially characterized in 2006 [13].

CL-11 was detected in the blood and shown to have specific lectin activity, binding to mannose- and fucose-containing microbial-derived products; CL-11 binds to various microorganisms, including bacteria, fungi and viruses, through partial calcium-dependent interactions and mediates complement activation [13,17–19]. CL-11 is also associated with MASPs and/or MASP-3 in plasma [14,15] and plays an important role in innate immune defense. In addition, CL-11 performs several functions in embryogenesis, waste removal, and developmental as well

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as other processes [20,21].

CL-11 is also involved in embryonic developmental processes in mice and zebrafish [22,23], serving as a guidance cue for migrating neural crest cells and other cell types during embryogenesis [23]. CL-10 is expressed at the basement membrane of the palate during murine embryo development. Both CL-10 and CL-11 are believed to regulate cell migration via cell attraction in 3 MC syndrome [22].

Overall, information about CL-11 is limited in fish species, with only two reports of CL-11 in fish, zebrafish (*Danio rerio*) and Nile tilapia (*Oreochromis niloticus*), published to date [23,24]. The former study determined the effects of loss of function of CL-11 protein during zebrafish embryogenesis; the latter examined expression and immune functional characteristics of CL-11 and reported that the prokaryotic recombinant CL-11 protein of Nile tilapia exhibits strong antibacterial activity [24]. Nonetheless, it remains unclear whether CL-11 plays a role in the host defense against pathogen infection in fish species. Half-smooth tongue sole (*Cynoglossus semilaevis*) is an important commercial aquaculture species in China, and an understanding of the immune genes involved in defense against pathogens is necessary for healthy aquaculture of this species. In a previous study, a partial cDNA sequence of *CL*-11 was obtained in the immune tissue transcriptome of half-smooth tongue sole [25] and was found to be significantly upregulated in immune tissues after challenge with *Vibrio anguillarum*. The objectives of the present work were to identify the full-length cDNA of CL-11 from *C. semilaevis* (*CsCL*-11), characterize the expression of this molecule at the mRNA and protein levels and determine the immune role of CsCL-11 in defense against pathogens.

2. Materials and methods

2.1. Fish samples

Half-smooth tongue sole individuals (mean weight of 300 g) were purchased from a local fish farm (Hai Yang, Yantai, China) and maintained in aerated 20 ± 2 °C seawater tanks with a flow-through water system. After 2 weeks of acclimation, the fish were randomly sampled to detect bacteria in the liver, spleen, blood, and head kidney. Only fish free of bacteria were used in experiments. After being anesthetized using MS-222, five healthy fish were randomly sampled and sacrificed. Twelve different types of tissues, including the blood, brain, gill, heart, head kidney, intestine, liver, spleen, ovary, metanephros, skin and stomach, were collected, flash frozen in liquid nitrogen and stored at −80 °C.

2.2. Cloning of CsCL-11

Total RNA was extracted from the kidney of *C. semilaevis* using RNA reagent (TaKaRa, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from the RNA template using M-MLV reverse transcriptase (Invitrogen, USA). For cloning of the *CsCL*-11 gene, PCR primers were designed according to the partial nucleotide sequences of *CsCL*-11 among *C. semilaevis* transcripts (http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0064485 Table 1). The amplified PCR product was cloned into P easy T Fast Vector (TIANGEN, China), and sequencing of both strands was carried out using T7 and SP6 primers. For the full-length cDNA sequence, 5′ and 3′-rapid amplification of cDNA ends (RACE) was performed using the Smart RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's instructions. Gene-specific primers for RACE PCR were designed based on the partial *CL*-11 sequence obtained (Table 1). After both strands were sequenced, the sequences were spliced with the previously known *CsCL*-11 sequence.

2.3. Sequence characterization, multiple sequence alignment and phylogenetic analysis of CsCL-11

The ORF of *CsCL*-11 was identified, and the amino acid sequence was analyzed using DNAStar and ORF finder at the NCBI website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The structure of the CsCL-11 peptide was predicted with the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). The genomic structure of CsCL-11 was analyzed using Splign at NCBI (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi). The Clustal W multiple alignment program was employed to compare the amino acid sequences of CsCL-11 with proteins in other species. The phylogenetic tree was constructed based on the neighbor-joining method using MEGA5.1 software with 1000 bootstrap sampling.

2.4. Experimental bacterial infection, tissue sampling, RNA extraction, and reverse transcription

Fish were randomly sampled and injected intraperitoneally with 100 μL of *V. anguillarum* at a dose of 2×10^7 cfu, as described in a previous study [26]. Control fish were injected intraperitoneally with 100 μL of axenic PBS. Experimental infection and tissues sample collection were performed under anesthesia using MS-222. The challenged fish and control fish were sampled at 0 h, 6 h, 12 h, 24 h, 48 h and 72 h post injection, and six tissues, including the liver, spleen, intestine, blood, head kidney and gill, were collected. Five individuals were randomly used for tissue preparation at each time point during the challenge experiment. All samples were flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Total RNA was extracted from tissues using RNA reagent (TIANGEN, China) following the manufacturer's protocol. All RNA samples were treated with RNase-free DNase I provided in the kit. The quantity, purity and integrity of RNA were assessed by 1.2% (w/v) agarose gel electrophoresis and on a Nanodrop-1000 spectrophotometer. Total RNA was resuspended in RNA-free distilled water and stored at −80 °C for qRT-PCR analysis. First-strand cDNA was synthesized from 2 μg total RNA with reverse transcriptase (TaKaRa, Japan) and random primers, and the resulting product was used as the template for qRT-PCR.

2.5. Quantitative analysis of CsCL-11 expression at the mRNA level

Quantitative qRT-PCR was performed using an ABI-7500 Real-Time System (Applied Biosystems, USA) with SYBR Green (Invitrogen, USA) as the fluorescent dye according to the manufacturer's protocols. Primers were designed according to the *CsCL*-11 sequence using Primer Premier 5 (Table 1). The specificities of all primer set in the PCR reactions were tested with dissociation curves. The qRT-PCR thermal cycling conditions for all reactions were 95 °C for 1 min 50 s followed by 40 cycles of 95 °C for 10 s and 61 °C for 33 s. All reactions were performed in biological triplicate, and the results were calculated relative to the expression levels of 18 S rRNA in each sample using the $2^{-\Delta\Delta CT}$ method [27]. Quantitative data are expressed as the mean value ± SD (standard deviation) representing the relative expression ratio. The significance of differences in expression was determined using two-way ANOVA followed by Duncan's multiple range tests, with a threshold significance level of $P < 0.05$. All statistical analyses were performed using SPSS 16.0.

