Upregulation of hepatopancreas associated lectin transcripts in *Macrobrachium rosenbergii* in response to *Vibrio harveyi* and *M. rosenbergii* nodavirus (MrNV)

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Abstract

Aim: The giant fresh water prawn, *Macrobrachium rosenbergii* is widely cultured in semi-intensive farming systems for commercial production. However, the major constraint to sustainable production practices include diseases due to bacterial and viral infections. The immunity related genes contribute towards prawn health by regulating the expression of antimicrobial peptides and promoting phagocytosis. In this study, we examined the expression of *Mr(T)Lec4* and *Mr(T)Lec2* associated with the hepatopancreas tissue of *M. rosenbergii* adults after challenge with *Vibrio harveyi* and *M. rosenbergii* nodavirus (MrNV).

Methodology: The transcript sequences were derived from the *M. rosenbergii* transcriptome database. Gene-specific primers were designed for qRT-PCR analysis of tissue-specific expression patterns of *Mr(T)Lec4* and *Mr(T)Lec2* in *M. rosenbergii*. Subsequently, the temporal expression of transcripts were examined in the hepatopancreas tissue following challenge of the adult prawn with bacteria *V. harveyi* and *M. rosenbergii* nodavirus (MrNV). The MrNV positive inoculum used for challenge experiments were obtained from infected post-larval stages of the prawn.

Results: The relative expression of *Mr(T)Lec4* and *Mr(T)Lec2* in the hepatopancreas showed an increase of about 23 and 28-folds, respectively, compared with other tissues. At post-*V. harveyi* challenge, the mRNA levels of *Mr(T)Lec4* in hepatopancreas was upregulated at 6 hr. Further, the expression pattern of *Mr(T)Lec4* and *Mr(T)Lec2* in hepatopancreas tissue at different time points in response to MrNV challenge was analyzed by qRT-PCR. Both the transcripts showed upregulation at 48 hrs post-challenge confirming induction after MrNV challenge.

Interpretation: This study for the first time reports involvement of hepatopancreas-associated lectin to *V. harveyi* and MrNV challenge. A direction towards its putative role in regulation of immune response of *M. rosenbergii* is been currently investigated using RNA interference and expression studies.

Key words: Antimicrobial challenge, Aquaculture, Carbohydrate-binding lectin domains, *Macrobrachium rosenbergii*, qRT-PCR

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Introduction

The status of giant fresh water prawn, *Macrobrachium rosenbergii* in the global aquaculture production is considered to be the most sustainable among the several cultivable finfish and shellfishes. There has been a moderate rise in *M. rosenbergii* production from 196,848 to 213,958 tonnes between 2004 and 2015 in many countries allowing for significant investments in the aquaculture sector (FAO, 2017). The revenue generated due to *M. rosenbergii* cultivation and processing in Asia alone touches >US \$1 billion annually (Schwantes et al., 2009). A rapid increase in global production of *M. rosenbergii* is attributed to developed socio-economic conditions, smart culture networks, and prosperous entrepreneurial ecosystems in most aquaculture-dominated countries (FAO, 2002; Ali et al., 2018). In the present context, a self-reliant economy based on sustainable freshwater prawn production seems imperative, provided viability of commercial aquaculture production continues by limiting the magnitude of disease outbreaks. Culture intensification and expansion, poor husbandry measures, transboundary movement of broodstock and post-larvae, and global climate change has brought about diseases of infectious and non-infectious aetiologies (Saurabh and Sahoo, 2008; Shailender et al., 2012; Dineshkumar et al., 2017). A thorough understanding of the host defense system through screening and characterization of genes involved in innate immune pathways is necessary towards addressing the issues to new pathogens and interactions with the host.

