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ORIGINAL RESEARCH

Production and purification of polyclonal antibody against shrimp lectin LvLTLC1

Phuong-Thao Thi Nguyen . Quoc-Gia Mai . Thuoc Linh Tran . Hieu Tran-Van

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Abstract Lectins play an important role in the shrimp immune response owing to the ability of recognising and eliminating invasive microorganisms. LvLTLC1, a recently discovered lectin, is able to resist the effects of *Vibrio parahaemolyticus* causing Acute Hepatopancreatic Necrosis Disease (AHPND) by its potential bacteria clearance of *Vibrio* spp. *in vivo*. In this research, the preparation of LvLTLC1 antigen by expressing and purifying the His-tagged recombinant protein, and production of LvLTLC1-specific mouse polyclonal antibodies were conducted. The produced mouse polyclonal antibodies were then used to determine the antiserum or purified antibodies titer by indirect ELISA and detect the LvLTLC1 expression in hepatopancreas tissues of both AHPND-infected and uninfected shrimps by SDS-PAGE and Western blot analysis, respectively. Results showed that LvLTLC1 expression in hepatopancreas tissues of AHPND-infected shrimps was significantly higher than that of healthy shrimps. Collectively, we reported for the first time the generation and evaluation of LvLTLC1-specific mouse antibody as a tool for further studies on understanding the nature of immune response in shrimp and developing immune-based applications.

Keywords Acute Hepatopancreatic Necrosis Disease . Litopenaeus vannamei . LvLTLC1 . Polyclonal antibody . Western blot

Introduction

Being the second shrimp-export country in the world with a turnover of billions of dollars each year, the farmed-shrimp industry in Vietnam has been growing rapidly in both acreage and quantity. This position, however, is now facing many challenges including weather, climate change, and diseases. The latter is the leading cause for the industry loss. Among them, Acute Hepatopancreatic Necrosis Disease (AHPND) has recently emerged as a potential threat not only for Vietnam's farmed-shrimp industry but also for global ones. Particularly, AHPND has caused the loss of hundreds of billion dollars in relation to 46,093-hectare damage (Vietnam Association of Seafood Exporters and Producers 2018).

Similar to other invertebrates, shrimp relies on innate immunity to protect them from invaders (OIE 2019). A large number of molecules called pattern recognition receptors (PRRs) are capable of recognizing a wide range of pathogen-associated molecular patterns (PAMPs) (Li et al. 2014), such as the lipopolysaccharides of Gram-negative bacteria, and triggering a series of reactions to fight infectious agents (Xu et al. 2014). Since lectin is a protein that can bind to glycoprotein or glycolipid on the microbial surface by non-covalent

Phuong-Thao Thi Nguyen
Department of Molecular and Environmental Biotechnology, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, Vietnam; Vietnam National University, Ho Chi Minh City, Vietnam; Tien Giang University, Tien Giang Province, Vietnam

Quoc-Gia Mai . Thuoc Linh Tran . Hieu Tran-Van(✉)
Department of Molecular and Environmental Biotechnology, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, Vietnam; Laboratory of Biosensors, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, Vietnam; Vietnam National University, Ho Chi Minh City, Vietnam
e-mail: tvhieu@hcmus.edu.vn

bonds with carbohydrates without altering the carbohydrate structure (Iwanaga et al. 2005), it is particularly classified as PRR. Lectin from shrimp has been shown to participate in a wide range of innate immune responses including pathogen identification, bacterial agglutination, microbicide and antivirus, enhanced opsonization, and cell encapsulation (Nyholm SV et al. 2012; Wongpanya et al. 2017; Liang et al. 2019). The discovery of new lectins from shrimp is crucial for developing tools and strategies for prevention and treatment of diseases in farmed shrimp.