2.6. Eukaryotic expression and purification of recombinant CsCL-11 (rCsCL-11)

The *CsCL*-11 gene was cloned and ligated into the pcDNA3.1 (+) vector after double restriction enzyme digestion with *Bam*H I and *Hind* III. The pcDNA3.1-CsCL-11 plasmid was then transformed into *E. coli* strain DH5α (Transgen, China), and clones were verified by PCR and sequencing (Personalbio, China). The linearized recombinant plasmid

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)	Utilization
CsCL-11-F1	AGACCCAGGTCCACAGG	qRT-PCR
CsCL-11-R1	TTCTCCTCCTTCACCAAC	qRT-PCR
CsCL-11-18 S-R	AGTGGGGTTCAGCGGGTTAC	qRT-PCR
Cs CL-11-18 S–F	GGTCTGTGATGCCCTTAGATGTC	qRT-PCR
CsCL-11-F2	CCGGAATTCCGGCACAACTTGGCAGAGCCGT	Recombinant expression *E. coli*, with *Eco*R I restriction site
CsCL-11-R2	TAAAGCGGCCGCAAATTCAGACGCTGTCCTTATCA	Recombinant expression in *E.coli*, with Not I restriction site
CsCL-11-GSP3-1	ACGAGGGAGCCAATGCCG	3′ RACE amplification
CsCL-11-GSP3-2	TCGTAGCTGAACCAGTCTG	3′ RACE amplification
CsCL-11-GSP5-1	TGTGTCCATGAGACGTCT	5′ RACE amplification
CsCL-11-GSP5-2	TCTTCTACATGGGGCAGAT	5′ RACE amplification
CsCL-11-293-F*	CGCGGATCCATGAGAGGCCAAAAGCTGTTGCTCC	Recombinant expression in HEK293Tcell, with *Bam*H I restriction site
CsCL-11-293-R*	CCCAAGCTTCATCATCATCATCATCATGACGCTGTCCTTATCAAATTCACAG	Recombinant expression in HEK 293Tcell, with *Hin*d III restriction site

Fig. 1. Nucleotide and deduced amino acid sequences of *CsCL*-11. Amino acid sequences are shown with one-letter codes below the coding regions. The signal peptide is underlined in bold. The initiation codon (ATG) and the termination codon (TGA) are marked. The collagen domain is labeled with a box. The CLECT domain is labeled using a gray box. The EPN, WTD, and four cysteine residues are shaded in bold.

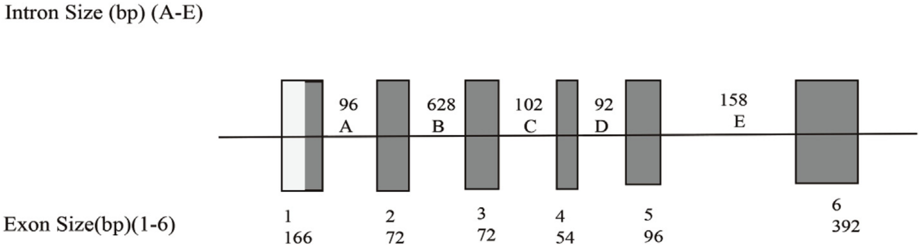

Fig. 2. The genomic structure of the *CsCL*-11 gene. Five introns were indicated by letters above the solid line (A–E), and six exons were numbered (1–6) under the lines. The first gray box contained the signal peptide and exon 1. The GenBank accession numbers of *CL*-11 from *C. semilaevis* genomic DNA sequence is NC_024,307.1.