Lectins play dynamic role in crustacean immunity and survival by participating in cell-cell interaction, cell-signaling, protein synthesis and pathogen recognition (Denis et al., 2016; Sanchez-Salgado et al., 2017). Crustacean lectins are more dynamic as they participate in humoral defence through recognition and antibacterial activity and cellular defense through encapsulation and phagocytosis (Vazquez et al., 2009; Zhang et al., 2016). Further, diversity in ligand specificities and the ensuing effector functions can be attributed to structural differences among the lectin classes. The lectins characterized from *M. rosenbergii* include membrane-spanning L-type lectin, specifically expressed in the hepatopancreas and intestine. This participates in bacterial clearance and inhibition of white spot syndrome virus (WSSV) replication *in vivo* (Huang et al., 2018). Earlier reports have characterized gills and intestine-specific Ca^{2+} -dependent lectins (C-type lectins) that can attach to lipopolysaccharide and peptidoglycan on Gram-negative and Gram-positive bacteria, respectively (Huang et al., 2016; Feng et al., 2016). A mannose-binding lectin from *M. rosenbergii* showed bactericidal action against most of the Gram-negative bacteria and few Gram-positive bacteria conferring resistance to microbes in the host (Arockiaraj et al., 2015). *M. rosenbergii* ficolins (containing fibrinogen-related domains) with greater expression in stomach were also found to possess pattern recognition function (Zhang et al., 2014). Other C-type lectins with dual

carbohydrate-recognition domains (CRDs) have also been characterized from giant fresh water prawn with multiple binding specificities (Ren et al., 2012; Zhu et al., 2013). Considering the diversity of lectins in the species and defense responses to multiple pathogens, the repertoire thus far characterized seems limited. Further, with nodavirus (causative virus related to white-tail disease) isolates reported from fresh water prawn hatcheries and nursery ponds of India (Sahul Hameed et al., 2004), Thailand (Yoganandhan et al., 2006), Indonesia (Murwantoko et al., 2016), China (Zhang et al., 2006), Taiwan (Hsieh et al., 2006) and Australia (Owens et al., 2009) causing economic loss upto 100%, a study on the defense strategy of the host with participatory role of lectins seems significant.

In the present study, two lectin transcripts [designated as *Mr(T)Lec4* and *Mr(T)Lec2*] were studied at the level of qPCR expression analysis. The transcript sequences were retrieved from the *M. rosenbergii* hepatopancreas transcriptome database (SRX097638). Relative quantification of *Mr(T)Lec4* and *Mr(T)Lec2* in different tissues were confirmed. Subsequently, we studied the time-course expression profile of the transcripts in the host after challenge of the host with *V. harveyi* and *M. rosenbergii* nodavirus (MrNV). This study for the first time showed that hepatopancreas-expressed lectin(s) in the fresh water prawn *M. rosenbergii* responds to the Gram-negative bacteria *Vibrio harveyi* and to the viral pathogen MrNV. To substantiate the importance of this work, a status report of lectin family transcripts expressed in tissues of various crustacean species including *M. rosenbergii* based on immune challenge studies was prepared. Molecular characterization of *Mr(T)Lec4* and *Mt(T)Lec2* envisages to understand the key regulatory function of the transcripts during MrNV infection in the adult prawns and deduce the susceptibility mechanisms of the host during the post-larval stages.

Materials and Methods

Experimental animals and microorganisms: Healthy adult prawns, *M. rosenbergii* (15-17 g body weight) were obtained from local aquaculture farm (Bhubaneswar, Odisha, India) and maintained in fresh water containers under laboratory conditions. The animals were fed daily with artificial pelleted diet. Healthy post-larval stage prawns were obtained from a hatchery and screened for White-tail disease (WTD) infection by RT-PCR using published primer information (Sahul Hameed et al., 2004). These prawns were maintained in the laboratory and fed twice a day with *Artemia nauplii*. WTD-infected post-larvae with morphological identification of whitish muscle in the abdomen were collected from nearby hatcheries reporting this infection. These were used as a source for infectivity experiments after preparation and filtration of viral inoculum. The filtrate was confirmed by RT-PCR (Ravi et al., 2010) and stored at -20°C. *V. harveyi* strain 639 were inoculated into 10 ml of Tryptic soy broth (TSB) + 2.5% NaCl for overnight incubation at 30 °C. The optical density of the bacterial

suspension was adjusted to 0.1 at 625 nm [equivalent to 10^8 colony-forming units (cfu ml⁻¹)]. For infectivity experiments, serially-diluted suspension of 10^6 cfu ml⁻¹ was used.

