



Original article

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Revealing localization and regulation of GTPase PmRab7 in lymphoid cells of *Penaeus monodon* after WSSV infection

Amrendra Kumar^{1*}, Vaishnavi Ramasubbu¹, Kiran D Rasal², Saravanankumar Ayyappan¹¹Center for Advances Studies in Marine Biology, Faculty of Marine Sciences, Annamalai University, Paranipttai, Tamilnadu 608502, India²ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar 751002, India

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ABSTRACT

Objective: To identify white spot syndrome virus (WSSV) entry into the host-cells of the cultured shrimp *Penaeus monodon*, we have attempted to localize PmRab7 (Ras-related in brain) which is playing a vital role in the WSSV internalization.

Methods: In this study, we have cloned PmRab7 and expressed in *Escherichia coli*, further purified rPmRab7 was used for antibody production, isolation of lysosomal sub-cellular fractions and western blot against lysosomal protein. Moreover, high fold-change in PmRab7 regulation with increasing copy number of WSSV has been studied by using real-time PCR.

Results: 651 bp amplicon size gene was successfully amplified, ligated amplicon with pTZ T-tail vector confirmed by colony PCR and restriction enzyme digestion on agarose gel. Subcloned (pRSET-B) 651 bp gene transformed successfully in *Rosetta* and after 6 h of induction expressed rPmRab7 was on SDS page, furthermore soluble fraction of rPmRab7 (26 kDa) was purified by ni-NTA column. AntiPmRab7 antibody was received by Merk Pvt. Ltd., and western blot analysis revealed that PmRab7 is present in the lysosomal sub-cellular fraction. Copy number of WSSV was increased 5 fold in 24 h and 20 fold in 72 h of infection and subsequently transcript of PmRab7 was Ct = 1.0 to Ct = 8.5.

Conclusions: Presence of PmRab7 on lysosome clearly indicating PmRab7 participating in lysosomal maturation, other hand WSSV may follow the same route of entry. WSSV internalization has directly linked with regulation of PmRab7.

1. Introduction

White spot syndrome virus (WSSV) is one of the major viral pathogens in shrimp farming since the early 1990s, which causing huge mortality and major harm to the shrimp industry resulting in loss of billions of the export[1,2]. Earlier studies implicated that envelope proteins like VP28, VP26, VP24, and VP19 are playing a crucial role during the early events of WSSV virus infection[3]. Further, VP28 have shown that, it is involved in systemic infection of WSSV and it binds to the surface of shrimp cells[4]. The complete sequence of WSSV useful for the molecular characterization

of the virus via genomic and proteomic methodologies, which subsequently helpful to identify WSSV genes such as latency-associated genes and many non-structural genes associated with infection[5]. The *in vivo* neutralization assays via raising antibodies against WSSV has identified that several envelope proteins were involved in virus infection. Although, several studies revealed the WSSV and host interaction, there is a lack of knowledge with regard to virus entry into host shrimp cells. The evidence suggested that Rabs (Ras-related in brain) act as molecular switches to control the trafficking of endocytic vesicles within cells, endosomes fusion, late autophagic vacuoles maturation and lysosome biogenesis[6-8]. Total 70 Rab isoforms have been identified in humans, among that Rab1, Rab5, Rab6, Rab7, and Rab11 are known as housekeeping Rabs, which are conserved from yeast to humans[9,10]. Rab7 proteins are found in all higher organisms and all members of the Rab family shown GTP-dependent activity[11,12].

Expression level and co-localization of Rab7 importantly pointed in several diseases such as echovirus into polarized epithelial cells, atherosclerotic plaque infection in rabbit and human, intracellular

*Corresponding author: Dr. Amrendra Kumar, PhD, Center for Advances Studies in Marine Biology, Faculty of Marine Sciences, Annamalai University, Paranipttai, Tamilnadu 608502, India