Although there have been several lectins identified in shrimp species including C-type, M-type, L-type, P-type, fibrinogen-like domain lectins, calreticulin/calnexin, the majority of shrimp lectin researches has only focused on characterizing C-type lectin. This prompts a need for an insight of other lectins on the understanding of shrimp immune response. With the exception of the newly identified LvLTLC1 from *L. vannamei,* and tiger prawn *Marsupenaeus japonicus* (Xu et al. 2014) is the only known L-type lectin has been characterized.

L-type lectins were found to interact with N-glycans of glycoproteins by a domain-recognizing luminal carbohydrate (Kamiya et al. 2008). Recently, a new L-type lectin, named LvLTLC1, has been proven to provide the immunity support on cultured shrimp contracted with some bacteria including *V. parahaemolyticus*, the causative agent of AHPND in shrimp. LvLTLC1, therefore, has demonstrated its potential in *Vibrio* spp. clearance *in vivo*, and could provide an option for AHPND treatments (Tian et al. 2017). Therefore, there is an unmet need for LvLTLC1-specific antibodies to study the nature of immune response against pathogens in shrimp and to develop immune-based methods such as ELISA or Western blot to deduce the pathogenicity. However, there are no LvLTLC1-specific antibodies commercially available.

In this research, the preparation of LvLTLC1 antigens by expressing and purifying the His-tagged recombinant LvLTLC1 proteins (Nguyen et al. 2020), and production of LvLTLC1-specific mouse polyclonal antibodies were described. The produced mouse polyclonal antibodies were then used to detect LvLTLC1 expression in hepatopancreas tissues of both AHPND-infected and uninfected shrimps.

Materials and methods

Materials and strain

Both naturally AHPND-infected and uninfected white leg shrimps *L. vannamei* collected from farms in Tien Giang Province, Vietnam were used for extraction of LvLTLC1 and identification of its tissue distribution. The AHPND status was confirmed using PCR and kindly provided by Mai-Hoang et al. (2021). The recombinant *E. coli* pET-LvLTLC1 plasmid BL21(DE3) strain (Nguyen et al. 2020) was used to express the recombinant LvLTLC1 protein.

LvLTLC1 antigens preparation

Optimized temperature and concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) (Biobasic, UK) were gained via survey to enhance the expression of LvLTLC1 protein synthesized by transforming the pET-LvLTLC1 plasmid into *E. coli* strains BL21(DE3) (data not shown). The final soluble recombinant protein was induced without IPTG for 20 hours at 16˚C (in Luria Bertani Broth-Ampicillin (LB-Amp) supplemented with 2% ethanol) (Nguyen et al. 2020). Centrifugation at 6,000 rpm for 5 minutes was applied to obtain the biomass, which was then resuspended in lysis buffer (0.5 M NaCl, 0.02 M NaH_2PO_4, 0.025 M imidazole, 2 mM dithiothreitol, 10% glycerol, and 1.5% Triton X-100) and disrupted by sonication (Microson Misonix incorporation, USA). The supernatant was collected through centrifuging at 10,000 rpm for 30 minutes at 4°C prior to transferring into a 5 ml His-Trap column connected with the FPLC (ÄKTA, GE Healthcare, USA) for recombinant LvLTLC1 protein purification. The binding buffer (0.5 M NaCl, 0.02 M NaH_2PO_4, 0.025 M imidazole, 2 mM dithiothreitol, 10% glycerol, and 1.5% Triton X-100) was used for washing step prior to eluting the recombinant LvLTLC1 proteins by binding buffer supplemented with 0.5M imidazole (Nguyen et al. 2020). The quality of eluted protein fractions after purification was confirmed by 15% SDS-PAGE gel electrophoresis and analyzed by Gel-Pro Analyzer. The Bradford protein assay was used to determine the concentration of total protein.

