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Identification of a novel C1q complement component in razor clam *Sinonovacula constricta* and its role in antibacterial activity

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ABSTRACT

The serum complement component C1q mediates a variety of immune regulatory functions. Herein, we identified a globular head C1q (*ghC1q*) gene in razor clam *Sinonovacula constricta*. The complete *Sc-ghC1q* gene was 872 bp long included an 81 bp 5′-untranslated region (UTR), a 95 bp 3′-UTR with a poly(A) tail, and an open reading frame (ORF) of 696 bp. The mRNA expression of *Sc-ghC1q* was upregulated in hepatopancreas and hemocytes. After *Staphylococcus aureus* or *Vibrio anguillarum* challenge, *Sc-ghC1q* mRNA transcript abundance was significantly upregulated in hemolymph. Recombinant *Sc-ghC1q* protein could bind lipopolysaccharide (LPS) and lipoteichoic acid (LTA), and it could agglutinate both Gram-positive and Gram-negative bacteria. Additionally, flow cytometry revealed that *Sc-ghC1q* strongly promoted phagocytosis in hemocytes. Together, these results demonstrated that *Sc-ghC1q* played an important role in innate immunity in *S. constricta*.

1. Introduction

The complement system is a connecting link between innate and acquired immunity, and plays a major role in innate immune responses. The complement system involves the inherent components, regulatory molecules, complement receptors, and activation of specific protein fragments, and totalling nearly 40 different types of protein molecules, which are triggered by the recognition of different substrates by complement component 1 (C1), C3, and lectins [1–3]. Three pathways activate the complement system: the classical pathway, the mannose-binding lectin pathway, and the alternative pathway, the latter of which plays a key role in the normal functional regulation of immune systems in organisms [4,5]. Each pathway can be activated by a recognition molecule combined with a specific set of ligands, but all three pathways share a common terminal pathway.

The C1q protein family includes several proteins containing C1q domains which named C1q domain-containing protein (C1qDC protein), and members contain a signal peptide, a collage-like region, and a globular C1q domain (gC1q). According to its structural characteristics, the C1q family can be divided into three types: 1) C1q which include collage-like region and gC1q, 2) C1q-like that have similar structure to C1q, and globular head C1q (ghC1q) which lack a collage-like region [6]. As a promoter of the complement classical pathway, C1q plays an essential role in recognising pathogens and activating the complement system [7], and also regulates numerous cellular processes independent of its role in complement activation [8]. C1q is a heteromeric hexamer composed of six subunits, each consisting of three polypeptide chains (A, B, and C) [9], and the C1q domain is considered an efficient pattern recognition domain [10]. This domain functions in signalling channels, binding various types of target proteins such as immunoglobulin G (IgG), lipopolysaccharide (LPS), and phospholipids [11]. C1q is mainly synthesised by myeloid cells, especially tissue macrophages and cultured monocytes, as well as dendritic cells (DCs) [12]. C1q can modulate DCs, platelets, microglial cells, and a diverse range of immune cells [13], and it also cleans apoptotic cells.

A variety of C1qDC proteins have been identified and well characterised in vertebrates, including the products of 31 and 52 C1qDC genes in human [14] and zebrafish [15], respectively. In recent years, many C1qDC genes have also been identified in invertebrates, and shown to affect their immune responses by performing functions

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Abbreviations

NCBI	National Center for Biotechnology Information
NJ	Neighbour-joining
ORF	Open reading frame
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
UTR	Untranslated region

including pathogen recognition [16] and agglutination of microorganisms [17]. In invertebrates, many contain only gC1q domain, and increasing evidence suggests that gC1q may directly interact with receptors on the cell outer membrane, and thereby contribute to the phagocytosis of pathogens [18]. C1qDC can bind various pathogen-associated molecular patterns (PAMPs), and the protein from bay scallop *Argopecten irradians* can agglutinate various microorganisms [17,19]. In Mediterranean mussel *Mytilus galloprovincialis*, MgC1q is strongly upregulated after challenge with Gram-positive or Gram-negative bacteria, indicating that this protein may act as a pattern recognition molecule in innate immune responses [20,21].

The razor clam *Sinonovacula constricta* is a member of the Veneroida family of Lamellibranch molluscs [22]. It is one of the four major aquaculture clams in China, with a high commercial and nutritional value. Due to the degradation of germplasm resources and pollution, this species has suffered a high mortality [23]. Although C1q had been well researched in vertebrates, less is known about the protein in molluscs, including *S. constricta*. In the present study, we identified the *Sc-ghC1q* gene in *S. constricta*, and studied its innate immunity functions. The findings expand our understanding of the C1q gene and protein in invertebrates, including its roles in the invertebrate immune system.

2. Materials and methods

2.1. Experimental samples

Razor clams for gene cloning and bacteria for challenge and other experiments were obtained from Donghang Farm, Sanmen City, Zhejiang Province, China, and used in accordance with the Guidelines on the Care and Use of Animals for Scientific Purposes set by the Institutional Animal Care and Use Committee of Shanghai Ocean University, Shanghai, China. The average body weight and length of adult clams were 9.0 ± 0.2 g and 5.0 ± 0.3 cm, respectively. Clams were cultured in an incubator at 25–27 °C with 2% salinity, and collected at different developmental stages using a silk screen.

2.2. Identification and cloning of the Sc-ghC1q gene

Total RNA was extracted from razor clams using an RNeasy Plus kit (Qiagen, Germany), in accordance with the manufacturer's instructions. Hepatopancreas (50 mg), hemolymph (1 mL), and gonad (50 mg) tissues were obtained from three adult clams and mixed before extracting total RNA. RNA concentration and integrity were determined using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA) and agarose gel electrophoresis.

Analysis of *Sc-ghC1q* gene sequence fragments from the cDNA library of *S. constricta* [24] revealed similarity with complement C1q proteins from *M. galloprovincialis*. Based on C1q sequences obtained in our laboratory [24], we used several pairs of forward and reverse primers (Table 1) for verification, and a full-length cDNA sequence of *Sc-ghC1q* was obtained by PCR using 5′-Full RACE and 3′-Full RACE kits (TaKaRa, Japan). The primers used for 5′-RACE and 3′-RACE are listed in Table 1. The product was analysed by agarose gel electrophoresis and sequenced by Sangon Biotech (Shanghai).

