

Short communication

Molecular characterization, expression and immune functions of two C-type lectin from *Venerupis philippinarum*

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ABSTRACT

In the present study, two C-type lectins (designated as VpClec-3 and VpClec-4) were identified and characterized from the manila clam *Venerupis philippinarum*. Multiple alignment and phylogenetic relationship analysis strongly suggested that VpClec-3 and VpClec-4 belong to the C-type lectin family. In nonstimulated clams, the VpClec-3 transcript was dominantly expressed in the hepatopancreas, while the VpClec-4 transcript was mainly expressed in gill tissues. Both VpClec-3 and VpClec-4 mRNA expression was significantly upregulated following *Vibrio anguillarum* challenge. Recombinant VpClec-4 (rVpClec-4) was shown to bind lipopolysaccharide (LPS) and glucan *in vitro*, whereas recombinant VpClec-3 (rVpClec-3) only bound to glucan. In addition, rVpClec-3 and rVpClec-4 displayed broad agglutination activities towards *Vibrio harveyi*, *Vibrio splendidus* and *V. anguillarum*, while no agglutination activities towards *Enterobacter cloacae* or *Aeromonas hydrophila* were observed in rVpClec-3. Moreover, hemocyte phagocytosis was significantly enhanced by rVpClec-3 and rVpClec-4. All the results showed that VpClecs function as pattern recognition receptors (PRRs) with distinct recognition spectra and are potentially involved in the innate immune responses of *V. philippinarum*.

1. Introduction

C-type lectins (CTLs) are generally multidomain proteins with diverse overall architecture and have at least one carbohydrate-recognition domain (CRD) of approximately 130 amino acid residues [1]. In the CRD, a characteristic double-loop stabilized by two highly conserved disulfide bridges is located at the bases of the loops, contributing to their immune functions, such as inflammation, opsonization, cell-cell and cell to extracellular matrix interactions, fertilization and regeneration [2–4]. Most dual-CRD C-type lectins include a QPD (Gln-Pro-Asp) motif and an EPN (Glu-Pro-Asn) motif which are specific for galactose and mannose binding, respectively [1].

To date, numerous C-type lectin genes have been identified in

mollusks [5–7]. These genes are involved in various biological immune processes, including nonself recognition, microbe agglutination, induction of phagocytosis and encapsulation, and anti-bacterial properties [7]. For example, CgClec-2 from *Crassostrea gigas* functions as a pattern recognition receptor (PRR) in immune recognition, serves as a regulatory factor in pathogen elimination, and plays a potential role in the activation of the complement system [5]. In addition, five C-type lectins from *Chlamys farreri* (CfLec-1–CfLec-5) and seven C-type lectins from bay scallop *Argopecten irradians* (AiCTL-1–AiCTL-7) have been characterized, and most of these lectins are involved in immune responses against certain Gram-positive or Gram-negative bacteria [8]. Generally, C-type lectins in bivalves often have a stronger affinity binding to carbohydrates on the surface of pathogen, and play essential roles in the

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Table 1
Primers used in the present study.

Primer	Sequence (5'-3')	Sequence information
P1	GGGTGTAGCGAAGATGTGGT	5' RACE primer
P2	GAATCTGTCGCTCAAACGC	5' RACE primer
P3	AGTGCTCCAGTCACCTTCCT	5' RACE primer
P4	GGAAGTCATAAGGCAGCAC	5' RACE primer
P5	ATGGCGTTGGAGCGACAGAT	3' RACE primer
P6	TCCGTGTTACCATCTTCGCTAC	3' RACE primer
P7	CTACGAGGCTCAGAGGTATTGC	3' RACE primer
P8	CCAACCCAAACAATGGAAGATG	3' RACE primer
P9	CCACATCTCGTACACCCACTTA	Real-time PCR
P10	AACTGTTCCGACTCAGACTAAC	Real-time PCR
P11	CCAACCCAAACAATGGAAGATG	Real-time PCR
P12	CGGACGTATCCACAGCAATTGCGC	Real-time PCR
P13	CGCACCTCCACGCCATCAT	β -actin primer
P14	GCAGCGCTCCATTCTTGTTC	β -actin primer
P15	GACAGATTGAGCGGGAGACG	Recombinant primer
P16	TCATCATCGGATTATTGCAATTTC	Recombinant primer
P17	GCATTTGATGACCTGTACAA	Recombinant primer
P18	TCATTATTCACTTGAGTTGAGTT	Recombinant primer
d _T	GGCACCGCGTCGACTAGTACT ₁₇	Oligo (d _T)-adaptor

early stages of infection by various microorganisms [9,10].

Venerupis philippinarum is distributed world-wide, is well adapted to the environment and has great commercial value. Mass mortalities in clam beds have occurred due to bacterial disease caused by environmental deterioration and pathogen invasion [11]. Therefore, characterization of immune-related molecular C-type lectins is urgently needed to elucidate the immune mechanism of this clam. To date, several C-type lectins have been cloned or purified from clam *Ruditapes philippinarum* [12], abalone *Haliotis discusdiscus* [13] and scallops *C. farreri* and *A. irradians* [14,15]. However, basic knowledge of their opsonic activities and antimicrobial properties in manila clam is lacking. In the present study, two C-type lectins (designated as VpClec-3 and VpClec-4) were identified from *V. philippinarum*, and their tissue-specific distribution, PAMP recognition and opsonization were also investigated to better understand the immune system in *V. philippinarum*.

2. Materials and methods

2.1. Clams and bacterial challenge

Healthy *V. philippinarum* clams (shell length of 3.0–4.0 cm) were purchased from a local culture farm, and acclimatized in aerated seawater at 20–22 °C for 10 days before processing. The clams were fed with an algae mixture of *Isochrysis galbana* and *Phaeodactylum tricornutum*, and the seawater was completely renewed daily.

Bacterial challenge experiments were performed according to our previous description [16]. Briefly, 300 clams were randomly divided into two groups and maintained in six aerated tanks (50 L). Three tanks served as the control, while the other three tanks were immersed with *V. anguillarum* at a final concentration of 1×10^7 CFU mL⁻¹. *V. anguillarum* was cultured in 2216E medium at 28 °C for 24 h, harvested by centrifuging at 5000 rpm for 10 min, washed with filter-sterilized sea water (FSSW) thrice, and resuspended in FSSW. The hemocytes of six individuals were randomly sampled at 0, 6, 12, 24 and 48 h post bacterial challenge. The clams cultured in the normal seawater were used as the control groups.

2.2. Gene cloning and sequence analysis of VpClec-3 and VpClec-4

Two manila clam ESTs were identified through large-scale EST sequencing of the constructed cDNA library [17]. To generate the full-length cDNA of VpClec-3 and VpClec-4, reverse primers P1–P4, and forward primers P5–P8 (Table 1), were designed based on the EST sequence. The nested PCR strategy was applied to the 3' and 5' RACE.

Nucleotide sequence and deduced amino acid analyses were

performed using the BLAST algorithm and the Expert Protein Analysis System. Protein domains were predicted using the simple modular architecture research tool version 4.0 [18]. Multiple alignments were performed using the ClustalW Multiple Alignment program and Multiple Alignment Show program.