Production of LvLTLC1-specific mouse polyclonal antibody

Animals were maintained in the experimental animal facility, and experiments were performed in accordance with the guideline approved by The Animal Care and Use Committee of University of Science in Ho Chi Minh City. Three male Swiss mice, whose weight ranged from 20 to 25 grams, were acclimated for 2 to 4 days. A solution consists of 100 µg purified LvLTLC1 recombinant protein (per mouse) previously dissolved in 200 µl phosphate-buffered saline (PBS) buffer, then emulsified with complete Freund's adjuvant (Santa Cruz, USA) at a ratio of 1:1 before injecting in the mice. During the next four weeks, mice were repeatedly immunized each week with 50 µg LvLTLC1 protein emulsified in incomplete Freund's adjuvant (Santa Cruz, USA). Mouse blood samples were collected seven days after the last injection, and antiserum was obtained by centrifugation at 3,500 rpm for 5 minutes at 4°C. In addition, prior to the immunization scheme, blood samples were collected and used as a negative control for indirect ELISA analysis.

Determination of antiserum or purified antibodies titer by indirect ELISA analysis

Firstly, purified LvLTLC1 antigen were diluted to 20 µg/ml in the coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 250 mM NaCl, 0.02% NaN_3, pH 9.5). Secondly, prepared antigen was coated overnight at 25°C on a 96-well ELISA plate with 100 µl per well. After that, the plate was washed three times with washing buffer (PBS supplemented with 0,1% Tween-20) and blocked with 200 µL of blocking buffer (3% bovine serum albumin BSA in PBST buffer), then incubated for 1 hour at 25°C. Subsequently, 100 µl of two-fold serially diluted mouse anti-LvLTLC1 serum or purified antibodies (1:500 to 1:256,000) was added to all wells and incubated at 25 °C for 3 hours prior to washing five times with a washing buffer. Then, incubating the plate with anti-mouse IgG conjugated horseradish peroxidase (HRP) (dilution 1:10,000). 3,3′,5,5′-Tetramethylbenzidine (TMB) conversion was detected by the visible blue-colored solution on the wells. The reaction was terminated by 50 µl of stop solution (2N HCl) after 30 minutes of development. Finally, the plate was analyzed on a microplate reader of 450 nm (Thermo Scientific, USA).

Purification of LvLTLC1-specific mouse polyclonal antibody

The antiserum was then purified by HiTrap rProtein A FF column (GE Healthcare, USA). The antibody purification was proceeded following the instruction manual. The antibody purity and concentration were determined using SDS-PAGE, Gel Pro Analyzer, and Bradford assay.

Extraction and tissue distribution of LvLTLC1

Collection of hepatopancreas, stomach, gills, heart, walking leg, and intestine tissues (5mg per sample) from healthy *L. vannamei* were frozen and homogenized in 300 µl of ice-cold lysis buffer (150mM NaCl, 1% Triton X-100, 0.1% SDS, 50mM Tris-HCl pH 8). During homogenization, a 300-600 µl lysis buffer was added and the mixture was continuously agitated for 2 hours at 4°C. Subsequently, the homogenate was centrifuged at 13,000 rpm for 30 minutes at 4°C. The obtained supernatant was placed on ice for the next steps.

Total lysate proteins were processed and separated by SDS-PAGE on 15% gel prior to immunoblotting. The transferred membrane was blocked with a blocking buffer (0.5% BSA (w/v) in PBST buffer) for 1 h and then incubated with LvLTLC1-specific mouse polyclonal antibody for 3 hours at 25°C. Next, the washing step was carried out five times with PBST buffer and the membrane was incubated for 3 hours at 25°C with anti-mouse IgG conjugated HRP. Primary and secondary antibodies were added at dilutions of 1:500 and 1:15,000, respectively. Later, repeating the washing step five times with PBST buffer and the targeted bands were detected by TMB solution (Thermo Scientific, USA).