2.3. Sequence analysis

The *Sc-ghC1q* gene sequence was analysed using the National Center for Biotechnology Information (NCBI) BLASTn (nucleotide) database, and amino acid sequences were retrieved from NCBI using the ORF finder tool. *Sc-ghC1q* protein domains were predicted and analysed using ExPASy, and signal peptide prediction was performed with SignaIP. BioEdit and MEGA 7.0 were used to carry out multiple sequence alignment and construct a phylogenetic tree, respectively, using the neighbour-joining (NJ) method with 10,000 bootstraps.

2.4. Analysis of Sc-ghC1q expression by quantitative real-time PCR (qRT-PCR)

Sc-ghC1q expression levels in adult tissues (hepatopancreas, gill, foot, hemolymph, mantle, gonad and siphon) were analysed by qRT-PCR. Optimal primers (Table 1), which could generate single PCR products and display an amplification efficiency close to 97.4%, were screened out by plotting standard curves. *S. constricta* 18S rRNA was used as an internal reference to verify the success of reverse transcription, and to calibrate the cDNA template, since 18S rRNA expression is more stable than that of other *S. constricta* housekeeping genes [23,25].

The qRT-PCR reaction system contained 500 ng cDNA, 6.8 μL nuclease-free water, 10 μL 2× SYBR Premix Ex Taq (TaKaRa, Japan), and 0.8 μL of each gene-specific primer (10 mM). Thermal cycling included an initial denaturation step at 95 °C for 30 s, followed by 35 cycles at 95 °C for 5 s and 55 °C for 30 s, and dissociation curve analysis at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s to verify the amplification of a single product.

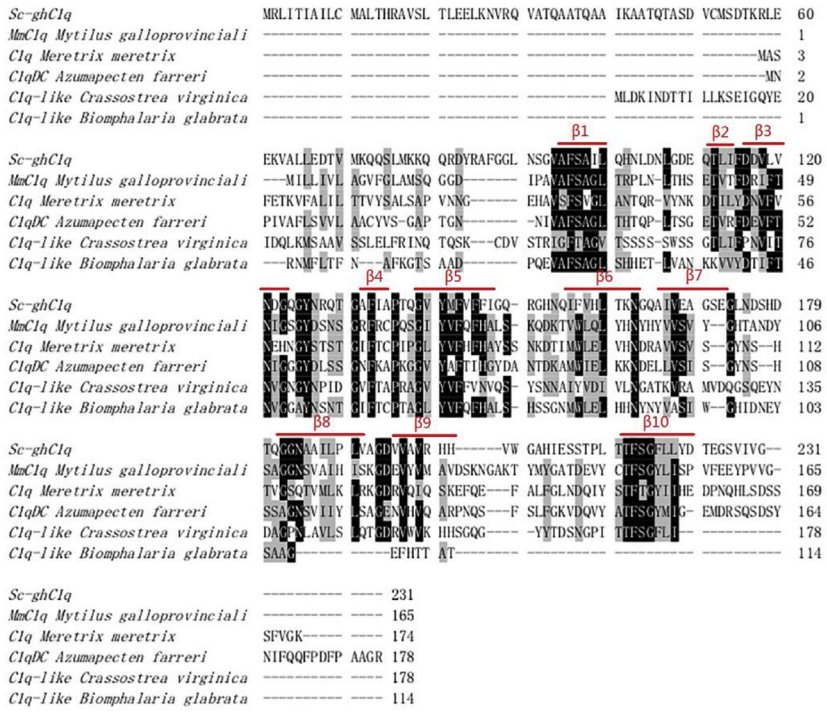
2.5. Bacterial challenge with Vibrio anguillarum and Staphylococcus aureus

For bacterial challenge with *V. anguillarum* and *S. aureus*, bacteria were obtained from the Aquatic Pathogen Collection Centre of the Ministry of Agriculture, China. *V. anguillarum* and *S. aureus* were cultured at 28 °C and 37 °C respectively for 12 h and adjusted to a cell density of 1×10^9 cells/mL with phosphate-buffered saline (PBS, Sangon Biotech, Shanghai). A total of 300 adult clams were selected for bacterial challenge and injected in the feet with 50 μL (1×10^8 cells/mL) of *S. aureus* or *V. anguillarum* suspension with a 1 mL sterile syringe. Clams in the control group were injected with 50 μL sterile PBS. Tissue

Table 1
Primers used to study *Sc-ghC1q*.

Primer	Sequence (5′ – 3′)	Purpose
Sc-C1q-F1	AGCACTTTTGGAGGATACCGT	EST sequence confirmation
Sc-C1q-R1	AGCCAGAGAAGGTCGTGAGAG	
Sc-C1q-F2	AGACTATCGTGCTTTTGGTGGT	EST sequence confirmation
Sc-C1q-R2	GTGAGAGGGGTGCTGGATTC	
Sc-C1q-F3	ATGAGGCTTATTACCATTGCG	EST sequence confirmation
Sc-C1q-R3	TACTGCCACGACATCACCG	
Sc-C1q-R4	CGAAGATGAGTGTTTGCTCGTCCCCT	5′-RACE
Sc-C1q-R5	TCGCTGAGAATGCCACACCCGAGTT	5′-RACE
Sc-C1q-F4	AATGATGGACAAGGCTACAACAG	3′-RACE
Sc-C1q-F5	ACCTTCTCTGGCTTCCTGTTAT	3′-RACE
Sc-C1q-F6	AGCAGGGAGCGAAGGTTTGA	Real-time PCR
Sc-C1q-R6	TGGTGACGTACTGCCACGAC	
Sc-C1q-F7	CGCGGATCCGCGCTGACACTCGAGGAACTAAAGA	Vector construction
Sc-C1q-R7	CGGAATTCCGCCCGACAATAACTGAGCCCTCC	
18S-F	TCGGTTCTATTGCGTTGGTTTT	qRT-PCR of controls
18S-R	CAGTTGGCATCGTTTATGGTCA	

(A)



(B)