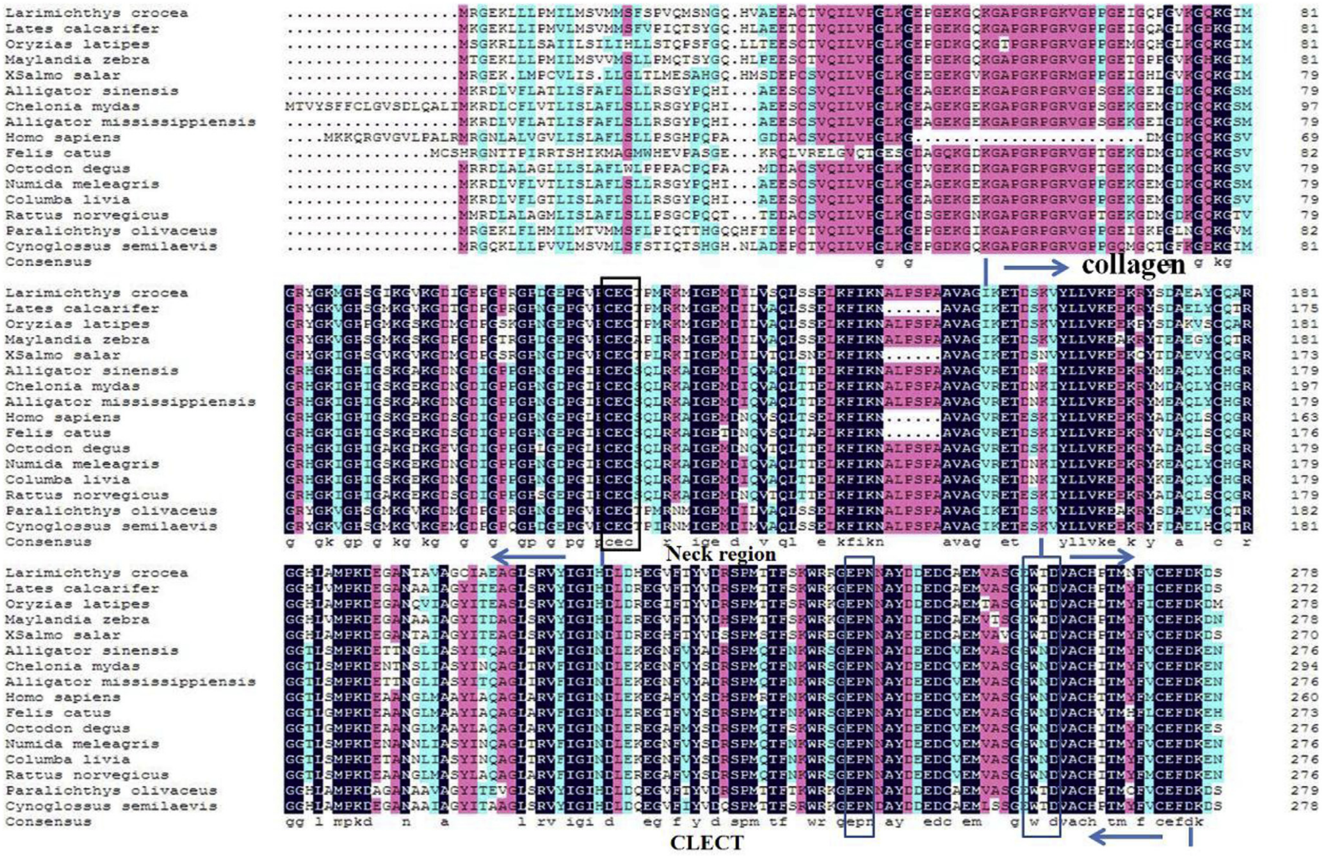

Fig. 3. Alignment of the amino acid sequences of CsCL-11 with the other CL-11 proteins using Clustal W method. The EPN, WT/ND are boxed. The sequences used are as follows: *Larimichthys crocea* (XP_019133008.1), *Latescal carifer* (XP_018539441.1), *Oryzias latipes* (XP_020569545.1), *Maylandia zebra* (XP_004540543.1), *Salmo_salar* (XP_014065661.1), *Alligator sinensis* (XP_014380446.1), *Chelonia myda s* (XP_007055703.1), *Alligator mississippiensis* (XP_014452821.1), *Homo sapiens* (XP_005263910.1), *Felis catus* (XP_019683523.1), *Octodon degus* (XP_004648109.1), *Numida meleagris* (XP_021247777.1), *Columba livia* (XP_021149170.1), *Rattus norvegicus* (XP_006240023.1), *Paralichthys olivaceu* (XP_019940823.1).

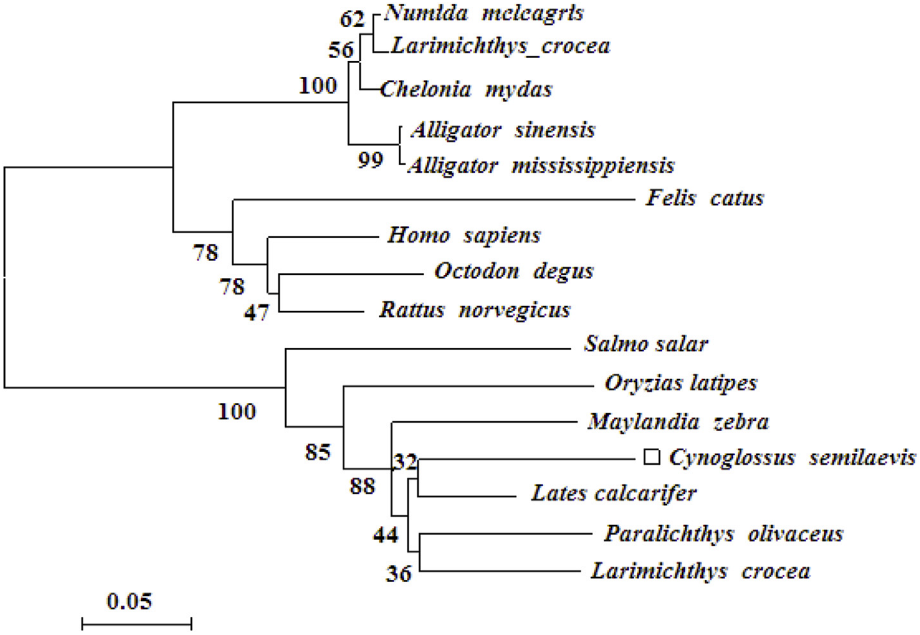

Fig. 4. Phylogenetic analysis of CsCL-11 with other homologous CL-11 sequences from teleosts, aves reptiles and mammalians. Sequences used here are the same with those for alignment analysis. The phylogenetic tree is constructed based on 1000 bootstrap replicates by neighbor-joining method. Number at each fork indicates the bootstrap.

pcDNA3.1-CsCL-11 was then transformed into the human embryonic kidney 293 T cellline (HEK-293 T) in 6-well plates using Lipofectamine 2000 (Invitrogen, Shanghai). The cells were then seeded in a 24-well plate, grown for 7–10 days at 37 °C. After transformation and selection, positive cell were incubated for at 37 °C using L15 medium with 1% penicillin-streptomycin (10% FBS, HyClone, Logan, UT, USA) in the 25 ML cell culture flask. Cells were collected by centrifugation (1500 rpm, 5 min) and treated with RIPA Lysis Buffer (Beyotime,

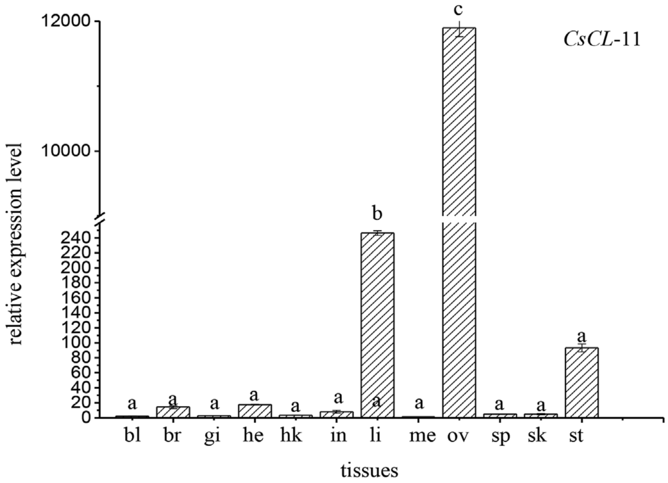

Fig. 5. Tissue distribution of *CsCL*-11in healthy *C. semilaevis*. The used tissues include bl (blood), br (brain), gi (gill), he (heart), hk (head kidney), in (intestine), li (liver), sp (spleen), ov (ovary), me (metanephros), sk (skin), and st (stomach), a and b indicate the Duncan grouping in SPSS.