Results and discussion

LvLTLC1 antigens preparation

LvLTLC1 antigens obtained from the soluble fraction were subjected to immobilized metal ion affinity

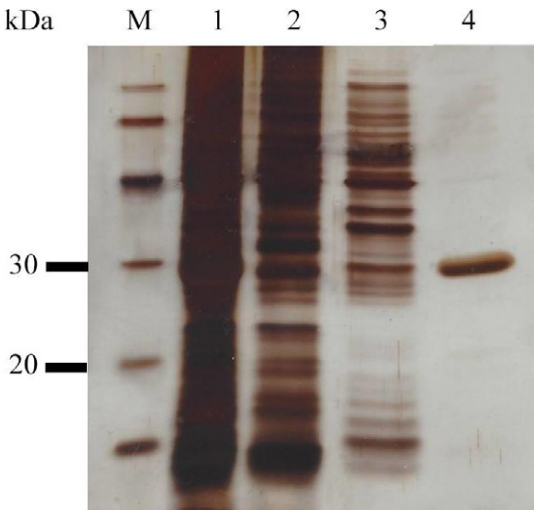

Fig. 1 Purification of LvLTLC1 antigens. M = protein marker, 1 = total protein samples before purification, 2 = flow-through sample, 3 = washing sample, 4 = elution samples

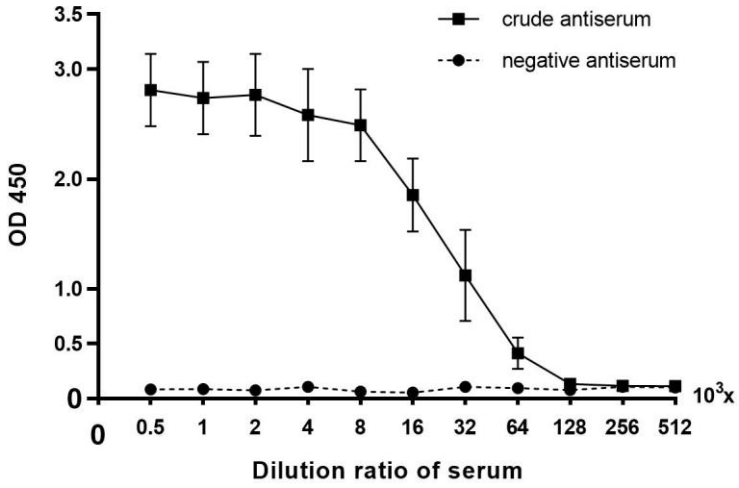

Fig. 2 Crude antiserum titration by indirect ELISA

chromatography (IMAC) to purify the target protein. Due to being fused to the 6x His-tag, LvLTLC1 protein had a high affinity with Ni^{2+} in the His-Trap column. Unbound proteins were discarded through the column, while weakly bound proteins were washed using low-concentration imidazole solution, leaving only His-tag fused proteins bound. Finally, the target protein was eluted from the column by an adequate imidazole elution buffer. Due to weak or no interaction with Ni^{2+}, the majority of protein in the soluble fraction flowed out of the column (Fig. 1, lane 2). Unwanted binding proteins were washed with imidazole solution with a concentration nearly as high as the elution buffer, hence a small amount of LvLTLC1 was washed out as well. However, this step was crucial to ensure the purity of the target protein (Fig. 1, lane 3). Despite the small amount of impurities, the purity of collected LvLTLC1 reached 92%. Therefore, it could conclude that the protein was purified (Fig. 1, lane 4).