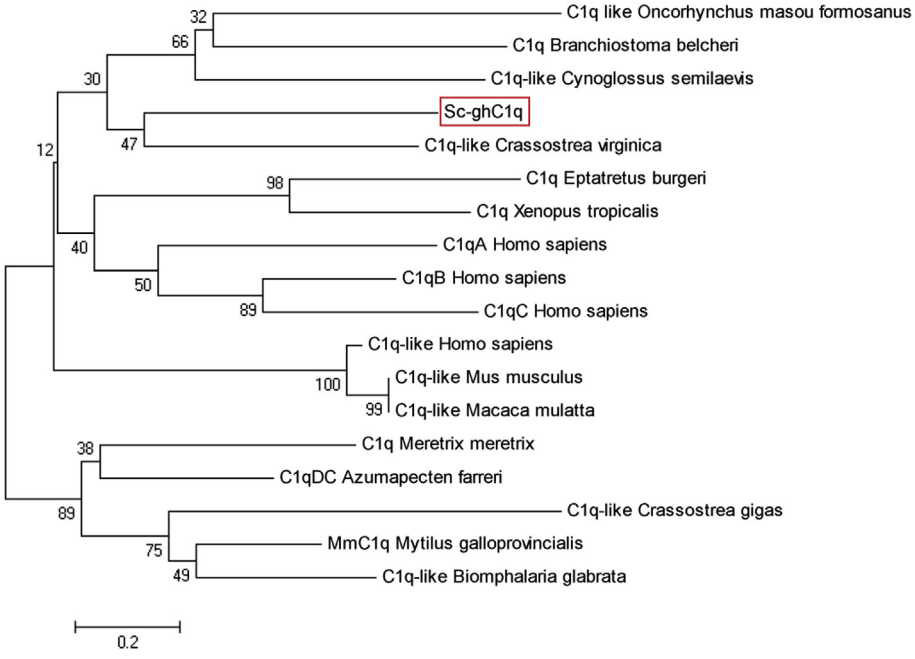


Fig. 1. (A) Multiple alignment of five C1qDCs homologs from invertebrates. Dashes (e) indicate gaps, black shadows indicate identical residues, and grey shadows indicate similar residues in the aligned amino acid sequences. MmC1q of *Mytilus galloprovincialis*, Accession No. CBX41690.1; C1q of *Meretrix meretrix*, Accession No. ADX99230; C1qDC of *Azumapecten farreri*, Accession No. ABS50435; C1q-like protein of *Crassostrea virginica*, Accession No. XP_022294892.1; C1q-like protein of *Biomphalaria glabrata*, Accession No. XP_013091954.1. (B) Phylogenetic tree analysis of *Sc-ghC1q* and C1qDC proteins from other organisms. GenBank accession numbers are as follows: C1q-like protein of *Oncorhynchus masou formosanus*, ABY55260.1; C1q of *Branchiostoma belcheri*, XP_019614074.1; C1q-like of *Cynoglossus semilaevis*, XP_008316912.1; C1q-like of *Crassostrea virginica*, XP_022294892.1; C1q of *Eptatretus burgeri*, BAM76761.1; C1q of *Xenopus tropicalis*, XP_002938623.2; C1qA of *Homo sapiens*, NP_001334395.1; C1qB of *Homo sapiens*, NP_000482.3; C1qC of *Homo sapiens*, NP_001334548.1; C1q-like of *Homo sapiens*, NP_001008224.1; C1q-like of *Mus musculus*, NP_694795.1; C1q-like of *Macaca mulatta*, NP_001252728; C1q of *Meretrix meretrix*, ADX99230; C1qDC of *Azumapecten farreri*, ABS50435.1; C1q-like protein of *Crassostrea gigas*, EKC25478.1; MmC1q of *Mytilus galloprovincialis*, CBX41690.1; C1q-like of *Biomphalaria glabrata*, XP_013091954.1.

samples from hemolymph were isolated for RNA extraction at 0, 4, 8, 12, 24, 48, and 72 h after inoculation with *S. aureus* or *V. anguillarum*. Hemolymph samples were obtained from nine clams, and samples from three individuals were mixed to reduce individual differences in experimental and control groups. For gene expression analysis, cDNA samples from tissue samples were analysed in triplicate using the $2^{-\Delta\Delta CT}$ method [26].

Data are presented as the mean ± SE. A T-test analysis was used for this experiment to assess the statistical significance between the control and challenge groups.

2.6. Expression and purification of recombinant Sc-ghC1q protein

A pair of primers (Table 1) with a *Bam*HI restriction enzyme site in

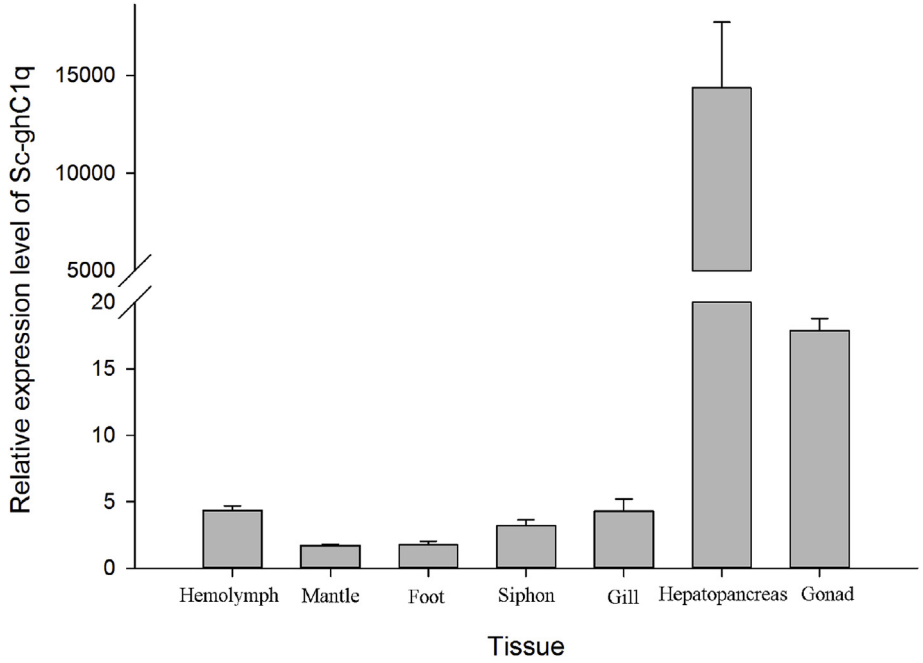


Fig. 2. Tissue expression of the *Sc-ghC1q* gene in seven adult tissues. The results are representative of three independent experiments. Bars represent the mean ± S.E.M. (n = 3) for each tissue.