2.3. Spatial and temporal expression patterns of VpClec-3 and VpClec-4 mRNA

Spatial and temporal expression profiles of VpClec-3 and VpClec-4 transcripts were analyzed using an Applied Biosystem 7500 Real-time PCR System. RNA extraction, cDNA synthesis and quantitative PCR analysis were performed as previously described [16]. Briefly, total RNAs from detected samples were extracted from six individuals using Trizol reagent (Invitrogen, USA). After reverse transcription, two pairs of gene-specific primers (P9, P10 for VpClec-3, P11, P12 for VpClec-4, Table 1) were designed to detect C-type lectin mRNA expressions. β -actin primers (P13 and P14, Table 1) were used to amplify a 121-bp fragment as an internal control. At the end of each PCR, dissociation curve analysis of amplification products was performed to confirm PCR product purity. The $2^{-\Delta\Delta CT}$ method [19] was used to analyze VpClec-3 and VpClec-4 expression levels. All data are reported as relative mRNA levels expressed as the mean \pm S.D. ($N = 6$). Statistical analysis was performed by one-way analysis of variance (one-way ANOVA) with a Duncan test using SPSS 16.0 software, and *P*-values less than 0.05 ($P < 0.05$) were considered statistically significant.

2.4. Recombinant expression of VpClec-3 and VpClec-4

Two specific primers (P15 and P16 for VpClec-3, P17 and P18 for VpClec-4, Table 1) were used to amplify the fragment encoding VpClec-3 and VpClec-4 mature peptides, respectively. The fragments were cloned into the pEASY-E1 simple vector (Transgen Biotech, Beijing, China), which was subsequently transformed into phage-resistant chemically competent cells, separately. The pEASY-E1-VpClec-3 and pEASY-E1-VpClec-4 plasmids were extracted and transformed into *E. coli* BL21 (DE3). Then cells were induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for protein expression. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the positive transformants after 4-hr induction with 1 mM IPTG. Recombinant proteins (rVpClec-3 and rVpClec-4) were purified using a Ni²⁺ chelating sepharose column and refolded in gradient urea-TBS glycerol buffer (6, 5, 4, 3, 2, 1, and 0 M urea in 20 mM Tris-HCl, 50 mM NaCl). The dialysate was changed every 12 h until 0 mM was reached. The concentrations of rVpClecs were measured via a BCA method [20].

2.5. Antibody preparation and western blotting analysis

The rVpCTLs (rVpClec-3 and rVpClec-4) were immunized into 6-week-old mice to prepare polyclonal antibodies against rVpCTLs. A total of 100 μ g rVpClec-3 and rVpClec-4 was intraperitoneally injected with complete Freund's adjuvant (Sigma, USA) in mice. Two weeks later, mice were inoculated with incomplete Freund's adjuvant. The third and fourth injections of 50 μ g rVpCTLs were given via tail vein at one-week intervals. Four days after the last injection, the mice were sacrificed to collect immunized serum [21].

The samples containing rVpCTLs were transferred onto nitrocellulose membranes electrophoretically at 200 mA for 45 min after SDS-PAGE. The blotted membrane was blocked with 3% BSA in PBS-T (PBS containing 0.05% Tween-20) at 37 °C for 1 h and incubated with 1:1000 diluted polyclonal antibody against rVpCTLs for 1 h at 37 °C. After three washes with PBS-T, goat-anti-mouse Ig-alkaline phosphatase conjugate (Southern Biotech, USA) secondary antibody was incubated with the membrane at 37 °C for 1 h. After three washes in PBS-T, positive bands were stained with NBT/BCIP substrate solution for 5 min. As

VpClec-3	P I D F V K H E S S C Y H I F A T P T L K W W D A M A Y C G I Y A D G E G S L A	71
VpClec-4	P D G W I Y F Q N S C Y T F G K D A - V Q F Y E A Q R Y C K - - - Q F D A G L V	144
<i>Pomacea canaliculata</i>	P A G W E D F Q S T C Y A F G T D K E - V K F V D A V T F C N - - - K H S A D V V	65
<i>Crassostrea virginica</i>	P G G W Y H F A T S C Y A F I D A E P L G W T E A M F Y C S - - - T L Q A K L V	59
<i>Crassostrea gigas</i>	P N H W K R H G N S C Y L F I Q D V P E D F I E A G S F C E - - - R R N S K L V	157
<i>Lottia gigantea</i>	A T G F T R F K T Q C Y V V P D A S - V T W G D A L E F C H - - - T F D S E L V	72
<i>Branchiostoma belcheri</i>	P A D Y Q K F N R T C Y R F S T D K - K P Y S E A R D T C H - - - G E G G I L A	121
<i>Mizuhoplecten yessoensis</i>	R N G W I Q F E S S C Y - F F S N D V E T W A E A S I I C - - - A D L K G H L A	63
VpClec-3	S I E S E S E Q L F L E K E L K T K Y P N P V Q K D F W L G A N D I T H E G T W	111
VpClec-4	N I E S S V E N F F L R N F L K E L K - - - A P N H W I G L T D V E Q E G D W	180
<i>Pomacea canaliculata</i>	E I T S E A E N K F V A E L V K S K G - - - G E D V W I G I S D L I Q E G H W	101
<i>Crassostrea virginica</i>	E I E S S P E N N F L K N H V I S L H K - - - N V S Y W I G L T D A L V E G K Y	96
<i>Crassostrea gigas</i>	E I E T A D E N N F L R V Q I L G T H P - - - Q E G Y W I G L S D I V F E G D W	194
<i>Lottia gigantea</i>	R V D D E E Q N F L E G Y L T K K R - - - A S H F W I A A T D L T F E G D W	108
<i>Branchiostoma belcheri</i>	T V K D E D T H N F L A N H V S S T T - - - H R N T W I G L S D I L A T E G L W	157
<i>Mizuhoplecten yessoensis</i>	T V N S A A E N S F L K A Q A N V L K - - - Q T G Y W L D G N D F E V E G S W	99
VpClec-3	V W I K K D E Y V Q M Y T N W A P G E P N S - - - G T G E N C L A L Y D M E	146
VpClec-4	R H Y P S I L K E V T F F - D W G K H Q P N N - - - G R V S N C A A F W E S F	214
<i>Pomacea canaliculata</i>	I Y L R S I L K E I T F S - K W E A H E P N N M - - - G G K E G C A L L H D - -	134
<i>Crassostrea virginica</i>	V W Q T T Q D D V A F V - D W S P P E P N D S - - - G H T E D C G A L W F A Y	131
<i>Crassostrea gigas</i>	V W T S S Q N S A S F T - D W S P T S P D N A - - - G N H Q D C A M F W V P D	229
<i>Lottia gigantea</i>	R W C N T M G P V V K T - F W G P Y Q P D N K M N R K G H V Q D C V V I L S D R T	147
<i>Branchiostoma belcheri</i>	V W D D G T L L L G Q G - I W G T G E P N G - - - G R G E N C A H I F P S K	191
<i>Mizuhoplecten yessoensis</i>	R W T T S D D L I T F N - D W H S G E P N Q - - - G A S A D C L A L W R D H	133
VpClec-3	Q Y K W N D A P C D L P E G F I C E V P V R N G N A I I G - - -	175
VpClec-4	Q H L W V D E P C T N K F R P L C E M K L N S S E - - -	239
<i>Pomacea canaliculata</i>	D G Y W N D I P C S A H F H I V C E L H - - -	154
<i>Crassostrea virginica</i>	R Y Q W N D A S C S N K L D F I C E K N I E G D N P N V I G - - -	161
<i>Crassostrea gigas</i>	H F H W N D H F C T V K A G Y I C E M E Y E G F G M L A K L Y H Q T	263
<i>Lottia gigantea</i>	H Y R W Y D E D C E Q K Y H F V C E K P I Y - - -	169
<i>Branchiostoma belcheri</i>	N Y R W N D G P C S R S Y N Y I C E I R G - - -	212
<i>Mizuhoplecten yessoensis</i>	A Y Q W A D E P C R H V W N F I C K T N I G D G E Q A I G - - -	162