China); protein was verified by10% SDS-PAGE. Purified protein was obtained using a Ni-NTA Purification System (GE, USA) follow the manufacturer's protocol. Finally, the purified protein was detected by discontinuous vertical 10% SDS-PAGE.

For western blotting, purified proteins were transferred to polyvinylidene difluoride (PVDF) membranes (180 mA, 1 h). The PVDF membranes were blocked with blocking buffer (TBST, 5% skim milk in TBS containing 0.05% Tween-20) for 1 h and incubated overnight at 4 °C with a primary purified Anti-His Tag Monoclonal Antibody (1:10,000 TransGen Biotech, Beijing). The membranes were subsequently incubated with goat anti-human IgG and HRP-conjugated secondary antibodies (1:5000 CWbiotech, Beijing) for 2 h, followed by visualization and detection with a DAB Chromogenic reagent kit (ZSGB-Bio, Beijing).

2.7. Antibacterial activity detection of the rCsCL-11 protein

The antimicrobial activity of the HEK-293 T cell-expressed rCsCL-11 protein against *E. coli*, *V. anguillarum*, *Vibrio parahaemolyticus, Pseudomonas aeruginosa*, and *Staphylococcus aureus* was determined using the inhibition zone method (Oxford cup method). All bacteria were cultured at appropriate temperature for 12 h, and then were diluted to 10^3 cfu/mL with PBS and transferred to coated plates. Three Oxford cup were punched into each plate, and 200 μL of PBS (negative control) or 0.5 mg/mL rCsCL-11 protein was added to each cup. While 50 mg/L of kanamycin sulfate was used as a positive control. The plates were cultured for 12 h–18 h at the optimal growth temperature for that bacterium, and the inhibition zone was observed and photographed.

2.8. Bacterial binding assays for the rCsCL-11 protein

The five kinds of bacteria (10 mL) described above (in 2.7 section) were cultured overnight; the cultures were then centrifuged (5000 × *g* for 5 min), and the pellets were washed three times with PBS and then resuspended with PBS to an OD value ($\lambda = 600$ nm) of 0.8. Next, 900 μL of the resuspended bacteria was mixed with 100 μL of rCsCL-11 protein for the test group. For the control group, 100 μL of BSA was used instead of 100 μL of rCsCL-11 protein. After incubation for 60 min at the optimal growth temperature for that bacterium, the cell pellet was washed 3 times with PBS to wipe off the remanent rCsCL-11 recombinant protein or BSA and then boiled for 10 min after adding 100 μL protein loading buffer. Western blotting was performed to determine bacterial binding with rCsCL-11, as described below.

2.9. Production and purification of the antibody

The cell-expressed rCsCL-11 protein was used as an immunogen to immunize rabbits. In the initial immunization step, the cell-expressed rCsCL-11 protein and equal volume of Freund's complete adjuvant were mixed and injected into a rabbit. In the subsequent booster immunizations (after 2-week), the immunogen was the same with the initial immunization step. After the third immunization step, blood from the tested rabbit were taken from the rabbits' ear veins. ELISA with rCsCL-11 as the antigen were deducted to reveal the antibody titers in the serum of immunized rabbits (to insure a perfect immune response, the antibody titers should be more than 1:1000), Three days after the last booster immunization, all blood was obtained from the immunized rabbits. The serum was separated from the blood, the affinity purification method (protein G-Sepharose) was used to purify the rabbit serum to obtain the antibody for rCsCL-11 protein.

2.10. Western blot assay of blood samples

Total protein from blood was extracted with RIPA Lysis Buffer (Beyotime, China). The mixture protein was detected by 10% SDS-PAGE. All different separated protein bands were transferred onto polyvinylidene difluoride (PVDF) membranes (180 mA, 1 h) and then PVDF membranes were blocked with blocking buffer for 1 h and incubated overnight at 4 °C primary antibodies against the cell-expressed rCsCL-11 protein (purified from rabbit) and GAPDH (Santa Cruz Biotechnology, CA, USA) at dilutions of 1:1000. The incubated membranes were washed and then incubated with 1:1000 secondary antibodies at room temperature for 4 h. The membranes were washed and visualized using an ECL Kit (Thermo Fisher, MA, USA).

3. Results

3.1. CsCL-11cDNA cloning and analysis

The full-length cDNA of *CsCL*-11 is 1220 bp in length and contains a 5′-UTR of 180 bp, a 3′-UTR of 218 bp and an open reading frame (ORF) of 819 bp (Fig. 1) that encodes 273 amino acids (Fig. 1). A putative signal peptide of 27 amino acids was identified at the N-terminus of the CsCL-11 protein. Online prediction of the deduced amino acid sequence showed that CsCL-11 contains two domains: a collagen domain (amino acids 55–114) and a CLECT domain (amino acids 153–273) (Fig. 1). There are 19 continuous Gly-Xxx-Yyy triplet repeats in the collagen domain. The CLECT domain, also called the C-type lectin domain or the carbohydrate-recognition domain (CRD), comprises 116 residues with four cysteines (C172, C244, C258, and C266). The mannose-type carbohydrate-binding motif EPN (Glu-Pro-Asn) and WTD (Trp-Asn-Asp) are also present in CsCL-11.

The *CsCL*-11 gene, which is located on half-smooth tongue sole chromosome 1, its genomic DNA is 2326 bp in length and consists of six exons (with lengths of 166 bp, 72 bp, 72 bp, 54 bp, 96 bp and 392 bp) and five introns (with lengths of 96 bp, 628 bp, 102 bp, 92 bp and 158 bp), as shown in Fig. 2.