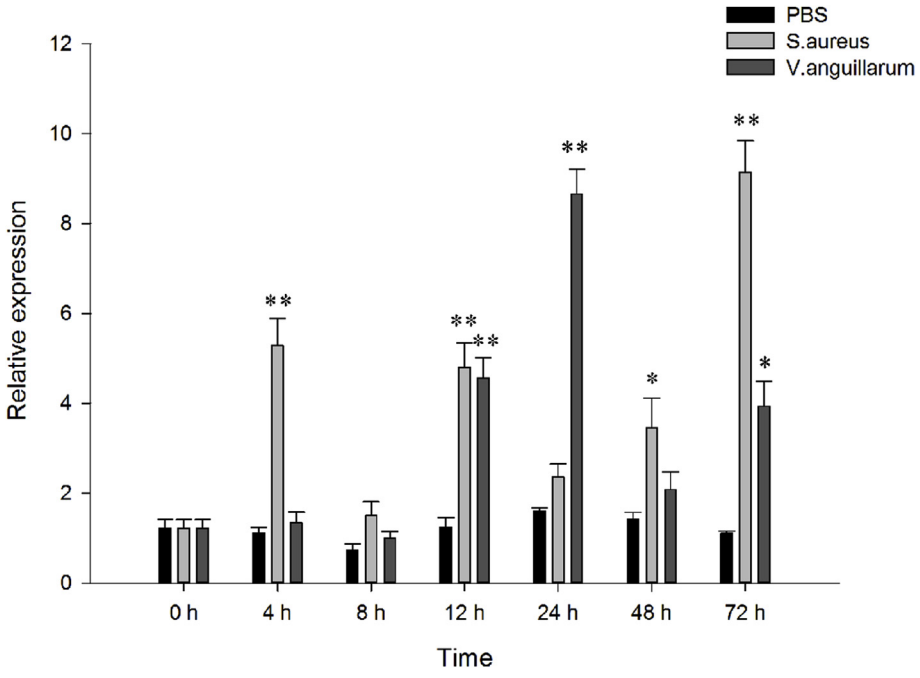


Fig. 3. RT-PCR analysis of *Sc-ghC1q* expression in the hemolymph of *S. constricta* following challenge with *Staphylococcus aureus* or *Vibrio anguillarum*. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$) compared with controls. Error bars represent mean ± S.E.M. (n = 3) from three independent PCR amplifications and quantifications.

the forward primer and an *Eco*RI site in the reverse primer were designed to amplify the sequence encoding the C1q domain of *Sc-ghC1q*. Both the gene fragment and the pGEX-4T-1 vector were digested by *Bam*HI and *Eco*RI (Takara, Japan), purified DNA fragments were ligated, and the resulting recombinant plasmid was transformed into competent *Escherichia coli* BL21 (DE3, Sangon Biotech, Shanghai) cells for expression analysis. After initial culturing, isopropyl-β-D-thiogalactoside (IPTG, Sangon Biotech, Shanghai) was added at a final concentration of 0.5 mM to induce protein expression, and recombinant C1q protein was purified using Glutathione Sepharose 4B resin (GE Healthcare) according to the manufacturer's instructions. The purified protein was analysed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Lipoteichoic acid (LTA) and LPS binding assays

LTA and LPS binding assays were performed by enzyme-linked immunosorbent assay (ELISA). Briefly, LTA and LPS (100 µL, 2 µg/mL) were added to separate well of a 96-well microtiter plate and incubated at 4 °C overnight to coat the wells. After washing three times with TBS solution containing 0.05% Tween-20 (TBST), the plate was blocked with 3% bovine serum albumin (BSA, Sangon Biotech, Shanghai) in PBS at 37 °C for 1 h. Next, 100 µL of recombinant protein (rSc-ghC1q-PBS) or glutathione-S-transferase (GST; negative control) was added after washing by TBST. Plates were incubated at 37 °C for 1 h, washed three times, and polyclonal antibodies recognising the GST-tag were added and incubated for 1 h. After washing, Horseradish peroxidase (HRP)-

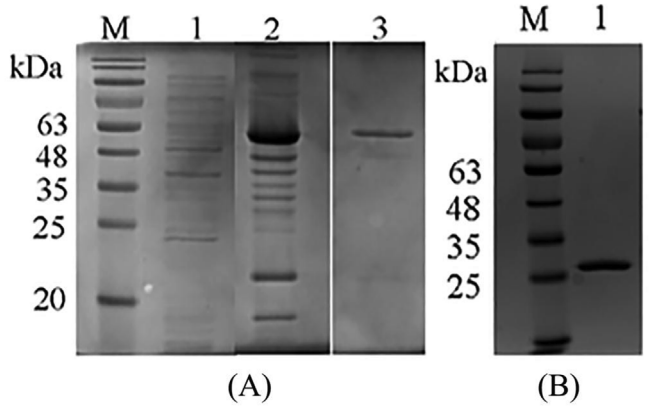


Fig. 4. SDS-PAGE analysis of the rSc-ghC1q protein. (A) Lane M, protein molecular weight standards (11–245 kDa); lane 1, non-induced total cell fraction; lane 2, supernatant of *Escherichia coli* cells harbouring the pGEX-4T-1-Sc-ghC1q construct following ultrasonication and centrifugation; lane 3, purified rSc-ghC1q protein. (B) Lane M, protein molecular weight standards (11–245 kDa); lane 1, purified GST-tag protein.

labelled goat anti-rabbit antibodies were added and incubated for 1 h to determine binding activity. Finally, 3,3′,5,5′-Tetramethylbenzidine (TMB) chromogenic substrate (100 μL; Sangon Biotech, Shanghai) was added to each well and samples were incubated at room temperature in the dark for 15 min. The reaction was stopped by adding 50 μL of H_2SO_4 and the absorbance was measured at 450 nm.

