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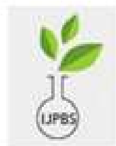
Research Article **Immunobiology**



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EXPRESSION OF TOLL PROTEIN GENE IN TISSUES OF TIGER SHRIMP (*PENAEUS MONODON*) EXPERIMENTALLY EXPOSED TO *VIBRIO HARVEYI*

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ABSTRACT

Toll protein is an important non-specific immune protein in crustaceans which rely on innate immunity. The expression of Toll protein gene was studied in healthy and experimentally exposed (*Vibrio harveyi*) *Penaeus monodon* (tiger shrimp) by reverse transcriptase polymerase chain reaction (RT-PCR) using self-designed Toll-specific PCR primers. Comparison of the expression profiles of Toll protein gene in gills, hepatopancreas and gut of *P.monodon* showed that experimental exposure to a gram-negative bacterial pathogen, *Vibrio harveyi* induces Toll protein gene expression in these organs. The identity of the PCR amplified *P.monodon* Toll protein gene was confirmed by nucleotide sequencing and analysis.

KEY WORDS: ***Penaeus monodon*** **– Toll protein gene expression – RT-PCR.**

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INTRODUCTION

Host defense in shrimp is believed to rely largely on innate immunity. Innate Immunity is a sensitive non-self recognizing system triggered by components of pathogens called pathogen associated molecular patterns (PAMPs). Toll receptors have been recognized as major pathogen recognition receptors (PRRs) that play an essential role in recognition of microbes during host defense[1-3]. Toll receptors are a typical type-I, membrane-spanning, non-catalytic receptor proteins consisting of leucine-rich repeats (LRRs) and an intracellular C-terminus domain with a Toll/interleukin (IL-1) receptor (TIR) domain[3]. The cytoplasmic TIR domain harbors conserved amino acids that have been shown to be involved in the signalling as well as in the localization of the Toll, while the LRR is involved in pathogen recognition[4]. The objective of this study was to assess the basal expression of Toll protein gene in healthy *P.monodon* and to compare the expression profiles in the tissues of healthy and *V.harveyi* exposed *P.monodon.*

MATERIALS AND METHODS

(i) Shrimp samples and bacterial challenge

Apparently healthy *P.monodon* (Tiger shrimp) juveniles of about 10$\pm$5g weight were collected from shrimp farms in and around Chennai, Tamilnadu, India. Shrimp samples were acclimatized to laboratory conditions for two weeks with good aeration and *ad libitum* feeding. Shrimps were maintained in groups of six numbers each for the control and treatment groups in triplicates. A virulent *V.harveyi* isolate (VH/SDDL/09) obtained from an infected shrimp was used in this study. Shrimps in the treatment group were experimentally exposed to inactivated *V.harveyi* (VH/SDDL/09) culture following the method described by Soonthornchai *et al*. (2010). Briefly, the bacterial isolate was inoculated in tryptone soya broth at 30°C for 24h. The bacterial culture was centrifuged at 5,000 xg for 15 min and the pellets were washed with phosphate buffer saline (PBS) and resuspended in the same solution. Inactivation of bacterial culture was carried out by the addition of 0.1 % formalin to the bacterial suspension followed by overnight incubation[5]. The shrimps were kept in inactivated bacterial solution with the concentration of $4x10^{7}$ CFU ml^{-1}. Shrimps of control group were maintained under identical conditions as that of treatment group but were not exposed with bacteria.

(ii) Tissue sampling, RNA isolation and cDNA synthesis

Samples aliquots of tissues from gill, gut and hepatopancreas were collected from the shrimps in treatment group, 24h after experimental exposure to *V. harveyi*. Tissue samples were also collected simultaneously from shrimps in the control group. Total RNA was extracted from the tissues using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol and quantified using a biophotometer (Eppendorf, Germany). About 2µg of total RNA from samples were transcribed separately using high capacity cDNA synthesis kit (Applied Biosystems Inc, USA).

(iii) Reverse Trancriptase (RT-PCR) assay

About 1 µl of cDNA was used as the template for PCR amplification of Toll protein using self-designed Toll-specific PCR primers (TPM506F and TPM506R) designed for this study based on the sequence information of Toll protein gene (EF460463, EF117252, EF407561, AB333779, DQ923424) from various species of shrimp that were available in the Genbank database (www.ncbi.nlm.nih.gov/genbank). The details of the PCR primers used in this study are presented in Table 1. PCR assay was carried out in a total volume of 25µl with 22µl of 1X PCR master mix (Banglore Genei, Banglore, India), 1 µl (20 pmoles) each of forward and reverse primers and 1µl of cDNA (100 ng) from samples. PCR amplification was performed in a PCR thermal cycler (Eppendorf, Germany) with an initial denaturation at 94°C for 3 min; 30 cycles of

denaturation at 94°C for 1 min; annealing at 57.8°C for 45 sec; extension at 72°C for 45 sec and a final extension at 72°C for 5 min. PCR amplified products were resolved in 1.5% agarose gel at 80V for 45 min. The separated PCR products were visualized under UV after staining with ethidium bromide and the results were documented in a gel documentation system (BioRad, USA).