Fig. 1. Multiple alignments of VpClec-3 and VpClec-4 with other C-type lectins from *Pomacea canaliculata* (XP_025099424), *Crassostrea virginica* (XP_022295064), *Crassostrea gigas* (EKC22069), *Lottia gigantea* (XP_009061655), *Branchiostoma belcheri* (XP_019639478) and *Mizuhoplecten yessoensis* (XP_021356222). Conserved amino acid residues were shaded in dark, and similar amino acids were shaded in gray. The letters in box indicate the motif for determining ligand binding specificity.

negative control, preimmunized serum was used instead of immunized serum.

2.6. PAMPs binding assay

Wells of the 96-well assay plate were coated with lipopolysaccharide (LPS), peptidoglycan (PGN) or glucan (Sigma-Aldrich, USA) and blocked with 3% BSA as previously described [10]. Then, 100 µL VpClec-3 and rVpClec-4 in TBS-Ca²⁺ buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl₂, pH 7.5) with or without EDTA (10 mM, chelating agent) at different concentrations (1.0, 2.5, 5.0 and 10.0 µg/mL) was added to each well. The plates were incubated for 1 h at room temperature. The plates were incubated with 100 µL diluted poly-clonal antibodies against rVpClec-3 and rVpClec-4 (1:1000 diluted in PBS) at 37 °C for 1 h, respectively. The wells were then incubated with rVpClecs antibody (1:5000) and goat-anti-mouse Ig-alkaline phosphatase conjugate (1:5000) (Southern Biotech, USA). Finally, pNPP substrate solution was added, and the plates were incubated in the dark at room temperature. The absorbance was measured at 405 nm with a microplate reader (Tecan M200, Switzerland). Wells containing 100 µL carbonate-bicarbonate buffer served as blanks. Preimmunized serum was used as the negative control instead of immunized serum. Each experiment was performed in triplicate.

2.7. Microbe agglutination assay

The microbial agglutination assay was performed in the presence of Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria (*Vibrio harveyi*, *Vibrio splendidus*, *Vibrio anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*) according to the previous report [22]. Briefly, bacteria undergoing exponential growth were harvested and stained by crystal violet. The labeled microbes were then suspended in

TBS-Ca²⁺ buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl₂, pH 7.5) with or without EDTA. Then, 10 µL microbe suspension was incubated with 25 µL rVpClec-3 and rVpClec-4 solution (final concentration of 1 µM) at room temperature for 1 h, respectively. Microbes dissolved in TBS buffer were selected as the control. Thereafter, 10 µL of the mixture was mounted onto a glass slide and observed by a light microscopy (BX51, Olympus, Japan).

2.8. Cell motility assay

Cell motility was examined on the soft-agar plates (0.3% agar). After Gram-positive bacterium (*S. aureus*) and Gram-negative bacteria (*V. harveyi*, *V. splendidus*, *V. anguillarum*, *E. cloacae* and *A. hydrophila*) were cultured to the stationary phase, bacteria mixed with rVpClec-3 and rVpClec-4 in TBS-Ca²⁺ buffer (final concentration of 10 µg/mL) were located on the soft-agar plates (1% tryptone, 0.25% NaCl and 0.3% agar). Bacteria not subject to rVpClecs treatment were used as the control groups. Motility halos were quantified after 16 h using at least three plates for each condition. The size of motility halos reflects the antibacterial activities of rVpClecs.

2.9. Antimicrobial activity assay

V. anguillarum were grown in 2216E broth and harvested at the logarithmic phase of growth. The bacterial cells were washed thrice with sodium phosphate buffer (pH 7.4) and diluted to 1.0×10^7 CFU/mL. The bacterial suspension (100 µL) was added to an equal volume of rVpClecs (at a final concentration of 100 µg/mL) with or without 10 mM ZnCl₂ in TBS. Cell suspensions without rVpClecs were prepared as a negative control. Each sample was incubated with aeration at 220 rpm. Then, the OD₆₀₀ was measured every 1 h.

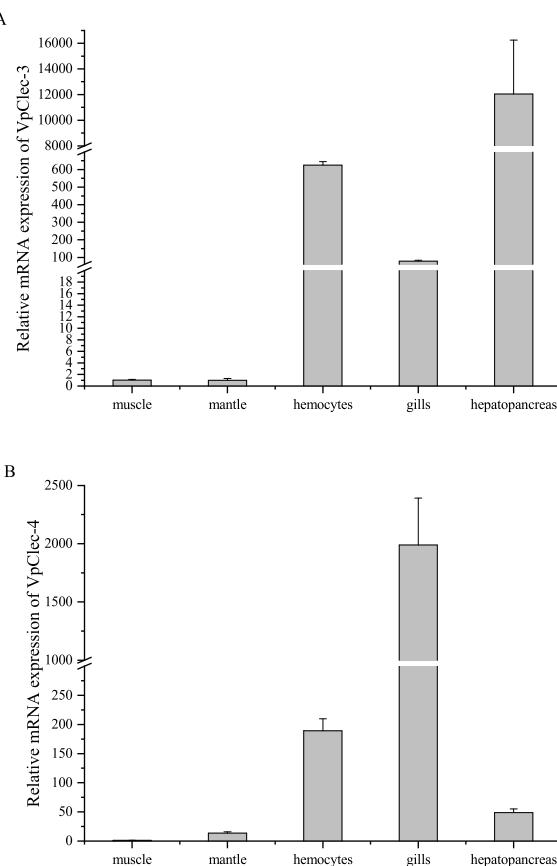


Fig. 2. VpClec-3 and VpClec-4 mRNA expression levels in different tissues detected by qRT-PCR. Transcript levels in mantle, gills, hemocytes, and hepatopancreas were normalized to that of muscle. Vertical bars represent the mean \pm S.D. ($N = 6$).