Each experiment was repeated in triplicate and the data were presented as mean ± SD (n = 3). Differences between control and experimental groups were assessed for statistical significance with one-way ANOVA.

2.8. Bacterial agglutination assay

Three Gram-positive bacteria (*S. aureus*, *S. agalactiae* and *B. subtilis*) and three Gram-negative bacteria (*V. anguillarum, E. coli* and *A. hydrophila*) were separately cultured overnight and washed twice with PBS. Bacterial suspensions were prepared in PBS at a cell density of 1×10^8 cell/mL, and fluorescein isothiocyanate (FITC) was added to obtain a final concentration of 1 mg/mL. Mixtures were then incubated for 30 min at room temperature in the dark. Bacterial suspensions in PBS (100 μL) were incubated with 20 μL of recombinant protein (rSc-ghC1q-PBS, 100 μg/mL) or GST (100 μg/mL; negative control) at room temperature for 1 h, and agglutination was observed by microscopy.

2.9. Analysis of phagocytic rate

V. anguillarum and *S. aureus* cells were cultured overnight at 28 °C and 37 °C respectively, the final bacterial suspension was adjusted to 2×10^8 cells/mL with PBS, and labelling of bacteria with FITC was performed. Fresh hemocytes from clams were washed three times with PBS by centrifuging at 1000 × *g* for 5 min, and adjusted to a cell density of 1×10^7 cells/mL using a flow cytometer (BD ACCURI C6 PLUS). Bacterial suspensions (200 μL) were mixed with 180 μL of hemocyte suspension and 20 μL of rSc-ghC1q (100 μg/mL) in a 1.5 mL Eppendorf tube. In the control group, GST-tag protein (100 μg/mL) or PBS was substituted for rSc-ghC1q. In the inhibited group, rSc-ghC1q was replaced by cytochalasin B (3 mg/mL). Mixtures were incubated in the dark at room temperature for 1 h with agitation every 5 min, and samples were analysed using a flow cytometer as described previously [27]. And the data were analyzed with one-way ANOVA.

3. Results

3.1. Gene cloning and sequence analysis of Sc-ghC1q

In this study, we cloned the *C1q* gene from *S. constricta* and named it *Sc-ghC1q* (MH894276). The full-length 872 bp cDNA includes an 81 bp 5′-untranslated terminal region (UTR), a 95 bp 3′-UTR with a poly(A) tail, and a 696 bp open reading frame (ORF; Fig. S1). The ORF encodes a polypeptide of 230 amino acids with a signal peptide (residues 1–19), a coiled-coil region (CCR) (residues 54–86), and a gC1q domain (residues 94–224; Fig. S1). The calculated isoelectric point (pI) is 5.70 and the predicted molecular weight is 25.02 kDa.

3.2. Multiple sequence alignment and phylogenetic analysis

To investigate the homology between *Sc-ghC1q* and C1q in other species, multiple sequence alignment and phylogenetic analysis were conducted using six C1qDC homologs from invertebrates (Fig. 1A). The results revealed low sequence identity with C1qDC from other invertebrates; *Sc-ghC1q* shares 43% sequence identity with C1q in *Crassostrea virginica*. Furthermore, multiple sequence alignment analysis

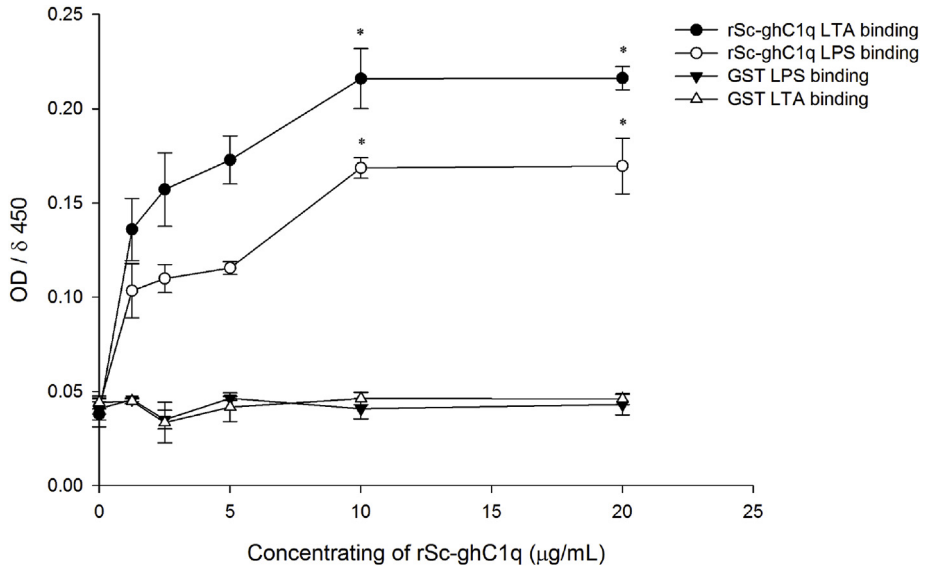


Fig. 5. Binding activity of rSc-ghC1q toward LTA and LPS. The absorbance of three samples was independently measured at 450 nm, and the data were presented as mean ± S.E.M. (n = 3) (*$p < 0.05$).