Tel: +91 8895668876

Fax: 0674 2466407

E-mail: amrendrambiotech@gmail.com

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trafficking of *Salmonella* to lysosome, maturation of *Mycobacterium bovis* bacillus calmette gue phagosomes, adenovirus infection. Rab7 was co-localized in the presence of Rab5 with endosome/lysosome marker[13-18]. An earlier study has shown, PmRab7 a small GTPase protein possibly involved in replication of several shrimp viruses[4]. It also has shown that silencing of dsRNA of PmRab7 could inhibit the replication of viral DNA in *Penaeus monodon* (*P. monodon*)[19]. The entry of WSSV is still under dim light, in that contest, we have attempted to localize the PmRab7 protein in lymphoid tissues sub-cellular fraction of *P. monodon*. We also performed an antibody production against PmRab7 for investigation of the localization of PmRab7 on the lysosome followed by real-time PCR. Our study revealed that PmRab7 could be utilized as a marker in the WSSV infection in *P. monodon*. Our work revealed the functions of endocytic PmRab7 during the multiplication of WSSV infection. This work led to further study for investigating regulatory network pathways with respect of WSSV infection.

2. Materials and methods

2.1. Experimental animal

The shrimps (*P. monodon*) weighing (11 ± 2) g were collected from a private pond of Maharashtra and acclimatized, maintained in the cement tanks of wet-lab facility of the Indian Council of Agricultural Research-Central Institute Fisheries Education, Mumbai, India. The experimental animals were tested for the presence of viruses such as monodon baculovirus and WSSV as a precautionary measure to avoid negative results[2].

2.2. Oral challenge test with WSSV

The quantity (10^3 copies/ μ L) of WSSV infected tissue was taken for oral challenge (lowest quantity of WSSV infected head soft tissues required for causing 100% mortality in *P. monodon* within 7 days of administration). The experimental animals were challenged with WSSV infected head soft tissues (at the rate of 2% of body weight of experimental animals) on the first day and third day post-administration of the appropriate feed type.

2.3. Total RNA extraction and cDNA synthesis

The total RNA was extracted using TRIZOL reagent (Invitrogen, USA) by following the manufacturer's protocol. The RNA purity was measured by NanoDrop, and RNA quality was calculated by 1% gel electrophoresis. The cDNA was synthesized from the RNA using the first strand cDNA synthesizing kit (Fermentas, USA) by using moloney murine leukemia virus reverse transcriptase (M-MuLV) following the manufacturer's protocol with the help of the RT-PCR.

2.4. Expression, purification and antibody preparation of recombinant PmRab7

PmRab7 gene was amplified from cDNA by using primer PmRab7-

1F 5' CGGGATCCCGTGTCTGCAGCTCGGATTTC -3' with BamHI RE and PmRab7-1R 5' GGAATTCCTGTTTAGCCT TGTTGTCATTGG -3' with EcoRI RE, purified amplicon was initially cloned in pTZ t-tail vector followed by sub-cloned in pRSET-B expression vector. Expression optimized by induction 100 mmol/L isopropyl β -D-1-thiogalactopyranoside on 37 °C in 6 h, expression was confirmed by western blot, rPmRab7 was purified by anti-His immobilized metal ion affinity chromatography column followed by Acta-purifier by using 100 mmol/L Tris, 20 mmol/L NaPI, 2 mg/mL deoxyribonuclease, 0.2% NP₄ and 2 mmol/L MgCl₂ buffer, purified rPmRab7 was further assist for anti PmRab7 antibody preparation and purification by Merk USA.

2.5. Lysosomal sub-cellular fraction of lymphoid tissues of *P. monodon*

Lysosomes were isolated from live shrimp dissected under sterile condition[20]. The lymphoid organs were further subjected to 0.1 mol/L ice cold sucrose solution. The lymphoid organ was homogenized at 4000 r/min for 3 min, post-nuclear supernatant were collected and incubated at 37 °C in the presence of a 2 mmol/L CaCl₂ for 10 min, solution were subjected to centrifugation for 10 min at 86711 r/min at 2 °C. The pellets were washed three times with 0.1 mol/L ice cold sucrose solution. Total lysosomal protein was measured using standard procedures[21]. Then, sodium dodecyl sulphate (SDS) page and western blot were performed for PmRab7 confirmation.