2.10. Phagocytosis assay

Hemocytes from manila clams were collected in an equal volume of prechilled anticoagulant (Tris-HCl 50 mM; glucose 2%, NaCl 2%; EDTA 20 mM; pH 7.4). After harvested by centrifugation, hemocytes were resuspended in TBS buffer (Tris-HCl 50 mM; CaCl₂ 5 mM) and incubated with 1 μ M rVpClec-3 and 1 μ M rVpClec-4 at 18 °C for 30 min. Then, 5 μ L of 3% fluorescence microspheres was added to each hemocytes suspension and incubated for additional 1 h at room temperature. Phagocytosis was analyzed using an Accuri C6 flow cytometer (BD) with BD CFlow® software. Differences were considered significant at $P < 0.05$ as assessed by *t*-test and marked by an asterisk. Each phagocytosis assay was repeated in triplicate. Data were subjected to Student's *t*-test, and differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. cDNA cloning and sequence analysis of VpClec-3 and VpClec-4

The complete cDNA sequences of VpClec-3 and VpClec-4 were deposited in the GenBank database under accession nos. MN653934 and MT680919, respectively. The open reading frame of VpClec-3 and VpClec-4 were 528 bp and 720 bp, encoding a polypeptide of 175 and 239 amino acid residues, respectively. The VpClec-3 peptide had a calculated molecular mass of 20 kDa and a *pI* of 4.51, while VpClec-4 peptide had a predicted molecular mass of 27.9 kDa and a *pI* of 5.26.

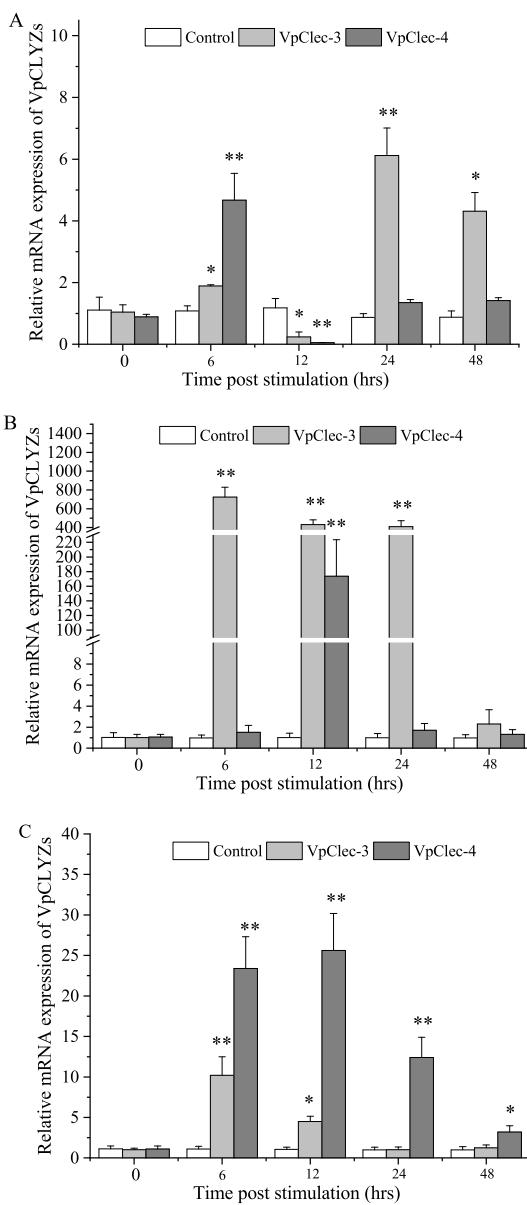


Fig. 3. Temporal expression of VpClec-3 and VpClec-4 mRNA in hemocytes (A), hepatopancreas (B) and gills (C) after *V. anguillarum* challenge. The values are shown as mean \pm S.D. ($N = 6$). (*: $P < 0.05$, **: $P < 0.01$).

3.2. Homologous analysis of VpClec-3 and VpClec-4

VpClec-3 and VpClec-4 exhibited relatively high identities with C-type lectins from other mollusks. For example, VpClec-3 shared 38.9% similarity with a C-type lectin from *Crassostrea virginica* (XP_022330927), while VpClec-4 was mostly homologous with a C-type lectin from *Pomacea canaliculata* (XP_025099424, 40.9% similarity). These two VpClecs shared 17% similarity with each other (Fig. 1).

3.3. Tissue distribution and temporal expression profiles after *V. anguillarum* challenge

The distribution of VpClec-3 and VpClec-4 transcripts in various tissues of unchallenged clams was analyzed by qRT-PCR. VpClec-3 mRNA transcripts were predominantly expressed in hepatopancreas and hemocytes, moderately expressed in gills, and marginally expressed in mantle and muscle (Fig. 2A). VpClec-4 mRNA transcripts were also prominently expressed in gills and highly expressed in hemocytes and

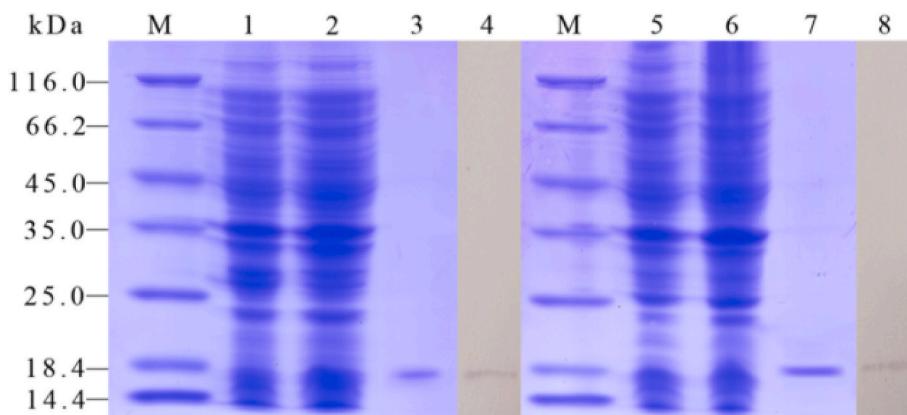


Fig. 4. SDS-PAGE analysis of the recombinant VpClec-3 and VpClec-4. Lane M: protein molecular standard; Lane 1 and 4: uninduced bacterial lysate; lane 2 and 5: bacterial lysate after induction with IPTG; lane 3 and 6: purified rVpClec-3 and rVpClec-4.

hepatopancreas (Fig. 2B).

The temporal expression profiles of VpClec-3 and VpClec-4 transcripts in hemocytes after bacterial challenge are shown in Fig. 3A. During the first 6 h after pathogen challenge, VpClec-3 mRNA expression levels increased up to 1.89-fold compared with the control group ($P < 0.05$). After a sudden decrease at 12 h (0.24-fold, $P < 0.05$), VpClec-3 mRNA expression levels were upregulated significantly at 24 h (6.12-fold, $P < 0.01$) and 48 h (4.31-fold, $P < 0.05$). After *V. anguillarum* stimulation, VpClec-4 mRNA expression was up regulated significantly and reached the maximum value (4.67-fold, $P < 0.01$) at 6 h. Then, the expression level was downregulated (0.05-fold, $P < 0.01$) at 12 h post stimulation (Fig. 3A). As time progressed, the expression of VpClec-4 transcripts recovered to the original level at 24 and 48 h. In hepatopancreas, expression of VpClec-3 was significantly ($P < 0.01$) up-regulated to 723.7, 431.6 and 410.6-fold compared with blank group at 6, 12 and 24 h post-challenge, respectively (Fig. 3B). Compare with VpClec-3, expression of VpClec-4 was only significantly (173.6-fold, $P < 0.01$) up-regulated at 12 h post-stimulation (Fig. 3B). In gills, transcripts of VpClec-3 was significantly up-regulated to 10.2-fold ($P < 0.01$), 4.5-fold ($P < 0.05$) of blank group at 6 h, 12 h post *V. anguillarum* stimulation, respectively (Fig. 3C). Expression of VpClec-4 was significantly up-regulated after stimulation, and it reached the maximal level at 12 h post stimulation, which was 25.6-fold ($P < 0.01$) compared with the blank group, and as time progressed it was significantly highly expressed to 12.4 ($P < 0.01$) and 3.2-fold ($P < 0.05$) of the blank level at 24 and 48 h post-stimulation (Fig. 3C).