Determination of antiserum titer by indirect ELISA analysis

Antiserum titer was determined by measuring O.D. at 450 nm (OD_{450nm}) with high OD_{450nm} value reflected strong antiserum-antigen reactions while low value indicated insignificant interactions. The OD values of antiserums and pre-immunized mouse serum dilutions were shown in Fig. 2 The OD_{450nm} result of post-immunized mice was significantly higher than compared to the pre-immunized samples, and the value

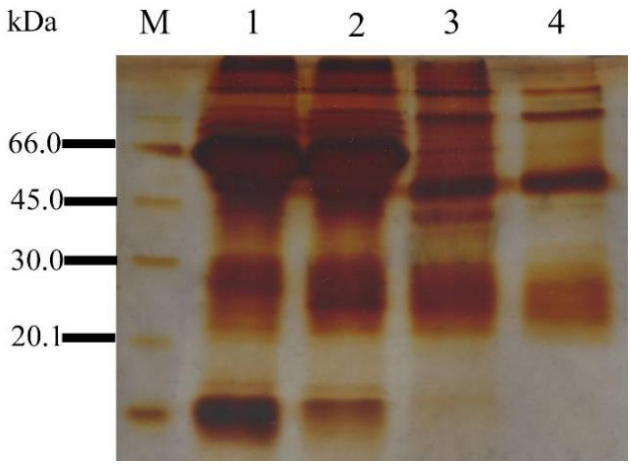

Fig. 3 Purification of LvLTLC1-specific mouse polyclonal antibodies. M = low molecular weight protein marker, 1 = protein samples before loading, 2 = flow-through sample, 3 = washing sample, 4 = elution samples

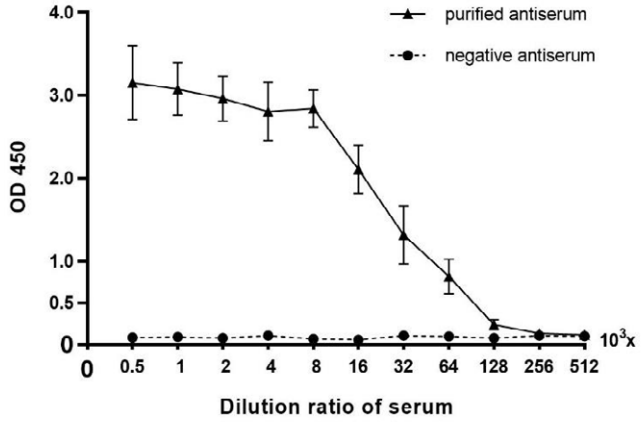

Fig. 4 Purified antiserum titration by indirect ELISA

gradually dropped in each dilution. Meanwhile, the OD_{450nm} level in pre-immunized mice was almost zero, and there was no fluctuation when increasing the dilution. The results indicated that LvLTLC1-specific mouse polyclonal antibodies in antiserum were able to specifically react with LvLTLC1 antigens while the negative control serum gave no significant signal. The titer of LvLTLC1-specific mouse polyclonal antibodies was calculated at 128,000 indicating that the polyclonal antibodies were highly sensitive to LvLTLC1 antigen.

Purification of LvLTLC1-specific mouse polyclonal antibodies

After collecting, there were still many impurities in the serum, which required a further purification step to achieve higher purity for efficient subsequent experiments. As antibodies bind to protein A through the Fc region; in this study, the polyclonal antibodies were purified by affinity chromatography with HiTrap rProtein A FF column. It could be observed that many impurities were removed at the sampling (Fig. 3, lane 2) and column washing (lane 3) steps. Thereby, after the purification step with protein A affinity chromatography column, protein impurities were removed significantly. Moreover, in lane 4 (protein in elution sample) there were two protein bands at the 45-66 kDa and 20.1 to 30 kDa bands. These two protein bands were sized corresponding to the heavy chains (about 50 kDa) and light chains (25 to 30 kDa) of antibodies. When compared with pre-purified antiserum, a large portion of contaminants was discarded, thereby enhancing the purity of the antiserum. This allowed concluding that polyclonal antibodies from mice were captured and purified.