3.2. Multiple sequence alignment of CsCL-11

Multiple sequence alignment of CL-11 amino acid sequences from *C. semilaevis* and fifteen other species revealed sequence conservation. Most of these sequences share some conserved residues and domains (Fig. 3). Moreover, they all have 14 completely conserved Gly-Xxx-Yyy triplet repeats located in the collagen domain and four cysteine residues in the conserved CLECT domain. Furthermore, the EPN and WT/ND motifs in the CLECT domain are conserved. The deduced protein sequence of CsCL-11 displays the highest identity (86.4%) with CL-11 of *Lates calcarifer* and the lowest identify (57.8%) with the *Felis catus* protein. CsCL-11 exhibited identities of 82.8%, 82.4%, 82.1%, 78.9%,

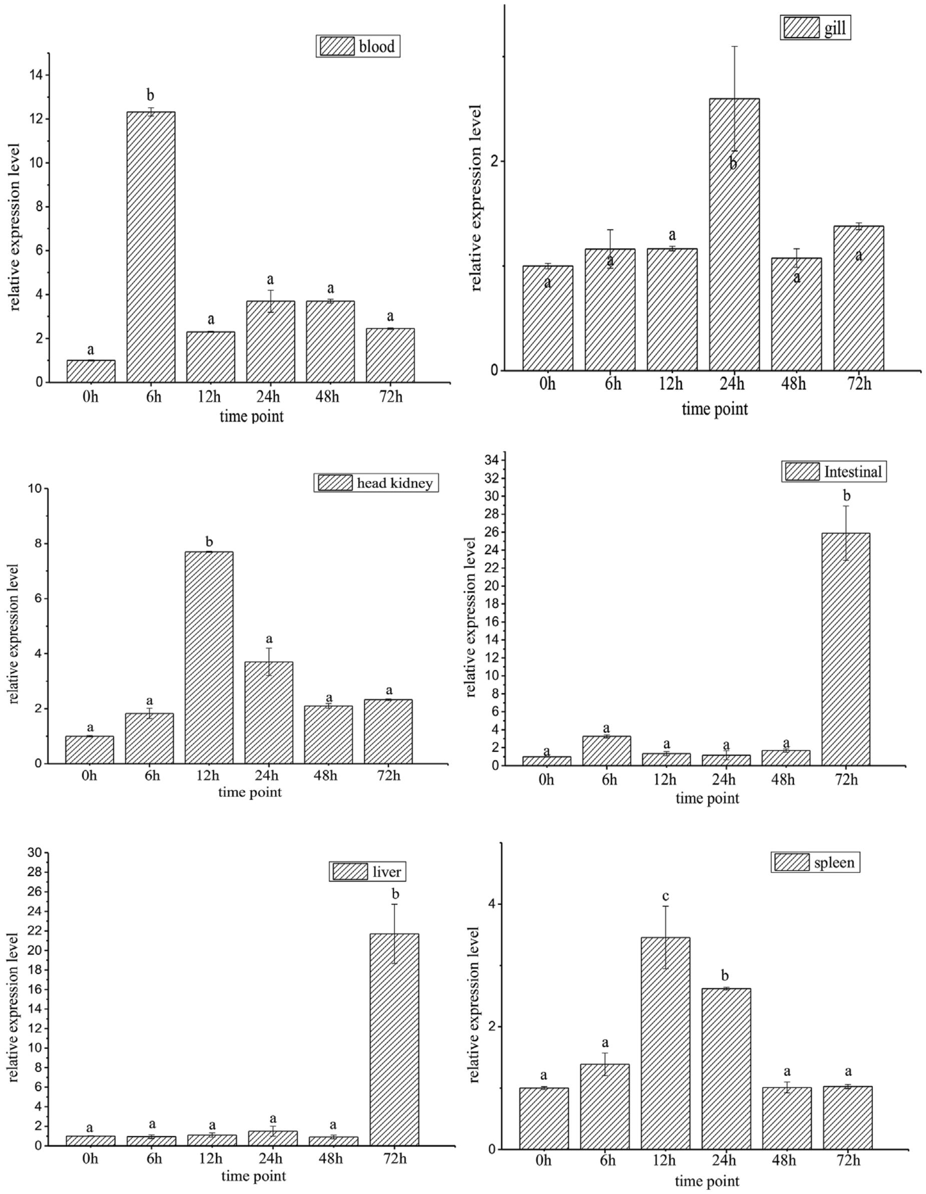

Fig. 6. Temporal expressions of *CsCL*-11 in the blood, gill, head kidney, intestine, liver and spleen after infected with *V. anguillarum*. Statistical comparison of the levels detected at different time points is carried out by Duncan method in SPSS, a, b and c suggest the Duncan grouping in SPSS.

75.3%, 64.3%, 63.9%, 62.3% and 61.7% with reported homologous sequences in *Paralichthys olivaceus*, *Maylandia zebra*, *Larimichthys crocea*, *Oryzias latipes*, *Salmo salar*, *Numida meleagris*, *Alligator sinensis*, *Alligator mississippiensis*, *Chelonia mydas*, *Columba livia, Octodon degus*, *Homo sapiens* and *Rattus norvegicus*, respectively.

The amino acid sequences of CL-11 and CL-10 in *C. semilaevis*, human and mouse (Supplement Fig. 1) were compared, showing the same conserved residues and domains. All contain 12 continuous Gly-Xxx-Yyy triplet repeats, as well as two conserved cysteines in the neck region. Furthermore, two cysteine residues joining the collagen-like region and the neck region are conserved in these six sequences.

3.3. Phylogenetic analysis of CsCL-11

To further clarify the phylogenetic relationships of CsCL-11 with the proteins in other species, we employed the neighbor-joining method to construct a phylogenetic tree based on the CL-11 sequences mentioned above (Fig. 4). These sequences clustered into two clades. CsCL-11 first clustered with *L. calcarifer*, *P. olivaceus* and *L. crocea* CL-11 sequences and then with CL-11 sequences from other teleosts, including *M. zebra*, *O. latipes* and *S. salar*. The clade branched separately from another that included sequences from birds (*N. meleagris* and *C. livia*), reptiles (*A. sinensis*, *A. mississippiensis* and *C. mydas*), human (*H. sapiens*) and other mammals (*F. catus*, *O. degus* and *R. norvegicus*).

3.4. Tissue distribution of CsCL-11

The tissue-specific expression profile of *CsCL*-11 mRNA was evaluated by qRT-PCR assays. As shown in Fig. 5, *CsCL*-11 mRNA expression was widely distributed and showed extreme tissue-specific variation in healthy half-smooth tongue sole, with detection in all 12 tissues examined. The relative expression of *CsCL*-11 in the blood was used as the first standard; the highest expression of *CsCL*-11 compared with this standard (12,000 times higher) was observed in the ovary, followed by the liver, stomach, heart and brain, which showed 240, 90, 20 and 15 times higher expression than did the blood, respectively. Among other tested tissues, *CsCL*-11 expression was slightly higher in the head kidney, skin and spleen than in the gill, metanephros and skin, with

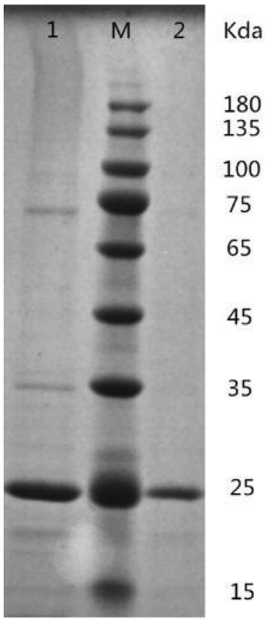

Fig. 7. SDS-PAGE analysis of the purified rCsCL-11protein expressed in HEK 293 T cells. Lane M: protein molecular weight marker, Lane 1: unpurified rCsCL-11 protein, Lane 2: purified rCsCL-11 protein.

minimum expression similar to that in the blood.