3.4. Recombinant expression of VpClec-3 and VpClec-4

Recombinant VpClec-3 and VpClec-4 were highly expressed in *E. coli* Origami (DE3) cells after induction by IPTG. Here, rVpClec-3 and rVpClec-4 expressed obvious protein bands at approximately 19.7 kDa and 19.5 kDa, respectively (Fig. 4). Protein quantification using the BCA method revealed that the concentrations of purified rVpClec-3 and rVpClec-4 were 387.3 µg/ml and 416.1 µg/ml, respectively. Western blotting was performed to identify the specificity of the antibodies against rVpClec-3 and rVpClec-4. Clear reaction bands with high specificity were observed, indicating that the antibodies could react with rVpClec-3 and rVpClec-4, respectively (Fig. 4, line 4). Negative bands were not observed in the study (data not shown).

3.5. PAMP binding assay of rVpClec-3 and rVpClec-4

ELISA was performed to detect the binding activity of rVpClec-3 and rVpClec-4 towards PAMPs. The results showed that rVpClec-3 only binds PGN in the presence of Ca^{2+} *in vitro*, and does not bind LPS or glucan (Fig. 5A). In addition, rVpClec-4 binds LPS and glucan in the presence of

Ca^{2+} *in vitro*, but does not bind PGN (Fig. 5C). The binding ability of rVpClec-3 and rVpClec-4 was dose-dependent (Fig. 5). Moreover, rVpClec-3 and rVpClec-4 could not bind any PAMPs in the absence of Ca^{2+} (Fig. 5B and D).

3.6. Microbial agglutination, swimming motility assay and antibacterial activities

After crystal violet staining, microbial agglutination was directly observed under light microscopy after incubated with rVpClec-3 and rVpClec-4. rVpClec-3 showed strong agglutinate activity against *S. aureus*, *E. coli*, *V. parahaemolyticus*, *V. harveyi*, *P. putida* and *P. mirabilis*, and *P. pastoris*. However, rVpClec-4 could not agglutinate *P. putida* and *P. mirabilis* (Fig. 6). In the absence of Ca^{2+} , rVpClec-3 and rVpClec-4 could not agglutinate these detected bacteria. Meanwhile, swimming motility assays were performed on low-salt, soft-agar plates. The motility of Gram-positive bacterium (*S. aureus*) and Gram-negative bacteria (*V. harveyi*, *V. splendidus*, *V. anguillarum*, *E. cloacae* and *A. hydrophila*) was limited after incubation with rVpClecs. Compared with rVpClec-3, rVpClec-4 showed increased antibacterial activity against these detected bacteria (Fig. 7). In addition, rVpClec-4 showed an increased inhibitory effect on *V. anguillarum* growth, especially the exponential phase (1–3 h) and the stationary phase (6–8 h), compared with rVpClec-3 (Fig. 8).

3.7. Phagocytosis assay

The phagocytic activity of clam hemocytes was significantly enhanced by rVpClec-3 and rVpClec-4, respectively. The phagocytic ability of hemocytes was 23.2% (1 µg/mL, $P < 0.05$) and 25.7% (10 µg/mL, $P < 0.05$) in the rVpClec-3 treatment group compared with only 13.4% in the control group. For rVpClec-4, the phagocytic ability of hemocytes was 19.1% (1 µg/mL, $P < 0.05$) and 21.3% (10 µg/mL, $P < 0.05$) (Fig. 9).

4. Discussion

As key components of the innate immune system, C-type lectins typically play important roles in immune responses against pathogen infections [7]. Although various C-type lectins have been discovered in mollusks [7,16], knowledge about C-type lectins in manila clams is limited. In the present study, two C-type lectins were characterized from manila clam, and their tissue distribution, temporal expression profiles, PAMP binding assay results, and antibacterial and opsonic activities were also investigated.

Multiple alignments revealed that VpClec-3 and VpClec-4 showed high similarity to C-type lectins from *C. virginica* and *P. canaliculata*,

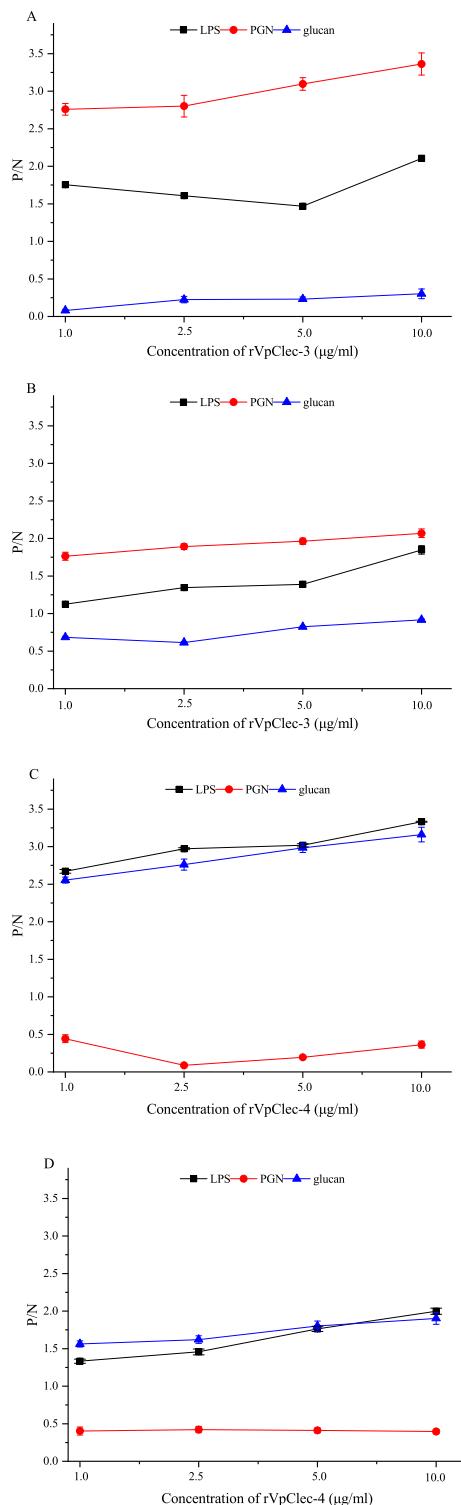


Fig. 5. ELISA analysis of rVpClecs in the presence of Ca^{2+} (A, C) or not (B, D). The interaction was detected with goat-anti-rat Ig-alkaline phosphatase conjugate at 405 nm. Samples with $\text{P}/\text{N} > 2.1$ were considered positive. Results are representative of average three such experiments.

respectively. In addition, the carbohydrate recognition domain (CRD) with a Glu-Pro-Asn (EPN) motif was identified in VpClec-3, while a Gln-Pro-Asn (QPN) motif was identified in VpClec-4. This finding suggested that VpClec-3 and VpClec-4 might have different functions in immune responses. CTls do not generally harbor a QPN or EPN motif [7]. Mutated motifs are also detected in the CRD, such as EPD, EPK, EPS,

EPQ, QPG, QPS, QPD, QPT, and YPT, and these motifs do not affect agglutination or specificity [7,23].

