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Research Article

Development of a Novel shRNA Construct pSh-IRAK-4 for Silencing of IRAK-4 Gene and Delineating TLR-Mediated Pathway in *Penaeus monodon In-Vitro*

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Abstract

In order to understand the TLR pathway defence mechanism of *Penaeus monodon*, the essential molecule involved in the transduction of Toll-pathway, Interleukin-1 (IL-1) receptor-associated kinase-4 (IRAK-4) was investigated by the mechanism of RNA interference (RNAi) by silencing of the mRNA expression of IRAK-4 gene. In this study we have developed short hairpin RNA (shRNA) construct in pSUPER vector targeting IRAK-4 gene silencing of TLR pathway in *P. monodon*. The silencing efficiency of pSh-IRAK-4 construct was first confirmed *in-vitro* in primary haemocyte culture by transfection using pSh-IRAK-4 plasmid, followed by induction with Lipopolysaccharides (LPS). Loss of expression of IRAK-4 was studied by relative gene expression in pSH-IRAK-4 group compared to LPS induced group, the maximum suppression of IRAK-4 gene in cell culture was recorded as 96% at 12h and 93.5% at 24h post LPS induction in pSh-IRAK-4 group. After confirmation of silencing efficiency of construct, the expression of TLR genes of IRAK-4 mediated pathway, was studied post LPS induction both *in vitro* using real-time qRT-PCR with β-actin as the internal reference gene. For this IRAK-4 downstream genes TRAF6, Dorsal and 4 Antimicrobial peptides (AMPs) (ALF, PEN, AST, Crustin) molecules were studied. Significant downregulation of mRNA expression level in downstream molecules of TLR pathway below IRAK-4 gene viz., TRAF6, Dorsal, and 4 AMPs (ALF, PEN, AST, Crustin) compared to LPS group in response to LPS stimulation was observed in pSh-IRAK-4 group ($P \leq 0.05$). Taking all these results together, it is confirmed that TLR pathway is governed by central mediator kinase molecule IRAK-4, when induced by LPS ligand, NF-kB activates the downstream cascades of AMPs of the Toll pathway in *P. monodon*. Our result confirms the designing of a novel pSh-IRAK-4 construct and its application in efficient silencing of IRAK-4 gene in *P. monodon*. Plasmid-based IRAK-4 knockdown approach would provide an insight to the role of IRAK-4 in shrimp immune system.

Keywords: RNA Interference; pSUPER Vector; shRNA; IRAK-4

Abbreviations

α: Alpha; β: Beta; Δ: Delta; µg: Microgram (10^{-6}g); µl: Microlitre (10^{-6}l); °C: Degree Centigrade; ALF: Anti-lipopolysaccharide Factor; AST: Astakine; bp: Base Pair; CT: Value Cycle Threshold Value; DNA: Deoxyribonucleic Acid; DNAase: Deoxyribonuclease; dsRNA: Double-stranded RNA; Fig.: Figure; H: Hour(s); L: Litre; M: Molar; Min: Minutes; Ml: Milliliter (10^{-3}L); mM: Milli Molar; mRNA: Messenger RNA; Myd88: Myeloid Differentiation Primary Response 88; N: Normal; NCBI: National Center for Biotechnology Information; NF-κB: Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells; Ng: Nano Gram; O/N: Overnight; PCR: Polymerase Chain Reaction; PEN: Penaeidin; RE: Restriction Enzyme; RNA: Ribonucleic Acid; Rpm: Revolution Per Minute; S: Seconds; shRNA: Short Hairpin RNA; siRNA: Small Interfering RNA; TRAF6: Tumor Necrosis Factor Receptor Associated Factor 6; U: Unit

Introduction

Immunity sensors play an essential role to recognize pathogen components called pathogen associated molecular patterns