3.5. Expression profiles of CsCL-11 after bacterial challenge

To investigate whether *CsCL*-11 expression is affected by bacterial infection, we challenged half-smooth tongue sole with the fish pathogen *V. anguillarum* and used qRT-PCR to analyze expression levels in the blood, gill, head kidney, liver, intestine and spleen at different time points (0 h, 6 h, 12 h, 24 h, 48 h, 72 h). In general, *CsCL*-11 gene expression was upregulated in all tissues examined, though the relative fold differences and peak point of expression varied in the different tissues. As shown in Fig. 6, expression of *CsCL*-11 in the blood after *V. anguillarum* infection was significantly induced at the acute stage (6 h post challenge), increasing by 12 times at the peak level ($p < 0.05$); expression gradually decreased at 12 h, 24 h, 48 h and 72 h, though the levels were still 2–4 times higher than normal levels at these time points. *CsCL*-11 displayed a similar expression pattern in the liver and intestine, with a peak value approximately 25 times higher than the normal value at 72 h in the intestine ($p < 0.05$). The second peak appeared in the liver at 24 h with a 2-fold change, and the intestine showed an increase of 3-fold at 6 h. *CsCL*-11 expression increased by 2.5 times in the gill at the peak ($p < 0.05$) at 24 h; at the other time points, the expression levels were similar to the normal levels. The qRT-PCR results indicated similar expression patterns of *CsCL*-11 mRNA in the head kidney and spleen after infection with *V. anguillarum*. In these two tissues, expression was increased at 6 h and peaked, with increases of approximately 8 times and 3.5 times, respectively, at 12 h after injection; the levels gradually decreased over the subsequent time points at 24 h, 48 h and 72 h, though they were still 1–2 times higher than the normal levels. These results show that pathogen infection induced specific patterns of expression and different changes in *CsCL*-11 levels in different tissues.

3.6. Expression and purification of rCsCL-11

rCsCL-11 protein was obtained using a pcDNA3.1 (+) vector system. In HEK-293 T cell lysate, an obvious band of 25 kDa was observed by Western blot analysis (Fig. 7, lane 1, 2). This result was consistent with the predicted molecular mass of the rCsCL-11 protein including the His-tag at the N-terminus. The rCsCL-11 protein was mainly present in cells and rarely found in culture supernatant. rCsCL-11 was purified and confirmed by western blotting. The concentration of purified rCsCL-11 obtained from HEK-293 T cells was 0.73 mg/mL.

3.7. Antibacterial activity of rCsCL-11

The recombinant protein expressed in HEK-293 T cells exhibited antibacterial activity against *E. coli*, *V. anguillarum*, *V. parahaemolyticus, P. aeruginosa* (gram negative) and *S. agalactiae* (gram positive) according to Oxford cup method result, as shown in Fig. 8. Based on the inhibition zones, rCsCL-11 displayed high antibacterial activity against these bacteria. An inhibition zone was observed on each plate with 0.5 mg/mL rCsCL-11 and on the plate with 50 mg/L kanamycin sulfate (Fig. 8); While the negative control (PBS) plate showed no inhibition zone.

3.8. Bacterial binding assays for eukaryotic expressedrCsCL-11

We also performed binding assays to determine whether rCsCL-11 directly interacts with bacteria. Western blot analysis after incubation of the protein with *V. parahaemolyticus*, *S. aureus*, *P. aeruginosa*, *E. coli* and *V. anguillarum* revealed target bands of 25 kDa (Fig. 9). As described above, the molecular weight of CsCL-11 is 25 kDa; thus, these findings indicated that rCsCL-11 directly bound to these bacterial cells.

3.9. Endogenous CsCL-11 protein in the blood of C. semilaevis

We next applied western blotting to evaluate the expression level of the endogenous CsCL-11 protein in blood samples from half-smooth tongue sole. High expression levels of the CsCL-11 protein were found in the blood. Moreover, higher levels of endogenous CsCL-11 protein in the blood were observed in animals challenged with *V. anguillarum* than in unchallenged animals (Fig. 10).

4. Discussion

In this study, we present the identification and characterization of the *CsCL*-11 gene from half-smooth tongue sole. The predicted CsCL-11 amino acid sequence mainly contains two domains: the collagen and CLECT domains. In addition, *CsCL*-11 mRNA was found to be widely distributed among 12 tissues, and induced expression was observed soon after bacterial infection. Our results also showed that rCsCL-11 expressed in eukaryotic systems had strong antibacterial activity against various bacteria and was able to bind to bacteria. CsCL-11 protein levels in the blood significantly increased after *V. anguillarum*

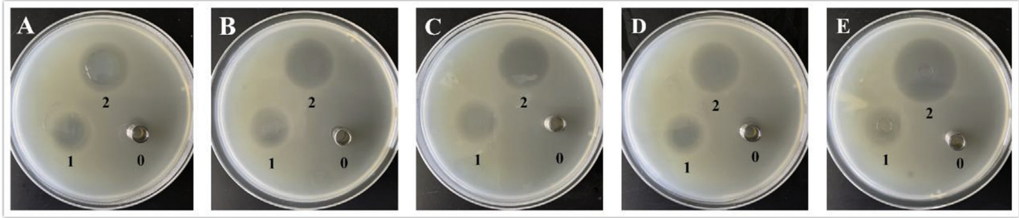

Fig. 8. Extracellular antimicrobial activity of eukaryotic expressed rCsCL-11 protein with the Oxford cup method. rCsCL-11 protein against *E. coli* (A), *V. anguillarum* (B), *V. parahaemolyticus* (C), *P. aeruginosa* (D) and *S. aureus* (E). 0: negative control (PBS), 1: 0.5 mg/mL rCsCL-11, 2: 50 mg/L kanamycin sulfate.