2.6. Absolute quantification of WSSV

Copy number was calculated by interruption of the experimentally determined threshold cycle[22] as previously described for infectious hypodermal and hematopoietic necrosis as per protocol. A viral copy number of experimental samples were determined by quantitative real time PCR using Maxima TH SYBR green qPCR master mix reagent (Fermentas and Applied Biosystem, USA). Briefly, Serial dilutions of WSSV plasmids of known copy numbers were prepared and used as templates for RT-PCR reactions. PCR primers were designed using GeneRunner software and the cyclic conditions were used are as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing and extension at 60 °C for 1 min. Each RT-PCR reaction generates a cycle threshold (Ct)[22] value, the cycle number at which the fluorescent intensity of PCR amplicons rises above that of the background level. Mean Ct values for each dilution were calculated. A standard curve was made by plotting plasmid copy numbers against their respective mean Ct values.

2.7. Relative quantification of PmRab7

The cDNA concentrations of all samples were adjusted to 100 ng/ μ L. Relative mRNA expression of PmRab7 by PmRab7-F 2 5' GCATTCTATCGAGGAGCTGATTG 3' and PmRab7-R 2 5' CATTGTTGTGCTCGCTTCGT 3' primer was measured by qPCR using

interferon-1 α primers F-5'-GGTGCTGGACAAGCTGAAGGC-3' and R-5'-GGTTCGGTGATCATGTTCTTGATG-3' as internal standard.

The amplifications were performed in a 96-well plate. The qPCR mixture contained 1.25 μ L of c-DNA, 10 μ L of nuclease free water, 12.5 μ L of 2X SYBR green PCR master-mix and 0.625 μ L of each gene specific primer (stock concentration of 0.25 μ mol/L). The amplification programme consisted of 95 $^{\circ}$ C for 10 min followed by 40 cycles of 95 $^{\circ}$ C for 15 seconds, 60 $^{\circ}$ C for 1 min. All the reactions were performed in duplicates (each well containing 10 μ L of master mix).

2.8. Statistical analysis

One-way ANOVA performed to fit the distribution normal level, hence, natural log of each copy number of WSSV, the correlation coefficient was determined plotting the log value of copy numbers of WSSV. The $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of PmRab7[23].

3. Results

3.1. RNA isolation and first strand c-DNA synthesis and PCR amplification

Extracted RNA samples with good quality and integrity were obtained based on NanoDrop analysis results. The optical density 260/280 ratio of purified RNA was between 0.788–1.830. The synthesized cDNA was successfully amplified by PCR reaction. Length of PmRab7 amplicon was 650 bp. The spectrophotometric analysis of the purified extracted DNA of PmRab7 was performed according to manufacturer protocol for the Fermentas Gene jet PCR purification kit and showed as absorbance as A260/A280 (quality check; ideally higher than 1.8) and concentration for PmRab7 as 1.8 and 24.6 ng/ μ L, respectively.

Ligation reaction was performed for the insertion of the digested PmRab7 (EcoR 1 and BamH 1) into the pTZ plasmid vector (pTZ: 2886 bp, PmRab7 650 bp). 100 ng reaction for restriction enzyme digestion had showed digestion by releasing PmRab7 650 bp DNA band or 3364 bp pRSET-B DNA band on agarose gel electrophoresis (Figure 1a, b).

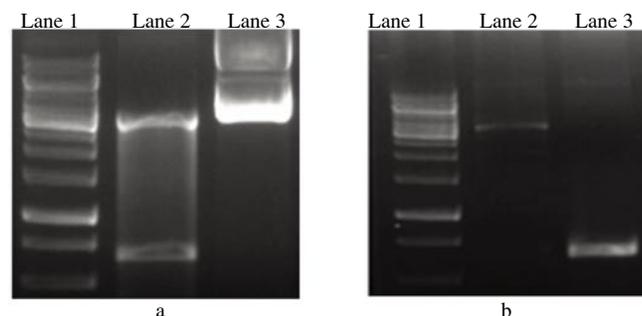


Figure 1 a: Restriction digestion of pTZ vectors containing PmRab7 (Lane 1 contains a 1kb DNA ladder, lane 2 PmRab7 insert and lane 3 undigested pTZ vector); **b:** Restriction digestion of pRSET-B vector with PmRab7 insert: Lane 1; 1 kb DNA marker, Lane 2 showing RE digested vector 2.9 kb, Lane 3 showing RE digested 650 bp PmRab7 inserts.