Table 1
PCR primers used in the study

Target gene	Primer sequence 5'-3'
Toll Protein gene	Forward – ACGCTCGATTTCAAAATGCT
	Reverse – CTCACCATCACTGGCACACT

(iv) Nucleotide sequencing and analysis

RT-PCR amplified product of Toll protein gene of *P. monodon* was sequenced and the identity was confirmed by nucleotide BLASTn analysis (www.ncbi.nlm.nih.gov). The sequence information of Toll protein gene of *P.monodon* generated in this study was submitted to Genbank, NCBI.

RESULTS

Basal expression of Toll protein gene was observed in gills, gut and hepatopancreas of healthy *P.monodon* in the control group (Fig. 1). Tissue- specific and induced expression of Toll protein gene was observed in these tissues in *P.monodon* when experimentally induced with *V.harveyi* at 24h post-exposure. Visual observation of PCR amplified products separated by gel electrophoresis showed that the induced Toll protein gene expression was the highest in gills followed by gut and hepatopancreas (Fig. 1). The PCR amplified products of Toll protein gene of *P.monodon* showed homology varying from 90% - 98% with other shrimp Toll protein gene sequences in the Genbank database.

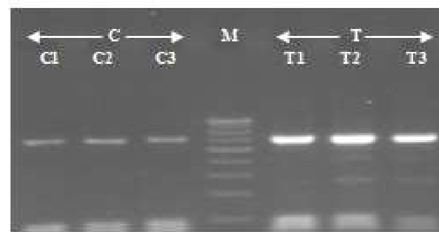

Fig 1. Expression of Toll protein gene in *P. monodon* (Tiger shrimp)

Lanes:
C - PCR amplified products from tissues of healthy control *P. monodon*
C1-Gut, C2-Gills, C3-Hepatopancreas.
M - 100bp molecular weight marker
T - PCR amplified products from tissues of *Vibrio harveyi* induced *P. monodon*
T1 - Gut, T2 – Gills, T3 – Hepatopancreas.

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DISCUSSION

Vibriosis is the most predominant bacterial disease reported to cause mortalities and production losses in shrimp culture worldwide[6]. Diseases caused by *V.harveyi* infection have been reported to be a serious problem in *P.monodon* farms and hatcheries[7]. Toll proteins are known to be the key components involved in innate immune response to various infections in crustaceans[8]. Recognition of bacterial or viral pathogens by Toll proteins in various penaeid shrimps has been documented[9]. *P. monodon* experimentally challenged with WSSV[10] and *Vibrio sp*[11] showed induced expression of Toll receptors. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis in *Litopenaeus vannamei* showed that Toll protein (lToll) was highly expressed in hemocyte, gill, heart, brain, gut, nerve, and muscle and less expressed in eye stalk and hepatopancreas[12]. In *Marsupenaeus japonicus,* Toll protein gene (MjToll) was constitutively expressed in the gill, gut, lymphoid organ, heart, hematopoietic organ, hemocyte and brain tissue. Significantly higher expression of Toll protein gene was observed in *M. japonicus* stimulated with peptidoglycan ligand[13]. Experimental exposure with *Vibrio sp.* induced the expression of Toll protein gene in the digestive organs of *P.monodon*[11].

In this study, basal expression of Toll protein gene was observed in gill and gut as reported previously in *M. japonicus*[13] and in hepatopancreas as reported in *L. vannamei*[12]. Post-PCR analysis revealed visually higher expression levels of Toll protein gene in the tissues of gills, gut and hepatopancreas at 24h post exposure to *V.harveyi*. This shows that these organs play an immunological role to resist pathogen invasion. Induction with WSSV in *P.monodon*[10] and *Vibrio anguillarum* in *Fenneropenaeus chinensis*[14] had also resulted in induced expression of Toll protein gene at 24h post induction similar to the observations of this study.

CONCLUSION

Reverse transcriptase PCR (RT-PCR) analysis showed that basal expression of Toll protein gene is present in various organs of *P.monodon viz*., gut, gills and hepatopancreas. The expression of Toll protein gene in these organs could be induced upon exposure to inactivated *V. harveyi,* a gram-negative bacterial pathogen that affects shrimps so as to improve the immune status in *P.monodon*.

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