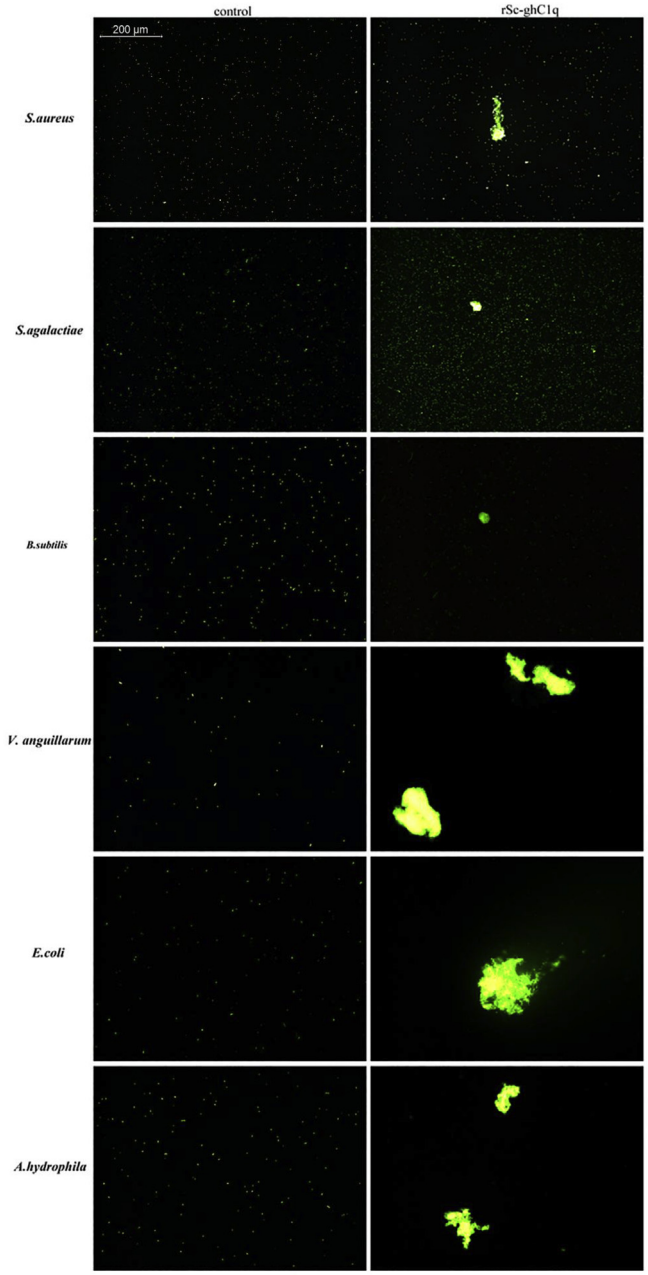


Fig. 6. Agglutination of *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus subtilis*, *Vibrio anguillarum, Escherichia coli* and *Aeromonas hydrophila* by rSc-ghC1q. Control, phosphate-buffered saline (PBS) with 1 mg/mL of GST-tag protein. rSc-ghC1q, PBS with 1 mg/mL rSc-ghC1q.

(Fig. 1A) revealed ten highly conserved β-sheets in the C1q domain. A NJ phylogenetic tree was constructed to investigate evolutionary relationships between *Sc-ghC1q* and its counterparts in other species (Fig. 1B). *Sc-ghC1q* clusters with C1qDC in *C. virginica* and *M. gallo-provincialis*, closer to the *C. virginica* protein, consistent with the results of multiple sequence alignment.

3.3. Tissue expression pattern of Sc-ghC1q

qRT-PCR analysis was carried out to investigate the expression of *Sc-ghC1q* in different tissues, and the results are presented in Fig. 2. *Sc-ghC1q* was widely expressed in the hemolymph, mantle, foot, siphon, gonad, hepatopancreas and gill. Furthermore, *Sc-ghC1q* expression was highest in hepatopancreas, and also high in hemolymph, but relatively lower in the foot compared with other tissues.

3.4. Sc-ghC1q mRNA expression level after bacterial challenge

We evaluated changes in *Sc-ghC1q* mRNA expression level in hemolymph in response to bacterial challenge to verify its function in innate immunity in response to microbes, using *S. aureus* and *V. anguillarum* as immune stimulants (Fig. 3). *Sc-ghC1q* mRNA expression level was upregulated in response to *S. aureus* from 4 h after injection ($p < 0.01$), peaking after 12 h. Similarly, after the calm were challenged with *V. anguillarum*, *Sc-ghC1q* mRNA expression level was significantly upregulated within 12 h ($p < 0.01$), then declined thereafter.

3.5. Expression of recombinant rSc-ghC1q protein

SDS-PAGE analysis indicated that a major protein (~51.02 KDa) was detected after induction, mainly expressed in soluble form and accumulated in the supernatant (Fig. 4A). The GST-tag protein was ~26 kDa as expected (Fig. 4B). This overexpressed protein with the predicted molecular weight was therefore presumed to be rSc-ghC1q, and the concentration of the purified protein was ~1 mg/mL according to the bicinchroninic acid (BCA) method.

3.6. Binding affinity of rSc-ghC1q to LTA and LPS

The results of LTA and LPS binding activity of rSc-ghC1q are shown in Fig. 5. rSc-ghC1q displayed high affinity toward both LTA and LPS, in a dose-dependent manner, while no apparent binding ability was observed for the GST-tag control group.

3.7. Bacterial agglutination properties of rSc-ghC1q

The effects of rSc-ghC1q on bacterial agglutination are shown in Fig. 6. In the experimental group, the rSc-ghC1q protein could agglutinate both Gram-positive (*S. aureus*, *S. agalactiae* and *B. subtilis*) Gram-negative (*V. anguillarum, E. coli* and *A. hydrophila)* bacteria, and displayed stronger agglutination of Gram-negative bacteria. No bacterial agglutination was detected in the control group.

3.8. Statistical analysis of phagocytosis

Phagocytosis in hemocytes was investigated by using flow cytometry, and the results are shown in Fig. 7. In the scatter plot obtained with *S. aureus* using 5 μL of phagocytes and hemocytes, the area of hemocytes is defined as Gate A (Fig. 7A), and all subsequent fluorescence data were limited to Gate A to ensure the accuracy of the analysis. In the fluorescence intensity graph, one part corresponded to untreated hemocytes, and the rest was defined as the phagocytic part, for which the fluorescence intensity reflects the number of phagocytic bacteria (Fig. 7B). Phagocytic ability (PA) is expressed as the proportion of hemocytes involved in phagocytosis in all cells. The more bacteria that are phagocytosed, the higher the fluorescence intensity. In the inhibited controls, cytochalasin B inhibited the phagocytosis of bacteria by hemocytes, which illustrated there was a real phagocytosis rather than only a non-specific adhesion to cells. Flow cytometry analysis revealed that rSc-ghC1q stimulated the phagocytic rate against both *S. aureus* and *V. anguillarum* (Fig. 7C), compared with GST-treated and untreated bacteria (Fig. 8). This result indicates that rSc-ghC1q enhances phagocytosis in hemocytes.