Tissue-specific expression patterns: Tissues such as gills, muscle, hepatopancreas, stomach, and intestine were harvested from the healthy prawns ($n = 3$) and stored in RNA later stabilization solution (Thermo Fisher Scientific, MA, USA) for qRT-PCR assay. Hemolymph was drawn by directly puncturing the heart using a sterile syringe attached with a 23-gauge needle containing 1 ml of ice-cold anticoagulant Alsever's solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 4.6). The hemolymph was centrifuged at $800 \times g$ for 10 min at 4 °C to collect the hemocyte pellet. Total RNA was prepared with 100 mg of different tissues (gill, muscle, hepatopancreas, stomach, intestine and hemocytes) using HiPurA™ Total RNA Miniprep purification kit (Himedia Laboratories, Mumbai, India) according to manufacturer's protocol. The concentration and purity of total RNA were determined by measuring the absorbance at 260 nm and the ratio of optical density OD_{260nm}/OD_{280nm} using a UV-Vis Spectrophotometer (Systronics India Ltd., Ahmedabad, India). The integrity of total RNA was determined using 1% Agarose gel electrophoresis. Only RNA with purity of 1.9 to 2.1 was used for cDNA synthesis. Total RNA (4 µg/µl) was used as a template for cDNA synthesis using Hi-cDNA synthesis kit (Himedia Laboratories) according to manufacturer's instructions. Quantitative changes in *Mr(T)Lec4* and *Mr(T)Lec2* transcripts were analyzed by qRT-PCR in an QuantStudio 3.0 real-time system (Thermo Fisher Scientific) using Hi-SYBR master-mix (HiMedia Laboratories Pvt. Ltd.). PCR was conducted in a 40 cycle amplification step comprising of

denaturation at 95 °C for 15 sec, annealing at 60 °C for 15 sec, and extension at 72 °C for 1 min. *M. rosenbergii* β -actin (*Mr β -actin*) was amplified as an endogenous control (qRT-PCR Fwd 5'-AATCGTGCGTGACATCAAGG-3' and qRT-PCR Rev 5'-TCTCGTTACCGATGGTGATGAC-3'). Gene-specific primers designed for qRT-PCR amplification of *Mr(T)Lec4* and *Mr(T)Lec2* were 5'-ACGTGCTTTTCGAGTGCAATG-3' (Fwd)/ 5'-GGTTCCCAGTAGCGAGAGATAA-3' and 5'-GGTTCCCAGTAGCGAGAGATAA-3' / 5'-GAAGGGTGCTTCACATAACA-3', respectively.

Temporal expression patterns after infection with microorganisms: Hundred microliters of bacterial suspension were injected intramuscularly to third abdominal segment of adult healthy prawns. Uninjected adult acted as negative control because phosphate-buffered saline injected control (wound-related effect) did not show a significant change in expression of the transcripts compared with uninjected controls. In another experiment, MrNV and extra small virus (XSV) were prepared from WTD-infected post-larvae at 10% suspension in TN buffer (20 mM Tris-HCl and 0.4M NaCl, pH 7.4) (Ravi et al., 2010). The presence of MrNV and XSV was confirmed by RT-PCR using MrNV primers (Fwd: 5'-GCGTTATAGATGGCACAAGG-3' and Rev: 5'-AGCTGTGAACTTCCACTGG-3'; 425 bp product size) and XSV primers (Fwd: 5'-CGCGGATCCGATGAATAAGCGCATTAATAA-3' and Rev: 5'-CCGGAATTCGGTTACTGTTCGGAGTCCCAA-3'; 546 bp product size) (Fig. 1).

MrNV and XSV inoculum (100 µl per animal) was injected intramuscularly at the third abdominal segment of adult *M. rosenbergii* with non-injected prawns acting as negative control.

Fig. 1 : RT-PCR analysis of white-tail disease (WTD) infected post-larvae of freshwater prawn, *M. rosenbergii*. MrNV (*M. rosenbergii* nodavirus); XSV (Extra small virus)-like particle. M, 100 bp marker; Lane-1, Negative control (healthy *M. rosenbergii*); Lane-2, 425 bp MrNV positive control; Lane-3, 425 bp MrNV from infected post-larvae; Lane-4, Negative control (healthy *M. rosenbergii*); Lane-5, 546 bp XSV positive control; Lane-6, 546 bp XSV from infected post-larvae.