As filter-feeding organisms, mollusks constantly encounter various potential pathogenic bacteria in the living environment. In the present study, VpClec-3 transcripts were primarily expressed in hepatopancreas, which were evolutionary forerunners in the integration of immunity and metabolism. In contrast, VpClec-4 transcripts were highly expressed in gills. Similarly, *Litopenaeus vannamei* LvLT is expressed exclusively in hepatopancreas tissues [24]. However, CFLec-1 transcripts in *C. farreri* are highly expressed in gills [25]. The different distribution of CTL transcripts perhaps contributes to its important roles in immune defense against pathogenic microorganism. Both VpClec-3 and VpClec-4 transcripts were highly expressed in hepatopancreas, gills and hemocytes after bacterial challenge. It suggested that the two VpClecs are important immune factors involved in elimination of invaded pathogens. Hemocytes typically play a central role in the mediation of immune capabilities via phagocytosis, encapsulation and nodule formation [26] and participate in tissues/shell repair processes [27,28] and detoxification [29] in invertebrates. After *V. anguillarum* challenge, both VpClec-3 and VpClec-4 transcripts were significantly upregulated in hemocytes. Similarly, CTLs transcript expressions in hemocytes was also significantly upregulated after Gram-negative bacteria challenge in oyster [30] and abalone [13]. These results suggested that both VpClec-3 and VpClec-4 were inducible acute-phase proteins involved in the immune responses of manila clams. However, 12 h after *V. anguillarum* challenge, VpClec-3 and VpClec-4 transcripts levels were sharply reduced compared with the control group. Similar results were also observed for VpClec-1 in manila clams [16]. The expression profiles might be influenced by changes in hemocytes types.

Nonself recognition is the first step of immune responses against pathogens. PRPs are secreted to identify the conserved PAMPs of pathogens, which are responsible for recognizing and defending pathogenic microbes [31], thus activating the signal pathways to synthesize immune effector proteins. Although C- and I-type lectins exhibit specific binding abilities, other nonself ligands can also be recognized by these lectins [32]. In the present study, PAMP binding assays suggested that both rVpClec-3 and rVpClec-4 function as PRRs and are involved in PAMP recognition. Notably, PGN is found in both Gram-positive and Gram-negative bacteria, whereas LPS and glucan are unique components of Gram-negative bacteria and fungi, respectively. The results that rVpClec-4 binds LPS and glucan and rVpClec-3 exclusively binds PGN indicate that these two CTls might play different roles in nonself recognition and downstream immune responses. Thus, VpClec-3 was hypothesized to recognize bacterial pathogens, whereas VpClec-4 initiates immune responses against both bacterial and fungal pathogens.

In the microbial agglutination assay, almost all the tested microorganisms were agglutinated by rVpClecs, indicating a broad PAMP recognition spectrum. Notably, rVpClec-3 exhibited agglutination activity against Gram-negative bacteria *V. harveyi*, *V. splendidus* and *V. anguillarum* even though it could not bind LPS. The bacterial agglutination of rVpClec-3 could be attributed to its PGN-binding ability [33]. Specifically, rVpClec-3 binds PGN, whereas rVpClec-4 does not. However, no agglutination activity against Gram-positive *S. aureus* was observed in rVpClec-3-treated groups. It was hypothesized that VpClec-3 is involved in the recognition of *S. aureus*, but it is possible that it requires the aid of other molecules to agglutinate the bacteria [24]. As a result, the broad microbial agglutinating abilities suggested that VpClec-3 and VpClec-4 participate in immune responses against pathogenic microbes.

It is important to understand the opsonization activity of lectins on hemocytes. In the present study, both rVpClec-3 and rVpClec-4 enhance the phagocytic activity of hemocytes against *E. coli* significantly, especially hemocytes induced by rVpClec-4. Similar results were also reported in the scallop *Chlamys farreri* [34]. In conclusion, opsonization of hemocytes mediated by VpClec-3 and VpClec-4, such as phagocytosis, was verified as part of the immune defense of manila clam.

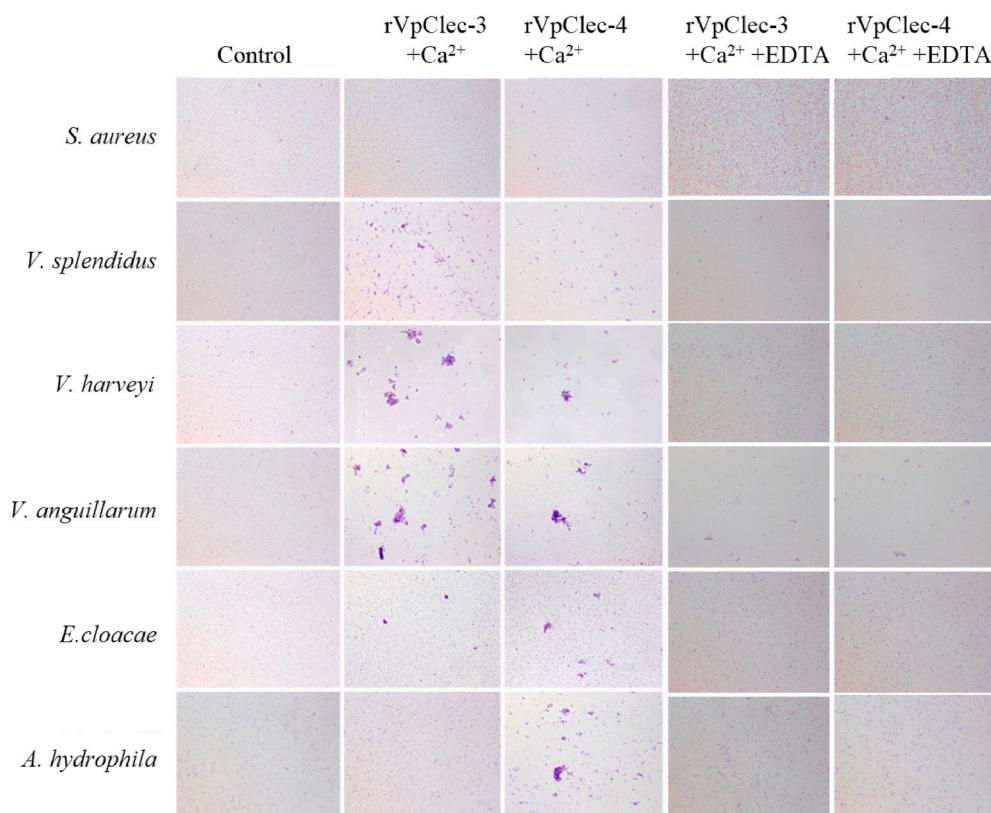


Fig. 6. Agglutination of bacteria by rVpClec-3 and rVpClec-4. Gram-positive bacterium (*Staphylococcus aureus*) and Gram-negative bacteria (*Vibrio harveyi*, *Vibrio splendidus*, *Vibrio anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*) induced by rVpClec-3 and rVpClec-4 with or without EDTA.