(PAMPS) which are recognised by pathogen recognition receptors and further generate a signal transduction leading to induction of host defence molecules. Toll-like receptor (TLR) and Toll-interleukin receptor (TIR) family members are such key immunity sensors, which induce an evolutionarily conserved intracellular signalling cascade in both vertebrates and invertebrates [1-3]. TLR/TIR signal transduction contains several interleukin-1 receptor-associated kinases (IRAKs) as downstream signalling mediators [4]. All IRAK members have two typical domain structures, a N-terminal domain containing a death domain (DD) and a central kinase domain [5]. The kinase domain is required for the serine/threonin kinase activity during signal transduction. Among the four mammalian IRAKs (IRAK-1, IRAK-2, IRAK-M and IRAK-4), only IRAK-1 and IRAK-4 have been shown to have active kinase activity [6,7]. IRAK-1 and IRAK-4 are orthologs of the Pelle serine/threonin kinase molecules, in the Drosophila Toll signaling cascade, which operates against Gram-positive bacteria and fungi [8,9]. Among IRAK family members, IRAK-4 shares the highest sequence homology with the Drosophila Pelle [7]. In contrast to Drosophila Pelle kinase, the mammalian IRAK-4 is also involved in response to Gram-negative bacteria infections [10,11]. IRAK-4 is an indispensable factor for TLR-downstream signalling activation of the host's immune functions subsequent to the different pathogen contact and ligands [6,12-14]. It is the central mediator in NF-kB activation and innate immunity signaling and its kinase activity is necessary for activation of IRAK-1 and perhaps other signal transducing substrates [9,15]. The IRAK-4 is a key kinase in signal transduction by the lipoprotein receptor Toll-like receptor (TLR) 2 and the lipopolysaccharide (LPS) receptor TLR 4 [16].

Studies performed by Deepika., *et al*. 2014, unravel the novel MyD88-dependent Toll-pathway by elucidating the activation of TLR, MyD88 and TRAF6 independently in response to various pathogens [17]. One important question for the TLR-induced MyD88 dependent pathway is the requirement of the kinase activity of IRAK-4 in various signalling events. We have generated shRNA construct targeting the IRAK-4 gene and found that the kinase activity of IRAK-4 plays a critical role in TLR mediated immune responses. Our results further indicate that inactivation of IRAK4 kinase activity deals to reduced mRNA stability and diminished production of cytokines and chemokines in response to LPS stimulation.

Gene silencing by RNA interference (RNAi) is a useful tool for the functional characterization of genes. RNA interference (RNAi) is the process of sequence-specific posttranscriptional gene silencing by which short interfering RNA (siRNA) or short hairpin RNA (shRNA) are used to inactivate the expression of target genes [18,19]. Although several attempts are made to vaccinate crustaceans by administering DNA constructs, which express viral capsid genes [20-22], as well as silencing compounds used (namely dsRNA, siRNA and shRNA). The major drawback of this method is that unlike temporal silencing compounds (dsRNA/siRNA/shRNA), DNA transfection might produce a genetically modified organism (GMO). In this respect, shRNA offers advantages over siRNA in silencing longevity, delivery options and cost. The shRNA is expressed as a DNA template and is transcribed in cells as a single-stranded RNA molecule (~50–100 bases) [23]. Complementary regions are spaced by a small 'loop' which causes the transcript to fold back on itself forming a 'short hairpin' in a manner analogous to natural microRNA. The recognition and processing of respective siRNAs (which is converted from shRNA to the corresponding siRNA) is done by RNAi machinery.

In the absence of a B cell lineage in arthropods, and hence no long-term vaccination strategy being available, the introduction of using RNAi in crustacean may serve as an effective tool to understand the defence mechanisms of crustacean [24]. This may also act as control and preventive measure for viral diseases for application in aquaculture [25]. Becoming a standard laboratory practice, RNAi technology has been increasingly used to unravel gene functions in *in-vivo*, providing new opportunities not only to understand in greater depth the nature of shrimp biological and physiological processes, but also to decipher host-pathogen interactions.