Total RNA isolated from hepatopancreas tissue of adult prawns (3 animals per time point at 6, 24, and 48 h post-injection) was converted to cDNA and amplified using qPCR as detailed earlier. As a normalizing control, *Mrβ-actin* was used. The relative expression levels were measured by the comparative Ct value method (Livak and Schmittgen, 2001). Results are expressed as mean $\pm$ SD from three independent biological replications.

Results and Discussion

Based on the lectin gene repertoire available in *M. rosenbergii* hepatopancreas transcriptome database (Zhu et al., 2013), *Mr(T)Lec4* and *Mr(T)Lec2* transcripts were identified

Fig. 2 : Tissue-specific expression analysis of *Mr(T)Lec-4* (A) and *Mr(T)Lec-2* (B). mRNA in the gill, hepatopancreas, muscle, stomach, intestine, and hemocytes of *M. rosenbergii*. Relative expression of *Mr(T)Lec-4* and *Mr(T)Lec-2* in adult tissues is shown. $\Delta\Delta C_t$ method was used to analyze tissue specificity. β -actin (*M. rosenbergii*) is used as an internal control to normalize levels between samples. Vertical bars represent standard errors ($n = 3$) $\pm$ SE. The results were statistically analyzed using one-way ANOVA and Tukey's multiple range test at 95% confidence level ($P < 0.05$). Different lowercase letters represent significant differences among groups.

showing greater expression in the hepatopancreas tissue compared with other tissues (Fig. 2).

Mr(T)Lec4 and *Mr(T)Lec2* transcripts were mostly localized to *M. rosenbergii* hepatopancreas tissue with relative expression of 23- and 28-folds, respectively. This was followed by a ~5-fold expression in the muscle tissue. Higher expression of lectin transcript (a L-type lectin; *MrVIP36*) in the hepatopancreas tissue of *M. rosenbergii* has been reported earlier (Huang et al., 2018). Surprisingly, the expression levels in hemocytes were significantly lower. Our results contradict the earlier finding where in *Marsupenaeus japonicus* C-type lectin (*MjHeCL*) showed hemocytes localization, and functioned by inhibiting the proliferation of hemolymph microbiota (Wang et al., 2014). In other tissues examined, *Mr(T)Lec4* and *Mr(T)Lec2* expression seemed considerably limited. The higher transcript abundance in hepatopancreas as compared to other tissues of *M. rosenbergii* is expected as the organ is involved in mediating strong innate immune responses in crustaceans against invading pathogens.

Fig. 3 : Temporal expression patterns of *Mr(T)Lec-4* (A) and *Mr(T)Lec-2* (B) in adult hepatopancreas tissue after *Vibrio harveyi* injection. *V. harveyi* strain 639 was injected into *M. rosenbergii* adults and samples were collected at 6 h, 24 h, and 48 hrs post-infection. Patterns of *Mr(T)Lec-4* were investigated, showing a significant increase 6 h post-injection. Uninjected adult prawns acted as the negative control. β -actin (*M. rosenbergii*) was used as an internal control. Results of three biological replications are represented with standard errors. * $P < 0.05$ (SAS, ANOVA).

Table 1 : Status survey (last five years) of lectin family transcripts expressed in tissues of *M. rosenbergii* and related genus based on immune challenge studies