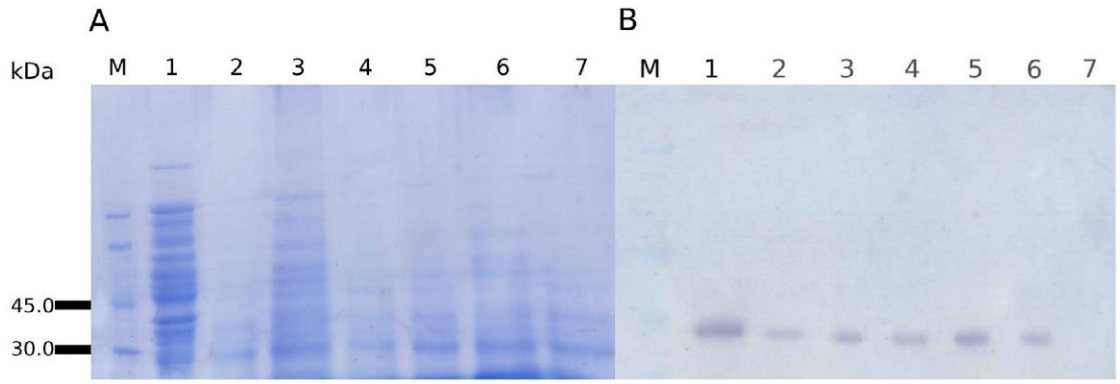

Fig. 5 Tissue distribution of LvLTLC1. (A) SDS-PAGE. (B) Western blot, M = low molecular weight protein marker, 1 = recombinant LvLTLC1, 2 = stomach, 3 = heart, 4 = intestine, 5 = gills, 6 = hepatopancreas, 7 = walking leg tissue samples

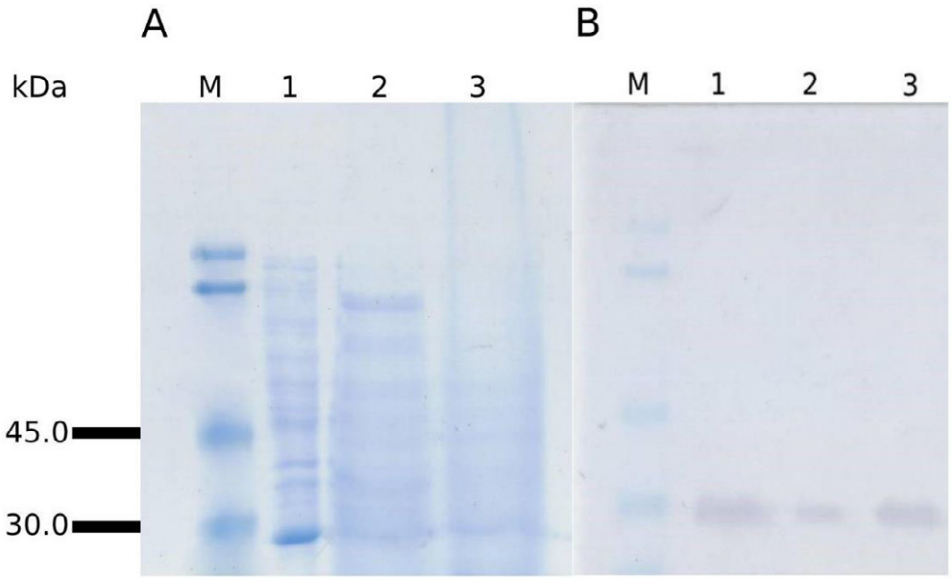

Fig. 6 Total protein of hepatopancreas tissues of AHPND-infected shrimps was analyzed by SDS-PAGE (A) and Western blot (B). M = low molecular weight protein marker, 1 = recombinant LvLTLC1, 2 = hepatopancreas of healthy shrimp, 3 = hepatopancreas of AHPND-infected shrimp

Determination of the purified antibody titer

The titer of the purified antibody was confirmed by the described method. Based on the obtained ELISA results, the OD values were significantly improved in comparison with the previous section as the antibody titer increased to 256,000. Obviously, the purified antibody would have been more effective in binding with the LvLTLC1 antigen. For that reason, the purified antibody would be used in the subsequent study on Western blot of LvLTLC1.