4. Discussion

As an important part of innate immunity in vertebrates and invertebrates, the complement pathway played a unique and irreplaceable role. Traditionally, C1q was considered to function in the

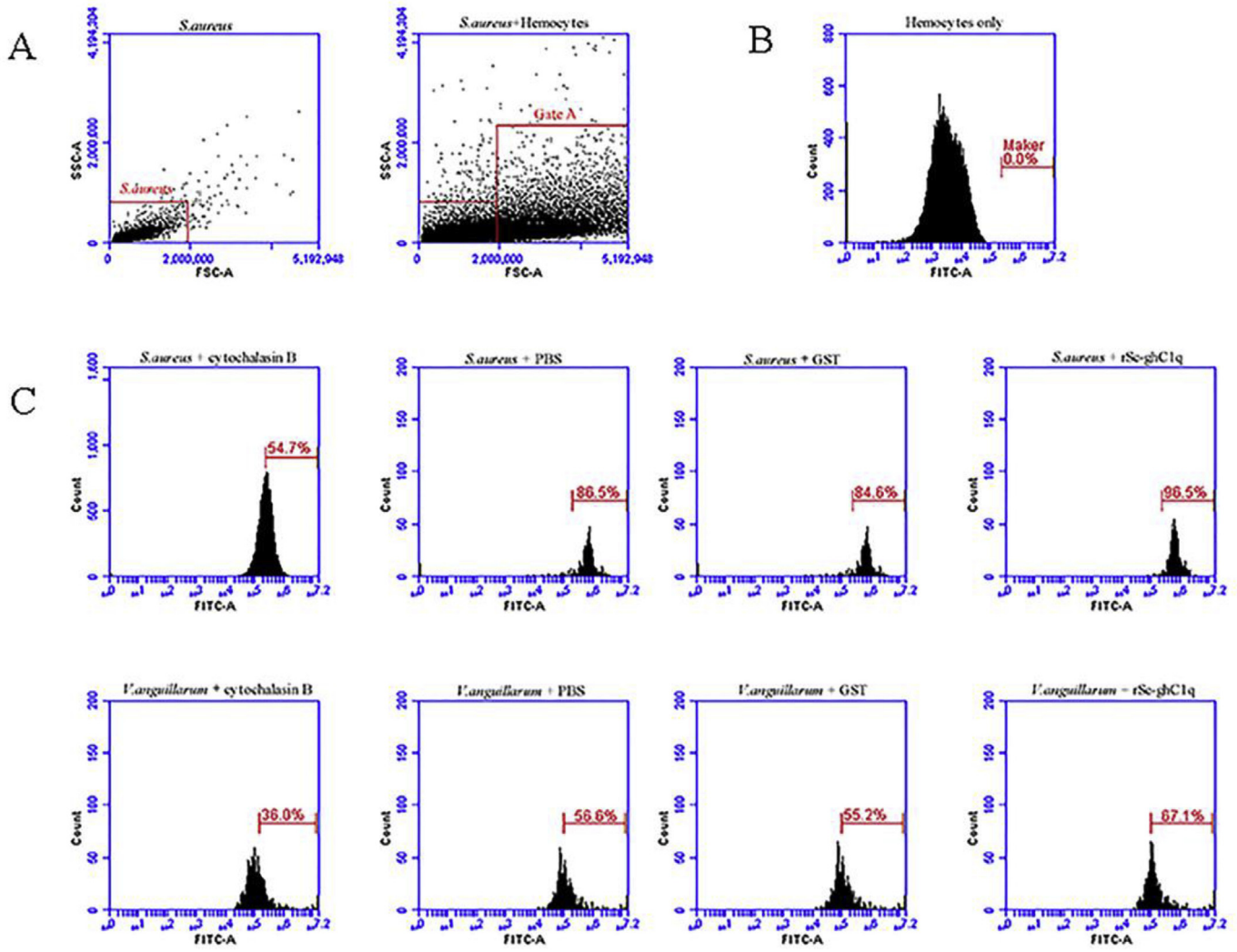


Fig. 7. Flow cytometry analysis of phagocytosis in hemocytes exposed to rSc-ghC1q-treated, GST-tag protein-treated, and PBS-treated *V. anguillarum* and *S. aureus*. (A) Dot plots of *S. aureus* and *S. aureus* + hemocytes. The area where hemocytes are located is defined as Gate A. (B) Histogram of hemocytes alone. The marker represents the phagocytosis part. (C) Histogram of hemocytes challenged with cytochalasin B-, PBS-, GST-, or rSc-ghC1q-treated *S. aureus* or *V. anguillarum*. The results shown in (C) are from one experiment from three separate experiments.

recognition of immune complexes and activation of the classical pathway. However, more recent studies have shown that C1q played a major role in inflammation and adaptive immunity [28,29].

The C1q molecule in vertebrates consisted of a globular C1q domain and a collagen domain, and some C1qDC molecules in invertebrates also had both these domains [30,31], although many C1q molecules in invertebrates, especially molluscs, only contained the globular domain [21,32]. For example, C1qDCs from Zhikong scallop *Chlamys farreri* lacked the collagen domain [33]. In our present study, we identified and characterized C1q in *S. constricta*, which had a typical globular head domain but lacked a collagen domain, making it a type of ghC1q [4,34], as found in other invertebrates. For example, 167 C1qDC proteins in *M. galloprovincialis* were found to be ghC1q proteins [20]. In the present study, the *Sc-ghC1q* protein was composed of 10 β-sheets without a collagen domain. In mammals, the collagen domain of C1q assisted homotrimerisation, which subsequently activated the downstream protease of the complement pathway [35]. Based on this knowledge, we inferred that the *Sc-ghC1q* protein may be present in monomer rather than trimer form, since without a collagen domain, the protein is not likely to trimerise or activate the downstream complement pathway. Thus, *Sc-ghC1q* probably functions as a pattern recognition receptor (PRR) to trigger immune responses in *S. constricta*. Mounting evidence suggested that the gC1q domain may interact with receptors on the cell outer membrane, which contributed to engulfing pathogens. In *Haliotis discus discus*, *AbC1qDCs* was found to act as pattern recognition receptors and played a potential immune defensive role in disk abalone againsted bacteria [36].