3.2. Expression and identification of rPmRab7 protein

In order to induce the protein in *E. coli Rosetta gami pLysS* cells at 37 $^{\circ}$ C, isopropyl β -D-1-thiogalactopyranoside 0.1 mmol/L under 6 hrs of incubation, also to avoid inclusion body formation, PmRab7 fusion protein was observed in comassive blue stained SDS gel as a 26 kDa band (Figure 2a, b). The soluble fraction was observed on SDS page after using detergent in lysis buffer, rPmRab7 confirmed on the nitro-cellulose paper of western blot (Figure 2c).

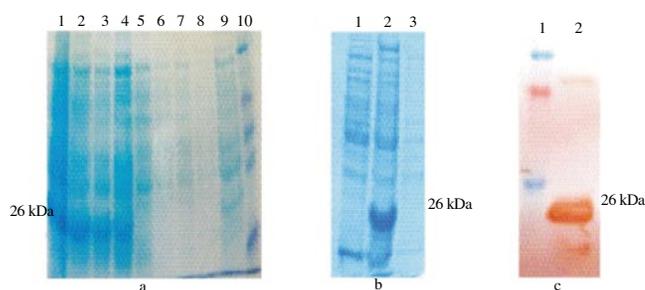


Figure 2. a: PmRab7 protein expression showing Lane 1, 2, 3 and 4 with induce pellet of heavy expression band of 26 kDa, Lane 5 showing control pellet, Lane 6, 7 and 8 induce supernatant, Lane 9 showing control supernatant and Lane 10 containing the protein marker; **b:** IPTG induction of PmRab7 on 37 $^{\circ}$ C at 6 h, showing expression band; **c:** Western blot; Lane 1 showing protein marker and Lane 2 showing recombinant expressed protein (26 kDa).

3.3. Purification and antibody production of rPmRab7 and western blotting of PmRab7 antiserum with sub-cellular lysosomal isolates

Purification of recombinant PmRab7 was also analyzed on SDS page (Figure 3a). His-Tag affinity in immobilized metal affinity chromatography was highly purified, minutes quality of impurities was removed by gel filtration Acta purifier, specific antibody production were shown highly pure light and heavy chain on SDS (Figure 3b).

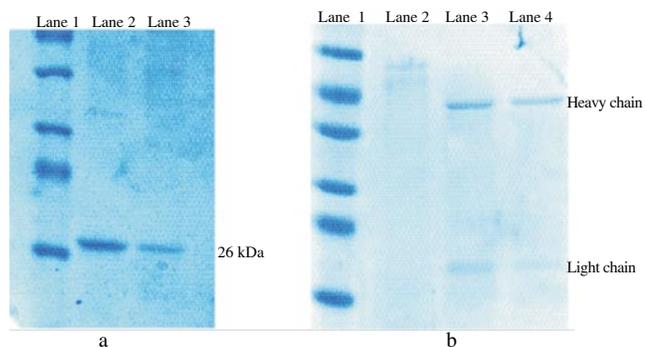


Figure 3. a: SDS image of purified PmRab7 (lane 1 marker, lane 2 and 3 purified PmRab7); **b:** SDS image of antibody raised against rPmRab7 (lane 1 protein marker, lane 2 negative control, lane 3 and 4 light chain and heavy chain of antibody).

Binding of recombinant PmRab7 (26 kDa) with partially purified WSSV (vP-28), binding of anti-PmRab7 antibody with lysosomal

fraction was clearly shown by western blot analysis (Figure 4a), and presence of wild type PmRab7 in the lysosomal fraction was also observed during western blot analysis (Figure 4b).

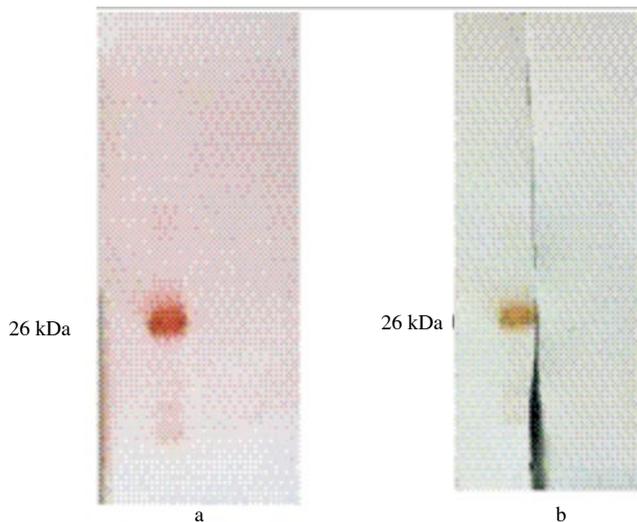


Figure 4. Western blot image of lysosomal PmRab7 with specific antibody.