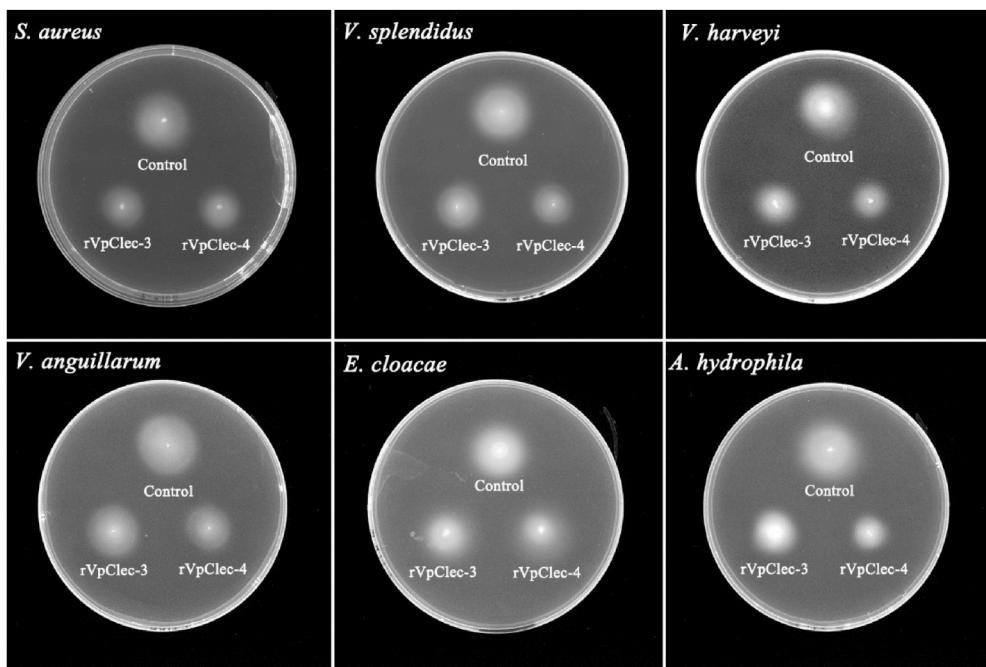


Fig. 7. Swimming motility of Gram-positive bacterium (*Staphylococcus aureus*) and Gram-negative bacteria (*Vibrio harveyi*, *Vibrio splendidus*, *Vibrio anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*) treated with rVpClec-3 and rVpClec-4.

CRediT authorship contribution statement

Zifan Xin: Writing - original draft. **Daode Yu:** Writing - original draft, Data supplement & Writing. **Bowen Yang:** Data curation. **Lizhu**

Chen: Data curation. **Zvi Hayouka:** Methodology, Validation. **Xingfu Chen:** Investigation, Software. **Yitong Gong:** Investigation, Software. **Haiyu Dai:** Investigation, Software. **Lei Wang:** Investigation, Software. **Yancui Zhao:** Supervision. **Xiaoli Liu:** Conceptualization, Writing -

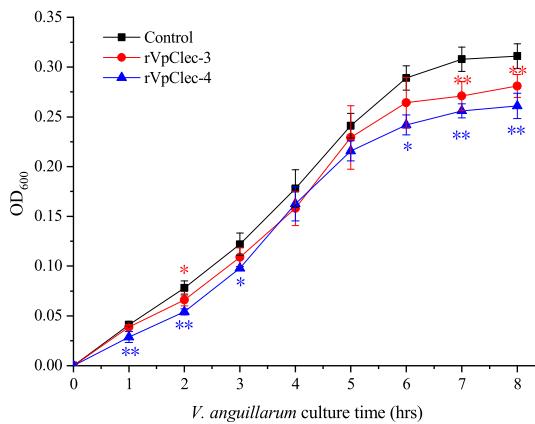


Fig. 8. Antibacterial activity of rVpClec-3 and rVpClec-4. Growth suppressive tests of rVpClec-3 and rVpClec-4 against *V. anguillarum* were measured at OD₆₀₀ every 1 h after starting the cultures. The values were shown as mean \pm S.D. (N = 3) (*: $p < 0.05$, **: $p < 0.01$).

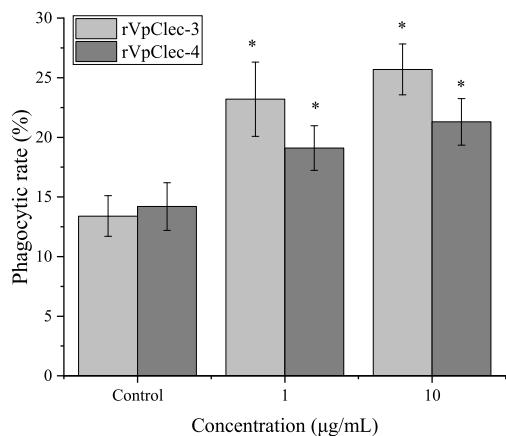


Fig. 9. Phagocytosis of hemocytes enhanced by rVpClec-3 and rVpClec-4. Phagocytosis was analyzed using an Accuri C6 flow cytometer (BD) with BD CFlow® software. The values were shown as mean \pm S.D. (N = 3) (*: $p < 0.05$, **: $p < 0.01$).