Organism	Lectin(s) family transcript	Designation	Sequence features	Immune challenge	Tissues expressed	Reference
<i>Macrobrachium rosenbergii</i> (Giant freshwater prawn)	Not known	Mr(T)-Lec4 Mr(T)-Lec2	Mr(T)-Lec4: 69 bp partial ORF sequence and 177 bp 3'-UTR (overlap sequence) Mr(T)-Lec2: Not reported	<i>M. rosenbergii</i> nodavirus (MrNV), <i>Vibrio harveyi</i>	Hepatopancreas	This study
<i>M. rosenbergii</i>	L-type lectin	MrVIP36	972 bp ORF encoding 323 aa protein, L-type CRD and a TM domain, Agglutinates Gram-positive and Gram-negative bacteria, Facilitates bacterial clearance <i>in vivo</i> and inhibits WSSV replication <i>in vivo</i>	<i>Staphylococcus aureus</i> , <i>Vibrio parahaemolyticus</i> , White Spot Syndrome Virus (WSSV)	Haemocytes	Huang et al., 2018
<i>Macrobrachium nipponense</i> (Oriental river prawn)	Ficolin(s)	MnFico1 MnFico2	Agglutinates Gram-positive and Gram-negative bacteria in presence of Ca ²⁺ , Facilitates clearance of <i>V. parahaemolyticus</i> <i>in vivo</i>	<i>V. parahaemolyticus</i>	MnFico1: Gills MnFico2: Heart	Li et al., 2017
<i>M. nipponense</i>	C-type lectin	MnCTL	774 bp ORF encoding 257 aa residues, Single CRD with an EPN motif, Agglutinates Gram-positive and Gram-negative bacteria in presence of Ca ²⁺	WSSV	Gills, Stomach	Huang et al., 2017
<i>M. rosenbergii</i>	C-type lectin	MrLec	1041 bp ORF encoding 346 aa, 18 aa signal peptide and single CRD, Agglutinates Gram-positive and Gram-negative bacteria in presence of Ca ²⁺	<i>V. parahaemolyticus</i> WSSV	Gills Intestine	Feng et al., 2016
<i>M. rosenbergii</i>	C-type lectin	MrCTL	657 bp ORF encoding 218 aa protein	<i>V. parahaemolyticus</i> WSSV	Gills, Intestine	Huang et al., 2016b
<i>M. rosenbergii</i>	C-type lectin	MrLec	801 bp ORF encoding 266 aa protein, Single CRD (EPN motif), Regulation of AMP expression, Agglutinates Gram-positive and Gram-negative bacteria in presence of Ca ²⁺	<i>Vibrio haemolyticus</i>	Gills	Huang et al., 2016a
<i>M. nipponense</i>	C-type lectin	MnCTLDcp1	999 bp ORF encoding 332 aa protein, LDLa and single CTLD	<i>Aeromonas hydrophila</i> <i>S. aureus</i>	Heart Haemocytes	Xiu et al., 2016
<i>M. nipponense</i>	C-type lectin(s)	MnCTLDcp2 MnCTLDcp3	MnCTLDcp2: 972 bp ORF encoding 323 aa protein, MnCTLDcp3: 555 bp ORF encoding 184 aa protein, Single CRD (in both), LDLa domain and signal peptide in MnCTLDcp2	Bacteria	Heart	Xiu et al., 2015a
<i>M. nipponense</i>	M-type lectin L-type lectin	MnMTL1 MnLTL1	MnMTL1: 1761 bp ORF encoding 586 aa protein; type-II TM region and GH family 47 domain MnLTL1: 1744 bp ORF encoding 323 aa protein; one CRD and type I TM domain	<i>A. hydrophila</i> <i>Aeromonas veronii</i>	All tissues	Xiu et al., 2015b
<i>M. rosenbergii</i>	Mannose-binding lectin(s)	MrMBL-N20 MrMBL-C16	Not reported	Virus, Bacteria	Gills	Arockiaraj et al., 2015
<i>M. rosenbergii</i>	C-type lectin(s)	MrCTL	Two CRD domains- MrCRD1 (QEP motif); MrCRD2 (EPD motif) Agglutinates Gram-positive and Gram-negative bacteria in presence of Ca ²⁺	<i>V. parahaemolyticus</i> WSSV	Hepatopancreas	Huang et al., 2015
<i>M. rosenbergii</i>	Ficolin(s)	MrFico1 MrFico2	Agglutinates Gram-positive and Gram-negative bacteria in presence of Ca ²⁺ , Clearance of bacteria <i>in vivo</i>	<i>V. anguillarum</i> WSSV	Hepatopancreas	Zhang et al., 2014

Fig. 4 : Temporal expression patterns of *Mr(T)Lec-4* (A) and *Mr(T)Lec2* (B) in adult hepatopancreas tissue after *M. rosenbergii* nodavirus (MrNV) injection. MrNV was injected into *M. rosenbergii* adults and samples were collected at 6 h, 24 h, and 48 h post-infection. Uninjected adult prawns acted as the negative control. β -actin (*M. rosenbergii*) was used as an internal control. Results of three biological replications are represented with standard errors. * $P < 0.05$ (SAS, ANOVA).