Extraction and tissue distribution of LvLTLC1

Polyclonal anti-LvLTLC1 antibodies were used to detect the protein in different organs. Protein was retrieved from these organs with the method as described, and subsequent SDS-PAGE and Western blot steps were performed. The SDS-PAGE result showed the presence of a 30-kDa band, equivalent to the recombinant LvLTLC1 mass. Also, the same size could be observed in almost all tissues tested except for walking legs. The Western blot result basically confirmed the prediction that these bands were shrimp LvLTLC1 with the expression level differed from organ to organ. Particularly, LvLTLC1 protein extraction was higher in gills, hepatopancreas, and intestines in comparison with stomach, heart, and intestine tissues (Fig. 5).

The high concentrations of LvLTLC1 protein expression in gills and especially hepatopancreas, along with its potential bacteria clearance of *Vibrio* spp. in vivo (Tian et al. 2017), prompt us to test the modulation of LvLTLC1 lectin expression in AHPND-infected shrimps. Therefore, hepatopancreas tissues of AHPND-infected shrimps were collected by the same method. This mixture was then extracted and evaluated for expression level of the LvLTLC1 targeted protein in comparison to healthy shrimp hepatopancreas tissues. Data on the SDS-PAGE and Western blot analysis showed LvLTLC1 expressing in hepatopancreas tissues of AHPND-infected shrimps was significantly higher than that of healthy shrimps.

Compared with previous publications, in the study by Ji PF et al., which demonstrated that lectin increased expression when Litopenaeus vannamei were immunized by lipopolysaccharides, the structural component of the outer membrane of Gram-negative bacteria in general and *Vibrio* spp. in particular, thus the results of this study were in line with the published data (Ji PF et al. 2009). It was worth noting that the lectin studied by Ji PF et al. was a C-type lectin, which is a well-known and long-discovered lectin rather than an L-type lectin. Therefore, the results provided more evidence for a new type of hepatopancreas-produced lectin played an important role in the shrimp immune response when it encounters pathogens. However, further researches are needed to warrant in detail the molecular mechanisms of LvLTLC1 in the *L. vannamei* immune response to pathogens, especially in shrimps infected with AHPND.

Conclusions

This research has purified the recombinant LvLTLC1 protein with 92% purity in order to produce polyclonal anti-LvLTLC1 antibodies. In addition, the titer of the purified polyclonal antibodies was 256,000 indicating that it was highly sensitive to LvLTLC1 antigen. Moreover, as can be observed on the SDS-PAGE and Western blot analysis, LvLTLC1 expression in hepatopancreas tissues of AHPND-infected shrimps was significantly higher than that of healthy shrimps. To our knowledge, this work documented for the first time the production of polyclonal antibodies against LvLTLC1.

List of abbreviations

AHPND	Acute Hepatopancreatic Necrosis Disease
PRRs	Pattern Recognition Receptors
PAMPs	Pathogen Associated Molecular Patterns
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB-Amp	Luria Bertani Broth-Ampicillin LB-Amp
PBS	Phosphate Buffered Saline
HRP	Horseradish Peroxidase
TMB	3,3′,5,5′-Tetramethylbenzidine

Conflict of interest The authors declare that they have no conflict of interest.

Authors' contributions PTTN and HTV carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. QGM carried out the immunoassays. HTV participated in the sequence alignment. TLT participated in the design of the study and performed the statistical analysis. HTV and PTTN conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

Iwanaga S, Bok LL (2005) Recent advances in the innate immunity of invertebrate animals. Int J Biochem Mol Biol 39(2):113. doi: 10.5483/bmbrep.2005.38.2.128

Kamiya Y, Kamiya D, Yamamoto K, Nyfeler B, Hauri HP (2008) Molecular basis of sugar recognition by the human L-type lectins ERGIC-53, VIPL, and VIP36. J Biol Chem 283(4):1857-61. doi:10.1074/jbc.m709384200