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References

- [1] A.N. Zelensky, J.E. Gready, The C-type lectin-like domain superfamily, *FEBS J.* 272 (24) (2005) 6179–6217.
- [2] N. Koizumi, M. Imamura, T. Kadotani, K. Yaoi, H. Iwahana, R. Sato, The lipopolysaccharide-binding protein participating in hemocyte nodule formation in the silkworm *Bombyx mori* is a novel member of the C-type lectin superfamily with two different tandem carbohydrate-recognition domains, *FEBS Lett.* 443 (2) (1999) 139–143.
- [3] X.Q. Yu, M.R. Kanost, Immulectin-2, a lipopolysaccharide specific lectin from an insect, *Manduca sexta*, is induced in response to gram-negative bacteria, *J. Biol. Chem.* 275 (48) (2000) 37373–37381.
- [4] Y.C. Liu, F.H. Li, B. Dong, B. Wang, W. Luan, X.J. Zhang, L.S. Zhang, J.H. Xiang, Molecular cloning, characterization and expression analysis of a putative C-type lectin (Fclectin) gene in Chinese shrimp *Fenneropenaeus chinensis*, *Mol. Immunol.* 44 (4) (2007) 598–607.
- [5] H. Li, H. Zhang, S. Jiang, W.L. Wang, L.S. Xin, H. Wang, L.L. Wang, L.S. Song, A single-CRD C-type lectin from oyster *Crassostrea gigas* mediates immune recognition and pathogen elimination with a potential role in the activation of complement system, *Fish Shellfish Immunol.* 44 (2) (2015) 566–575.
- [6] D.D. Li, H.T. Nie, K. Jahan, X.W. Yan, Expression analyses of C-type lectins (CTLs) in Manila clam under cold stress provide insights for its potential function in cold resistance of *Ruditapes philippinarum*, *Comp. Biochem. Physiol. C* 230 (2020).
- [7] L. Wang, L. Wang, M. Huang, H. Zhang, L. Song, The immune role of C-type lectins in molluscs, *Isj-Invert Surviv J.* 8 (2) (2011) 241–246.
- [8] C.K. Mu, L.L. Chen, J.M. Zhao, C.L. Wang, Molecular cloning and expression of a C-type lectin gene from *Venerupis philippinarum*, *Mol. Biol. Rep.* 41 (1) (2014) 139–144.
- [9] M.M. Huang, X.Y. Song, J.M. Zhao, C.K. Mu, L.L. Wang, H. Zhang, Z. Zhou, X. L. Liu, L.S. Song, A C-type lectin (AiCTL-3) from bay scallop *Argopecten irradians* with mannose/galactose binding ability to bind various bacteria, *Gene* 531 (1) (2013) 31–38.
- [10] M.M. Huang, L.L. Wang, J.L. Yang, H. Zhang, L.L. Wang, L.S. Song, A four-CRD C-type lectin from *Chlamys farreri* mediating nonself-recognition with broader spectrum and opsonization, *Dev. Comp. Immunol.* 39 (4) (2013) 363–369.
- [11] B. Allam, C. Paillard, S.E. Ford, Pathogenicity of *Vibrio tapetis*, the etiological agent of brown ring disease in clams, *Dis. Aquat. Org.* 48 (3) (2002) 221–231.
- [12] Y.S. Kang, Y.M. Kim, K.I. Park, S.K. Cho, K.S. Choi, M. Cho, Analysis of EST and lectin expressions in hemocytes of Manila clams (*Ruditapes philippinarum*) (Bivalvia : Mollusca) infected with *Perkinsus olseni*, *Dev. Comp. Immunol.* 30 (12) (2006) 1119–1131.
- [13] N. Wang, I. Whang, J. Lee, A novel C-type lectin from abalone, *Haliotis discus discus*, agglutinates *Vibrio alginolyticus*, *Dev. Comp. Immunol.* 32 (9) (2008) 1034–1040.
- [14] L. Zhu, L.S. Song, W. Xu, P.Y. Qian, Identification of a C-type lectin from the bay scallop *Argopecten irradians*, *Mol. Biol. Rep.* 36 (5) (2009) 1167–1173.
- [15] H. Zhang, H. Wang, L.L. Wang, X.Y. Song, J.M. Zhao, L.M. Qiu, L. Li, M. Cong, L. S. Song, A novel C-type lectin (Clec-3) from *Chlamys farreri* with three carbohydrate-recognition domains, *Fish Shellfish Immunol.* 26 (5) (2009) 707–715.
- [16] J.N. Zhang, Y.F. Zhang, L.Z. Chen, J.M. Yang, Q.Y. Wei, B.W. Yang, X.L. Liu, D. L. Yang, Two c-type lectins from *Venerupis philippinarum*: possible roles in immune recognition and opsonization, *Fish Shellfish Immunol.* 94 (2019) 230–238.
- [17] J.M. Zhao, C.H. Li, A.Q. Chen, L.Y. Li, X.R. Su, T.W. Li, Molecular characterization of a novel big defensin from clam *Venerupis philippinarum*, *PloS One* 5 (10) (2010).
- [18] J. Schultz, F. Milpetz, P. Bork, C.P. Ponting, SMART, a simple modular architecture research tool: identification of signaling domains, *P Natl Acad Sci USA* 95 (11) (1998) 5857–5864.
- [19] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)-Delta Delta C method, *Methods* 25 (4) (2001) 402–408.
- [20] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M. D. Provenzano, E.K. Fujimoto, N.M. Goede, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, *Anal. Biochem.* 150 (1) (1985) 76–85.
- [21] S.F. Cheng, W.B. Zhan, J. Xing, X.Z. Sheng, Development and characterization of monoclonal antibody to the lymphocystis disease virus of Japanese flounder *Paralichthys olivaceus* isolated from China, *J. Virol Methods* 135 (2) (2006) 173–180.
- [22] Y.H. Shi, X.L. Zhao, Z.H. Wang, Y.N. Shao, W.W. Zhang, Y.B. Bao, C.H. Li, Novel Ca²⁺-independent C-type lectin involved in immune defense of the razor clam *Sinonovacula constricta*, *Fish Shellfish Immunol.* 84 (2019) 502–508.
- [23] X.W. Wang, J.X. Wang, Diversity and multiple functions of lectins in shrimp immunity, *Dev. Comp. Immunol.* 39 (1–2) (2013) 27–38.
- [24] T.H.T. Ma, S.H.K. Tiu, J.G. He, S.M. Chan, Molecular cloning of a C-type lectin (LvLT) from the shrimp *Litopenaeus vannamei*: early gene down-regulation after WSSV infection, *Fish Shellfish Immunol.* 23 (2) (2007) 430–437.
- [25] H. Wang, L.S. Song, C.H. Li, J.M. Zhao, H. Zhang, D.J. Ni, W. Xu, Cloning and characterization of a novel C-type lectin from Zhikong scallop *Chlamys farreri*, *Mol. Immunol.* 44 (5) (2007) 722–731.
- [26] V. Matozzo, L. Ballarin, D.M. Pampanin, M.G. Marin, Effects of copper and cadmium exposure on functional responses of hemocytes in the clam, *Tapes philippinarum*, *Arch Environ Con Tox* 41 (2) (2001) 163–170.
- [27] T. Suzuki, R. Yoshinaka, S. Mizuta, S. Funakoshi, K. Wada, Extracellular-matrix formation by amebocytes during epithelial regeneration in the pearl oyster *Pinctada-fucata*, *Cell Tissue Res.* 266 (1) (1991) 75–82.
- [28] A.S. Mount, A.P. Wheeler, R.P. Paradkar, D. Snider, Hemocyte-mediated shell mineralization in the eastern oyster, *Science* 304 (5668) (2004) 297–300.
- [29] L. Giamberini, M. Auffret, J.C. Pihan, Haemocytes of the freshwater mussel, *Dreissena polymorpha* pallas: cytology, cytochemistry and X-ray microanalysis, *J. Molluscan Stud.* 62 (1996) 367–379.
- [30] X. Jing, E.P. Espinosa, M. Perrigault, B. Allam, Identification, molecular characterization and expression analysis of a mucosal C-type lectin in the eastern oyster, *Crassostrea virginica* (vol 30, pg 851, 2011), *Fish Shellfish Immunol.* 30 (4–5) (2011), 1207–1207.
- [31] C.A. Janeway, R. Medzhitov, Innate immune recognition, *Annu. Rev. Immunol.* 20 (2002) 197–216.

- [32] X. Du, G.H. Wang, Y.L. Su, M. Zhang, Y.H. Hu, Black rockfish C-type lectin, SsCTL4: a pattern recognition receptor that promotes bactericidal activity and virus escape from host immune defense, *Fish Shellfish Immunol.* 79 (2018) 340–350.
- [33] X.M. Wei, X.Q. Liu, J.M. Yang, S. Wang, G.H. Sun, J.L. Yang, Critical roles of sea cucumber C-type lectin in non-self recognition and bacterial clearance, *Fish Shellfish Immunol.* 45 (2) (2015) 791–799.
- [34] L.L. Wang, L.L. Wang, H. Zhang, Z. Zhou, V.S. Siva, L.S. Song, A C1q Domain containing protein from scallop *Chlamys farreri* Serving as pattern recognition receptor with heat-aggregated IgG binding activity, *PloS One* 7 (8) (2012).