Materials and Methods

Selection of target sequence for designing sense and antisense strands

Sequences available for crustacean IRAK-4, were retrieved from NCBI GenBank (KR136276.1, JN180645.1). The death domain sequence of the gene, a region unique to IRAK-4 was selected by multiple alignment of *P. monodon* and *L. vannamei* sequence by using CLUSTAL W (1.83) programme (Supplementary file: Figure 1). Sense and antisense strands were designed using Oligoengine

2.0 Software (Table 1) and the oligonucleotides were synthesized by the Eurofins services, India.

Supplementary Figure 1: Multiple alignments sequence of IRAK4 gene of *P. monodon* and *L. vannamei*. Blue colour indicates unmatch sequences, black colour indicates identical residues sequences.

Sense strand:	5' GATCCCCGTTGAAGAATGTGCTC-GAGTTCAAGAGACTCGAGCACATTCTTCAACTTTTTA 3'
Anti-Sense strand:	5' AGCTTAAAAAGTTGAAGAATGTGCTC-GAGTCTCTTGAACTCGAGCACATTCTTCAACGGG 3'

Table 1: shRNA Oligonucleotides sequences designed by online program (http://www.oligoengine.com).

Designing of shRNA sequences

shRNA-expressing plasmid was constructed by cloning shRNA duplex into pSUPER vector by *BglII* and *HindIII* enzyme sites. shRNA vector targeted against IRAK4 gene were designed by online tool oligoengine (http://www.oligoengine.com/) and the sequences were selected for this study (Table 1). The target region was selected from the open reading frame of a desired cDNA sequence, of 60 nucleotides downstream from the start codon. The Basic local alignment search tool (BLAST) search (www.ncbi.nlm.nih.gov/BLAST) was performed for shRNA sequences as the input against libraries or mRNA sequences of *P. monodon* and *L. vannamei* to ensure the uniqueness of the target gene and no other crustacean genes were targeted. Several shRNA duplexes were synthesized to control for the specificity of the knockdown experiments [26,27]. The sequence of the oligo chains was designed as *BglII*+sense chain+loop+antisense chain+termination signal+*HindIII* (Figure 1).

Figure 1: Schematic diagram of plasmid construct. (A) pSUPER vector; (B) The structure of the oligo chains transcribed in pSUPER vector to finally form pSh-IRAK4 construct.

Phosphorylation and annealing of the shRNA template oligonucleotides

Two equal amount of oligo sequence of sense and antisense strands were annealed and phosphorylated [28]. Briefly, the shRNA template oligonucleotides were dissolved in deionized water to a final concentration of 20 µM. The phosphorylation/annealing reaction for each shRNA template was set up in 20 µl of total volume reaction (Supplementary file: Table 1). The phosphorylated product was incubated at 37 °C for 30 min then heated to 95 °C for 2 min in a thermocycler. The reaction mix was allowed to cool at room temperature. 2 µl of aliquot from the reaction (1 µM shRNA template) was taken and it was diluted in 1:5 ratio by adding 8 µl of 1X T4 Kinase buffer and mixed well. 0.5 µl of the diluted shRNA was used as template (0.2 µM) for cloning reaction.

Sl. No.	Required Materials	Concentration
1.	Top strand shRNA template oligo (20 µM)*	4 µl
2.	Bottom strand shRNA template oligo (20 µM)*	4 µl
3.	10X T4 Polynucleotide Kinase Buffer	2 µl
4.	10 mM ATP	2 µl
5.	Deionised water	7 µl
6.	T4 Polynucleotide Kinase (10 U/µl)	1 µl
	Total Volume	20 µl

Supplementary Table 1: Phosphorylation and annealing reactions of the shRNA template oligonucleotides.

*For the insert-minus control, 8 µl of deionised water used in place of the top and bottom strands.

Cloning of shRNA template into shRNA expression vector (pSUPER)

RNA duplex was cloned into the BglII/ HindIII sites of the pSUPER vector. The directional cloning was accomplished using T4 DNA ligase. The ligated plasmid DNA was transformed into competent DH5α cells and then seeded on Luria Burtani (LB) agar medium plates containing 0.05 g/L ampicillin and incubated at 37°C O/N. The most prominent colonies were picked up and seeded in 5 ml LB broth containing 0.05 g/L ampicillin and incubated at 37°C O/N in a shaking incubator. The plasmid was extracted using Thermo Scientific GeneJET plasmid miniprep kit following the manufacturer's instruction. Confirmation of recombinant clones was firstly screened by performing colony PCR using vector specific M13 primers (498 bp) and then the isolated plasmid was confirmed by restriction enzyme digestion of selected plasmids with Bglll and HindIII and finally by sequencing (Eurofins, India). The plasmid construct thus obtained was designated as pSh-IRAK4. The concentration and purity of the plasmid was determined by NanoDrop spectrophotometer and agarose gel electrophoresis. The plasmid was stored at -20 °C for subsequent experiments.