3.4. WSSV copy number in lymphoid organ after infection

For each run, a standard curve was generated from samples of purified cloned WSSV plasmid ranging from 2.4×10^{-3} copies. The results were expressed as the mean copy number of WSSV per nanogram of total DNA. Quantification and copy number determination of WSSV using real-time PCR were carried out upon WSSV infection of lymphoid cells at different time-points. The viral load in WSSV showed more than fivefold increase after 24 h post-infection compared to 2 h post-infection and more than 20 fold higher after 72 h of infection when exposed to a diluted WSSV inoculum in the ratio 1:10 (Figure 5).

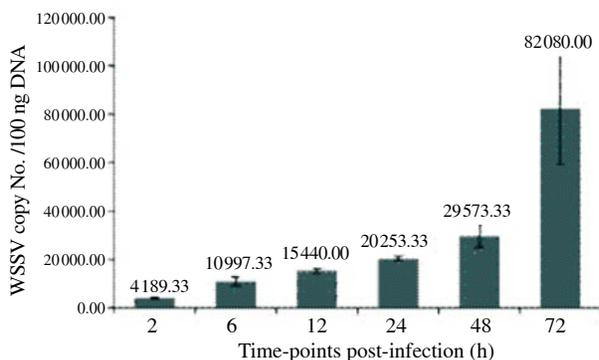


Figure 5. WSSV copy number in *P. monodon* lymphoid tissue from 2 to 72 h of infection.

3.5. Relative quantification of PmRab7 after WSSV challenge

Optimum amplification of PmRab7 by varying the annealing temperature of primers from 45 °C to 58 °C, the highest expression of PmRab7 mRNA was in 72 h of infection, and to lesser in 2 h

of infection. Significantly all-time point has shown up-regulation of PmRab7 after infection, minimum in 2 fold increase and significantly increased up to 8 fold in 72 h infection (Figure 6). The differential gene expression of PmRab7 with IFN1 transcript as endogenous reference (housekeeping gene) were obtained. The PmRab7 mRNA transcript expression level increased when the specimen was infected with WSSV Ct = 1.0 to Ct = 8.5. So this result strongly suggests that WSSV enable to induce expression of PmRab7 in *P. monodon* with increasing WSSV infection at certain time points and also simultaneously increases the expression of PmRab7.

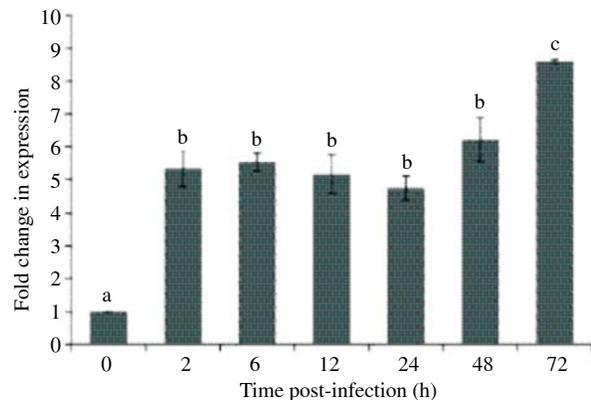


Figure 6. Regulation of PmRab7 gene, differential expression in lymphoid tissue of *P. monodon* after WSSV infection under the reference of IFN α gene from 0 to 72 h.

4. Discussion

WSSV has been emerged as the most prevalent devastating pathogen and resulted in a considerable loss in the shrimp industry. In order to understand WSSV infection in the shrimp, several researchers attempted to investigate WSSV genomic structure and its mechanism of infection during the spread of diseases. However, still there is a lack of adequate information with regards to mode of infection of WSSV into host cells. Here, we have attempted to localize PmRab7 protein on lysosome and check regulation of the PmRab7 gene in lymphoid tissues sub-cellular fraction of *P. monodon* upon WSSV infection using SDS, western blotting and qPCR.