Ji PF, Yao CL, Wang ZY (2009) Immune response and gene expression in shrimp (*Litopenaeus vannamei*) hemocytes and hepatopancreas against some pathogen-associated molecular patterns. Fish Shellfish Immunol 27(4):563-570. doi:10.1016/j.fsi.2009.08.001

Li M, Li C, Ma C, Li H, Zuo H, Weng S, Chen X, Zeng D, He J, Xu X (2014) Identification of a C-type lectin with antiviral and antibacterial activity from pacific white shrimp *Litopenaeus vannamei*. Dev Comp Immunol 46(2): 231-240. doi:10.1016/j.dci.2014.04.014

Liang Z, Yang L, Zheng J, Zuo H, Weng S, He J, Xu X (2019) A low-density lipoprotein receptor (LDLR) class A domain-containing C-type lectin from *Litopenaeus vannamei* plays opposite roles in antibacterial and antiviral responses. Dev Comp Immunol 92:29-

34. doi:10.1016/j.dci.2018.11.002
Mai-Hoang TD, Tien HL, Chau-Hoang HM, Nguyen-Phuoc KH, Pham HQ, Tran TL, Tran-Van H. (2021) A novel PCR method for simultaneously detecting Acute hepatopancreatic Necrosis Disease (AHPND) and mutant-AHPND in shrimp. Aquaculture 534:736336. doi: doi:10.1016/j.aquaculture.2020.73
Nguyen PTT, Nguyen LVH, Nguyen TT, Nguyen KLT, Tran-Van H (2020) Cloning, Expression and Purification of *Vibrio parahaemolyticus* L-type Lectin from White Leg Shrimp *Litopenaeus vannamei* for Bacterial Agglutinating. SSR Inst Int J Life Sci 6(3):2544-2551. doi:10.21276/SSR-IIJLS.2020.6.3.3
Nyholm SV, Graf J (2012) Knowing your friends: Invertebrate innate immunity fosters beneficial bacterial symbioses. Nat Rev Microbiol 10(12):815-827. doi:10.1038/nrmicro2894
OIE (2019) General information. In: Manual of Diagnostic Tests for Aquatic Animals. https://www.oie.int/en/what we do/standards/codes and manuals/aquatic manual online access/general information. Accessed 12 Apr 2021
Tian Y, Chen T, Huang W, Luo P, Huo D, Yun L, Chaoqun H, Cai Y (2018) A new L-type lectin (LvLTLC1) from the shrimp *Litopenaeus vannamei* facilitates the clearance of *Vibrio harveyi*. Fish Shellfish Immunol 73:185-191. doi:10.1016/j.fsi.2017.12.011
Vietnam Association of Seafood Exporters and Producers (2018) Vietnam shrimps export: look back 20 years. http://seafood.vasep.com.vn/key-seafood-sectors/shrimp/news/vietnam-shrimp-exports-look-back-20-years-16016.html. Accessed 20 Mar 2021
Wongpanya R, Sengprasert P, Amparyup P, Tassanakajon A (2017) A novel C-type lectin in the black tiger shrimp *Penaeus monodon* functions as a pattern recognition receptor by binding and causing bacterial agglutination. Fish Shellfish Immunol 60:103-113. doi:10.1016/j.fsi.2016.11.042
Xu H, Yang J, Gao W, Li L, Li P, Zhang L, Gong Y, Peng X, Xi JJ, Cheng S, Wang F (2014) Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. Nature 513(7517):237-241. doi:10.1038/nature13449
Xu S, Wang L, Wang XW, Zhao YR, Bi WJ, Zhao XF, Wang JX (2014) L-Type lectin from the kuruma shrimp *Marsupenaeus japonicus* promotes hemocyte phagocytosis. Dev Comp Immunol 44(2):397-405. doi:10.1016/j.dci.2014.01.016