This is ensured through its diverse lectin profiles (Cerenius et al., 2010; Du et al., 2013). The results are consistent to the observed transcript abundance in hepatopancreas, stomach and intestine of the crustacean, *Eriocheir sinensis* (Jin et al., 2013). Highest expression of lectin transcripts in hepatopancreas compared to other tissues were also recorded from the mud crab, *Scylla paramamosain* lectin isoforms (Jiang et al., 2012).

We were interested to understand the expression profiles of *Mr(T)Lec4* and *Mr(T)Lec2* in the *V. harveyi* and MrNV challenged hepatopancreas tissue of adult *M. rosenbergii*. A literature survey of last five years using PubMed with the search string 'crustacean lectins' (<https://www.ncbi.nlm.nih.gov/pubmed/?term=crustacean+lectins>) identified more than 40 references with immune challenge experiments. These included immunity studies in *M. rosenbergii* infected with Gram-positive bacteria *S. aureus*, Gram-negative *Vibrio* species like *V. haemolyticus*, *V. parahaemolyticus*, *V. anguillarum*, and the WSSV. The status survey report of lectin family transcripts expressed in various species of *Macrobrachium* genus with

reference to immune challenge studies has been succinctly presented in Table-1. The survey suggested no studies concerning *M. rosenbergii* interactions with pathogenic MrNV and *V. harveyi*. With no potent anti-MrNV agents developed till date to prevent or control the pathogenicity of virus, understanding the requirement of lectins as an inhibitor for the development of viral pathogenicity would provide useful leads for antiviral discovery. Considering the future perspectives, this study was considered purposeful.

qRT-PCR experiments showed that *Mr(T)Lec4* transcript level was upregulated ($P < 0.05$, relative to the uninjected adult prawns) at 6 hrs (1.83 fold) and subsequently down-regulated at 12 hrs and 24 hrs upon infection by the pathogenic Gram negative bacterium, *V. harveyi* (Fig. 3).

No induction was noticed in the mRNA expression profile of *Mr(T)Lec2* upon *V. harveyi* infection. In fact, the expression of transcript was significantly down-regulated during the time-course of bacterial challenge. In another study, the expression of *Marsupenaeus japonicus* C-type lectin (MjHeCL) remained unaffected by *V. harveyi* or *V. anguillarum* challenge (Wang et al., 2014). Contrary to the results obtained for *Mr(T)Lec2*, the C-type lectin transcripts (C-type; dual carbohydrate recognition domain type) of *M. rosenbergii* challenged with *V. anguillarum* showed an upregulation (Ren et al., 2012). Further, a mannose-receptor was found to be induced upon *V. anguillarum* infection in red swamp crayfish, *Procambrus clarkii* (Man et al., 2018). In this context, it will be interesting to note the promiscuity of *Mr(T)Lec4* and *Mr(T)Lec2* identified in this study with homologous sequences available in the databases.

MrNV challenge also increased the expression of *Mr(T)Lec4* in adult *M. rosenbergii* (compared with uninjected group) (Fig. 4). Post-MrNV challenge, at 6 hr, the expression of transcript was down-regulated, but upregulated at 48 hr ($P < 0.05$, relative to uninjected control group) to ~2.06 fold. MrNV challenge also significantly increased the expression of *Mr(T)Lec2* transcripts to 3.9 fold at 48 hr time-course.

Recognition of virus by multiple species of lectins could attribute to the strong immune eliciting capability of the virus in the host. Considering the resistance of the adult *M. rosenbergii* to MrNV and its clearance after 25 days' post-infection (Sarathi et al., 2008), the present study would be significant towards understanding the lectin repertoire in conferring innate immunity. Although functional studies on the same are forthcoming, the present study will shed focus on the correlation of lectins with antiviral immune response. This will be critical to study the post-larval stages of *M. rosenbergii* known to be more susceptible to virus injury and subsequent mortality. As the exact mechanism of MrNV clearance is still unclear in most crustaceans, including prawns and shrimps, the present report on *M. rosenbergii* is crucial in the perspective of host-pathogen interactions.

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