Several studies have attempted to demonstrate WSSV infection into shrimp host cells. The WSSV consist of around 35 viral envelope proteins, among that it has shown that, VP28 and VP26 are being most abundant (consisting of about 60%). Studies suggested that VP28 is the major envelope protein playing vital role in the WSSV infection. Using different molecular approaches it has reported that VP28 is involved in the process of binding virus to host cells of shrimp and it may contribute to the recognition of receptors at the cell surface of the shrimp during infection.

Further, Rab proteins were identified and revealed their role during viral infection in several organisms. Similarly, PmRab7 might be receptor for VP28, which is reducing mortality of shrimp

after WSSV challenge[24]. In earlier studies, it has also been shown that WSSV-binding activity with the expressed rPmRab7 where Rab7 plays a vital role in the trafficking of endocytosis vesicles within the cell, subsequent fusion of early endosome to late endosome and further in lysosome. Anti-VP28 antibody mediated neutralization had significantly revealed that, VP28 antibody boosting the life span of shrimp[5], and shown mortality reduction by using recombinant VP28, in response to the WSSV challenge[25].

PmRab7 gene with housekeeping elongation factor 1-alpha gene[26] has shown significantly up-regulated with certain fold changes in the lymphoid tissues with the post WSSV infection till 72 h of post infection. Interestingly, it has reported no significant fold changes found in the PmRab7 expression after WSSV challenge in the earlier study, while it has also been reported that significant upregulation of all GTPase family of gene after WSSV internalization by subtractive hybridization in *Penaeus japonicus*[27]. The role of Rab7 during the viral infection leads to internalized virus via catherin dependent endocytosis process of all most enveloped virus and are trafficked through early endosome to late endosome/lysosome[28]. Mechanism to release viral particle from lysosome is still unknown, some hypothesis suggest that it might be due to lysis or leakage of the endosomal compartment[29]. This result clearly indicates that, the localization of PmRab7 on lysosomal compartment and binding of PmRab7 with WSSV, hence WSSV may be involved in endocytosis process for internalization.

Expression of dominant negative Rab7 prevents the infection of the influenza virus, whereas over expression of wild type Rab7 could not able to show any strong evidence in virus entry. Similarly in case of Semliki forest virus and vesicular stomatitis virus expression of dominant negative Rab7 does not show any potentiality during viral entries into cells[30].

Transcriptional upregulation of the Rab7 during mycoplasma infection accumulating autophagic flux by purging endocytic membrane with autophagosome marker LC3-11 and cargo protein p62 during lysosomal degradation process suppressed by mycoplasma infection[31]. In the present study we have successfully confirmed the transcriptional upregulation of the PmRab7 during WSSV infection, same as in mycoplasma infection. It was in line with earlier studies, which shown Rab7 upregulated transcriptionally and other cell molecule like LC3-11 and p62 upregulating by post translation mechanism, it was resulted due to inhibition of autophagic flux[32,33]. PmRab7 was upregulated by WSSV infection during the entry of certain enveloped viruses into host cells[30]. This is because of the overexpression of Rab7 which has been found in acceleration of few cargo molecules on endosome and lysosome[34]. Rab7 and late endosome plays a crucial role in antigen processing and presentation in the major histocompatibility complex class II pathways. Its upregulation in B-cells during stimulation with lipopolysaccharide or CD40 increases the effectiveness of antigen presentation in B cells with the help of other cellular endocytotic components[11,35]. It has

also reported that the up-regulation of Rab GDIa during brain differentiation before pre-synaptic activity in mouse playing crucial role in brain development disease[36,37].

In conclusion, we have successfully detected the presence of PmRab7 on the lysosomal compartment and which helps to transport WSSV inside the host cells. Expression profiling of PmRab7 in lymphoid tissue of *P. monodon* by real time PCR against WSSV infection after different time points clearly indicated the WSSV internalization in the lymphoid organ. The present study also showed significant change in PmRab7 expression among different time point of infection. Therefore, PmRab7 could be utilized as a target gene for defeating WSSV infection in the shrimp industry in future.

Conflict of interest statement

We declare that we have no conflict of interest.

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