Sc-ghC1q was found to be widely expressed in different tissues, with highest levels in hepatopancreas and gonad, similar to findings in scallops [17,33]. At the mRNA level, four C1qDC genes of *Hyriopsis cumingii* were widely distributed [37]. *Sc-ghC1q* expression in immune organs (hepatopancreas and hemolymph) may indicate a role in innate immunity. The qRT-PCR results demonstrated that *Sc-ghC1q* expression was highest in hepatopancreas, confirming that the hepatopancreas is the primary site for the production of immune recognition molecules in invertebrates [38]. Molluscs had open circulatory systems, and the hemolymph played an essential function in immune defenses [39].

For marine animals living in intertidal regions such as *S. constricta*, microbial pathogen was a tremendous challenge. In the present study, *S. aureus* or *V. anguillarum* infection increased *Sc-ghC1q* mRNA transcript levels in the hemolymph, indicating that *Sc-ghC1q* participates in immune response. *Sc-ghC1q* expression in the hemolymph was upregulated at 4 h and 12 h after challenge with Gram-positive and Gram-negative bacteria, and expression peaked at 24 h and 48 h. A similar

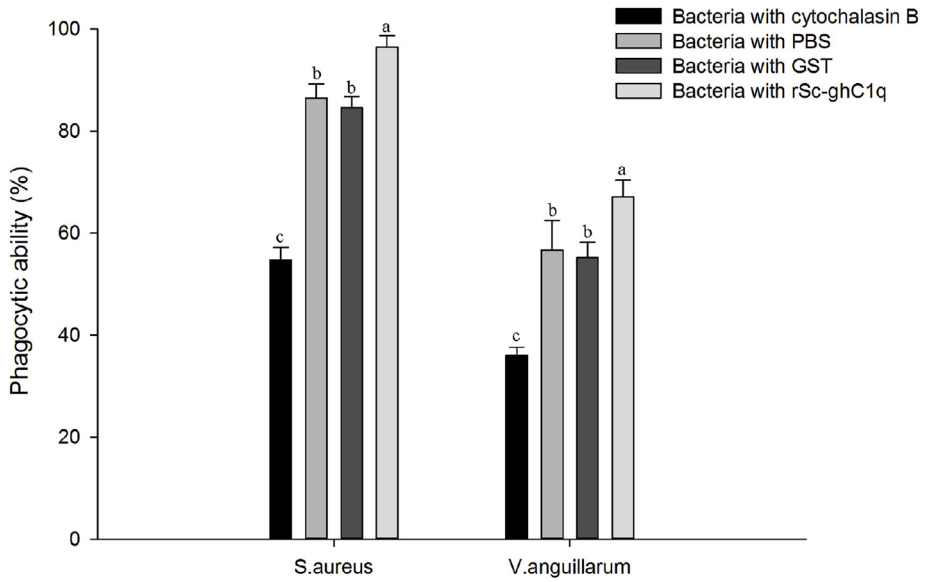


Fig. 8. Flow cytometry analysis of phagocytosis in hemocytes treated with rSc-ghC1q against *S. aureus* and *V. anguillarum*. GST-tag, PBS and cytochalasin B served as controls, and results are means ± S.E.M. (n = 3).

result was reported in a study on *Cynoglossus semilaevis*; *CssghC1q* expression was detected in most tissues, highest expression was observed in the liver, and there was a noticeable transformation in *CssghC1q* expression in the blood following bacterial challenge [40]. In the present study, the rapid response of hemolymph to bacterial challenge indicated that *Sc-ghC1q* might be an important immune factor, and its high expression following challenge implied that it might be associated with the identification of pathogens and enhancing phagocytosis to clear invading pathogens from the organism.

Immune recognition of common or conserved residues derived from pathogens is the first and crucial step in innate immunity, which discriminated self from the potentially harmful non-self, and activated a series of immune responses. In *C. farreri*, *CfC1qDC* bound LPS, peptidoglycan (PGN) and β-glucan, which indicated that *CfC1qDC* could recognise and bind various PAMPs [41]. In the present study, the *Sc-ghC1q* protein could bind LTA and LPS, major constituents of the outer membrane of Gram-positive and Gram-negative bacteria, respectively [42,43]. *Sc-ghC1q* appeared to be involved in bacterial recognition, and it participated in immune responses. In *C. farreri*, C1qDC bound strongly to LPS [33], and in some invertebrates, C1qDC could also cause bacterial and fungal agglutination, and even bound IgGs. The results of bacterial agglutination experiments in the present study showed that rSc-ghC1q could agglutinate both Gram-positive (*S. aureus*, *S. agalactiae* and *B. subtilis*) and Gram-negative (*V. anguillarum, E. coli* and *A. hydrophila*) bacteria. This result indicated that *Sc-ghC1q* recognised and bound various microorganisms, and triggered a series of immune responses. The proteins *AiC1qDC-1* and *AiC1qDC-2* in *Argopecten irradians* possessed strong yeast agglutination ability [17,44], and they acted as PRRs that played important roles in immune defences. To determine whether *Sc-ghC1q* could regulate phagocytosis by hemocytes in *S. constricta*, phagocytosis tests were performed, and the results showed that *Sc-ghC1q* enhanced phagocytosis. Thus, *Sc-ghC1q* appeared to recognise and agglutinate bacteria, and stimulated phagocytosis of bacterial pathogens. In *Crassostrea gigas*, *CgC1qDC-1* protein drastically enhanced the phagocytosis of oyster hemocytes which confirmed that *CgC1qDC-1* was mainly involved in the Gram-negative bacteria recognition and opsonization [18].

In conclusion, a novel full-length C1q gene from *S. constricta* was identified and characterised, and named *Sc-ghC1q*. Expression of *Sc-ghC1q* was enhanced in response to bacterial challenge. Furthermore, *Sc-ghC1q* could bind LTA and LPS, agglutinated both Gram-negative and Gram-positive bacteria, and stimulated the phagocytosis clearing process. Together, these results indicated that *Sc-ghC1q* is an important immune gene in the protection of *S. constricta* from bacterial infection.

Competing interests

The authors have no competing interests to declare.

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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.2019.01.014.

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