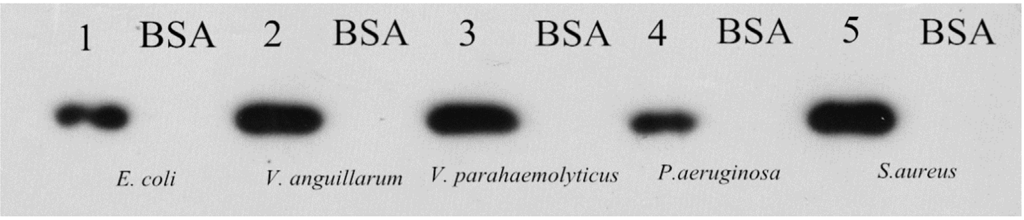

Fig. 9. Bacterial binding assays of eukaryotic expressed rCsCL-11proteinto various bacteria. BSA was incubated with bacteria as negative control, and binding assays of rCsCL-11 to *E. coli* (1), *V. anguillarum* (2), *V. parahaemolyticus* (3), *P. aeruginosa* (4) and *S. aureus* (5).

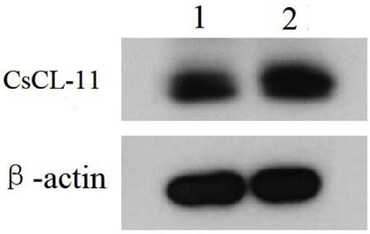

Fig. 10. Western blot analysis the expression of CsCL-11 protein from the blood of *C. semilaevis* after *V. anguillarum* infection. 1: control group; 2: individual infected with *V. anguillarum* after 6 h.

infection. To our knowledge, this is the first study on *CL*-11 in marine fish.

The predicted CsCL-11 protein is mainly composed of four regions: a signal peptide, a collagen domain, a neck region and a CLECT domain. In this study, the Collagen domain included 19 Gly-Xxx-Yyy structure (without interruption), which was similar to the CL-11of human and Nile tilapia, but there was a different in the number of repeated triplets, such as the human CL-11 with 24 Gly-X-Y repeats, Nile tilapia with 20 Gly-Xxx-Yyy structure [13,24]. *CsCL*-11 possesses the typical C-type lectin CRD, the mannose-binding motif EPN and WTD, the conserved residues involved in calcium binding. Multiple sequence alignment showed that the predicted CL-11 sequences from various species are highly similar. This analysis also clarified that the residues presumed to be important for the structure and function of each module, especially the four Cys residues, are well conserved.

We further compared CL-11 and CL-10 from *C. semilaevis*, human and mouse and found high conservation of the two cysteine residues in the neck region among six sequences. The two Cys residues in the neck region comprise a CXC motif that is found in CL-11 and CL-10 but not in other collectins, such as MBL [28]. Indeed, multiple sequence alignments of the MBL amino acid sequences of human, mouse, and chicken did not reveal two cysteines in the neck region [29]. Similar to CL-11, CL-10 is highly conserved. Both CL-10 and CL-11 include motifs that are very similar to the classical motif, such as continuous Gly-Xxx-Yyy triplet repeats and two conserved cysteines in the neck region; a previous phylogenetic analysis of CRDs (CLECT) from various collectins has revealed that CL-11 and CL-10 evolved from an ancestral collectin [16]. CL-11 is a soluble protein that forms complex heteromeric structures ranging from dimers to hexamers of subunits in tight association with another structurally related collectin, CL-L1, forming what is now designated CL-LK [30]. CL-LK interacts with all three MASPs, and the oligomeric form of CL-LK is important for MASP interaction, particularly MASP-2 interaction. MASP-1 and MASP-3 have also been shown to be involved in the activation of complement factor D and thereby the alternative complement pathway [31,32].

CsCL-11 mRNA was detected in the ovary, liver and stomach of half-smooth tongue sole, a result that is similar to that for *CL*-11 from Nile tilapia, which was reported to be highly expressed in the liver and widely expressed in other tissues, including the kidney, intestine and spleen [24]. However, the *CsCL*-11 expression pattern was different from that of the mammal gene, and human *CL*-11 mRNA expression is high in the adrenal glands, liver and kidney [13], whereas mouse *CL*-11 is expressed in the liver and kidney [33]. We also assessed time-course expression of *CsCL*-11 mRNA after *V. anguillarum* challenge, and the results revealed that pathogen infection induced tissue-specific expression of the gene. Indeed, significant upregulation of *CsCL*-11 was observed in the blood, spleen, head kidney and gill. In Nile tilapia, expression of *CL*-11 is significantly upregulated in the spleen and anterior kidney following challenge with *S. agalactiae* and *Aeromonas hydrophila* [24]. Collectively, the results of bacterial challenge suggest that *CsCL*-11 is involved in host defense against bacterial pathogen infection.

We successfully expressed the rCsCL-11 protein in HEK-293 T cells. The rCsCL-11 protein showed obvious antibacterial activity against various bacteria. Furthermore, binding assays showed that the rCsCL-11 protein from HEK-293 T cells could bind to bacterial cells (Fig. 9), suggesting that the antimicrobial activity of rCsCL-11 likely occurs through a mechanism that involves direct interaction. We also assessed the endogenous CsCL-11 protein and demonstrated by Western blot analyses that its production increased significantly after infection with bacterial pathogens. Our results correspond with those for the rCL-11 protein from Nile tilapia (rOnCL-K1), whereby pET-32a-OnCL-K1 showed a significant antibacterial effect against *S. agalactiae* and *A. hydrophila*, whereas the pET-32a control protein exhibited no antibacterial effects [28]. The CL-11 protein of Nile tilapia acts as an acute-phase protein. Involved in the immune response, the CL-11 protein in mammals is normally secreted. Hwang found that recombinant human CL-11 could bind to *S. pneumonia* in a calcium-dependent manner and induce complement activation [34]. Nonetheless, the molecular mechanism requires further investigation.

In summary, our results prove that *CsCL*-11, as a typical CL-11, is widely expressed in immune-related tissues of half-smooth tongue sole and may play a role in the immune response. Furthermore, rCsCL-11 exhibited high antimicrobial activity in eukaryotic systems against both gram-negative and gram-positive bacteria. The endogenous CsCL-11 protein was detected in the blood tissues of the fish. These findings provide new insight into the function of teleost *CL*-11.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.058.

References

[1] W. Weis, M. Taylor, K. Drickamer, The C-type lectin superfamily in the immune system, Immunol. Rev. 163 (1) (2010) 19–34.
[2] V.D.W. J Koenraad, L.M.G. Golde, Van, J.J. Batenburg, Collectins: players of the innate immune system, Eur. J. Biochem. 271 (7) (2004) 1229.
[3] K. Drickamer, A.J. Fadden, Genomic analysis of C-type lectins, Biochem. Soc. Symp. 69 (69) (2002) 59–72.

[4] K. Drickamer, M.E. Taylor, Recent insights into structures and functions of C-type lectins in the immune system, Curr. Opin. Struct. Biol. 34 (2015) 26–34.

[5] K. Drickamer, Two distinct classes of carbohydrate-recognition domains in animal lectins, J. Biol. Chem. 263 (20) (1988) 9557–9560.

[6] U. Kishore, et al., Surfactant proteins SP-A and SP-D: structure, function and receptors, Mol. Immunol. 43 (9) (2006) 1293.

[7] K. Ohtani, et al., Molecular cloning of a novel human collectin from liver (CL-L1), J. Biol. Chem. 274 (19) (1999) 13681.

[8] H. U, T.S. JC, Collections and ficolins: humoral lectins of the innate immune defense, Annu. Rev. Immunol. 21 (1) (2003) 547–578.

[9] A.M. Kerrigan, G.D. Brown, C-type lectins and phagocytosis, Immunobiology 214 (7) (2009) 562–575.

[10] S.P. Madhukaran, et al., Role of collectins and complement protein C1q in pregnancy and parturition, Immunobiology 221 (11) (2016) 1273–1288.

[11] F. Vieira, J.W. Kung, F. Bhatti, Structure, Genetics and Function of the Pulmonary Associated Surfactant Proteins A and D: the Extra-pulmonary Role of These C Type Lectins, Annals of Anatomy-anatomischer Anzeiger, 2017, pp. 184–201.

[12] K. Ohtani, et al., The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells, J. Biol. Chem. 276 (47) (2001) 44222–44228.

[13] H. Keshi, et al., Identification and characterization of a novel human collectin CL-K1, Microbiol. Immunol. 50 (12) (2006) 1001.

[14] M.L. Henriksen, et al., Heteromeric complexes of native collectin kidney 1 and collectin liver 1 are found in the circulation with MASPs and activate the complement system, J. Immunol. 191 (12) (2013) 6117–6127.

[15] Y.J. Ma, M.-O. Skjoedt, P. Garred, Collectin-11 (CL-K1):MASPs complex formation triggers activation of the complement lectin pathway—the fifth lectin pathway initiation complex, J. Innate Immun. 5 (3) (2012) 242–250.

[16] L. Selman, S. Hansen, Structure and function of collectin liver 1 (CL-L1) and collectin 11 (CL-11, CL-K1), Immunobiology 217 (9) (2012) 851–863.

[17] S. Hansen, et al., Collectin 11 (CL-11, CL-K1) is a MASP-1/3-associated plasma collectin with microbial-binding activity, J. Immunol. 185 (10) (2010) 6096–6104.

[18] S. Hansen, Collectin 11 – a new complement-associated pattern recognition molecule, Mol. Immunol. 56 (3) (2013) 270-270.

[19] S.W. Hansen, et al., The collectins CL-L1, CL-K1 and CL-P1, and their roles in complement and innate immunity, Immunobiology 221 (10) (2016) 1058–1067.

[20] X. Dong, et al., Collectin-11 has a critical role in the regulation of phagocytosis of photoreceptor outer segment fragments and cytokine production by retinal pigment epithelial cells, Immunobiology 221 (10) (2016) 1162–1163.

[21] L. Selman, et al., An enzyme-linked immunosorbent assay (ELISA) for quantification of human collectin 11 (CL-11, CL-K1), J. Immunol. Methods 375 (1–2) (2012) 182.

[22] M.M. Munye, et al., COLEC10 is mutated in 3MC patients and regulates early craniofacial development, PLoS Genet. 13 (3) (2017) e1006679.

[23] R. Caroline, et al., Mutations in lectin complement pathway genes COLEC11 and MASP1 cause 3MC syndrome, Nat. Genet. 43 (3) (2011) 197–203.

[24] L. Mu, et al., Expression and functional characterization of collection-K1 from Nile tilapia (Oreochromis niloticus) in host innate immune defense, Mol. Immunol. 103 (2018) 21–34.

[25] X. Zhang, et al., Transcriptome analysis revealed changes of multiple genes involved in immunity in Cynoglossus semilaevis during Vibrio anguillarum infection, Fish Shellfish Immunol. 43 (1) (2015) 209–218.

[26] Z.X. Sha, et al., Identification and expression analysis of goose-type lysozyme in half-smooth tongue sole (Cynoglossus semilaevis), Fish Shellfish Immunol. 32 (5) (2012) 914–921.

[27] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (6) (2008) 1101–1108.

[28] L. Mu, et al., Identification and characterization of a mannose-binding lectin from Nile tilapia (Oreochromis niloticus), Fish Shellfish Immunol. 67 (2017) 244.

[29] Z. Hao, et al., Characterization of a mannose-binding lectin from channel catfish (Ictalurus punctatus), Res. Vet. Sci. 92 (3) (2012) 408–413.

[30] A. Troegeler, et al., Collectin CL-LK is a novel soluble pattern recognition receptor for Mycobacterium tuberculosis, PLoS One 10 (7) (2015) e0132692.

[31] I. Daisuke, et al., The role of mannose-binding lectin-associated serine protease-3 in activation of the alternative complement pathway, J. Immunol. 187 (7) (2011) 3751–3758.

[32] T. Minoru, et al., Essential role of mannose-binding lectin-associated serine protease-1 in activation of the complement factor D, J. Exp. Med. 207 (1) (2010) 29–37.

[33] W. Motomura, et al., Immunolocalization of a novel collectin CL-K1 in murine tissues, J. Histochem. Cytochem. Off. J. Histochem. Soc. 56 (3) (2008) 243–252.

[34] I. Hwang, et al., In vitro and in vivo roles in Collectin Kidney 1 (CL-K1) with innate immunity against Streptococcus pneumoniae, Immunobiology 221 (10) (2016) 1217-1217.