Primary culture of haemocytes of *P. monodon* for in-vitro evaluation of silencing effect

For evaluating silencing efficiency of the short hairpin construct pSH-IRAK4 construct was transfected into primary cell culture of shrimp hemocytes (Supplementary file: Figure 2). The live and healthy shrimps were first kept in sterile sea water with 1X antibiotic-antimycotic solution (HiMedia, India) for 1 h prior to the experiment. Haemolymph was collected aseptically from the heart of the live and healthy shrimp and transferred gently to a sterile 15 mL tube containing equal volume of serum-free Leibovitz L-15 medium. The tube spinned at 1500 rpm for 3 min to settle the haemocytes and separate the haemolymph. The supernatant containing haemolymph was then discarded and 2 mL fresh serum-free medium was added to the pelleted haemocytes. The wash was repeated to ensure removal of all traces of the anticoagulant. After discarding the supernatant, growth medium containing 10% FBS was added to the pellet. After counting of the number of viable cells, concentration was adjusted to about 10^4 cells per mL using growth medium. The cells were then seeded in 6-well plates (growth area: 9 mm per well) at the rate of 2 mL per well and incubated at 28°C to allow the cells to attach to the substrate and multiply. Once the cells adhered, the monolayer was washed using serum-free L-15 medium and is further subjected to transfection studies [17].

Transfection of haemocytes of *P. monodon* with pSh-IRAK-4 plasmid

Haemocytes grown for 24h in a six-well plate were washed with serum-free medium. Lipofectamine TM 2000 transfection reagent (Thermo Scientific, USA) was used for transfection

Supplementary Figure 2: Transfection of haemocyte cells with pSH-IRAK4 construct, subsequently exposed to LPS induction, 12 h post transfection. A: Untreated haemocytes (Ht) cells; B: Transfected with pMock; C: Transfected with LPS; D: 3 h post transfection; E: 12 h post transfection; F: 24 h post transfection.

following manufacturer's protocol. To test the silencing effect of pSH-IRAK-4 construct, in two 6-well plates hemocytes cells were transfected with 2 µg of the silencing construct treatment plasmid (pSh-IRAK-4), while one plate was kept untreated to be used later for LPS induction (control), subsequently each plate exposed to LPS induction (10 µg/mL) (Sigma, USA), 12h post transfection. Untreated cells induced with LPS were kept as control. The transfected and induced cells were incubated at 37 °C and harvested after 3, 12 and 24h by pooling cells from two wells for each group. After this the cells were washed briefly with PBS and harvested directly into 1 ml TRIzol® (Life Technologies, USA) reagent for total RNA isolation as per manufacturer's protocol. Single stranded cDNA was prepared using 1 µg of DNaseI treated total RNA with oligo(dT) and MuLV reverse transcriptase (Thermo Scientific, USA) in a final volume of 20 µl [73]. The resulting cDNA was stored at -20°C for further use.

Confirmation of pSH-IRAK4 mediated silencing *in-vitro*

Silencing effect of the construct was determined by quantitative real-time PCR of PmIRAK-4 transcripts using primers IRAK4-RT-F/R, normalized to β-actin gene Q-F/R (Table 2). The relative transcription of PmIRAK-4 amplicon was calculated using the comparative Ct method with the formula $2^{-\Delta\Delta CT}$ [29] where the transcription of PmIRAK4 in the control group was set as the calibrator and β-actin was used as house-keeper gene. The inhibition ratio of IRAK4 expression was calculated by the following formula:

S. No.	Primer	Sequence (5′ to 3′)	Size (bp)	Reference
1.	PmIRAK-4 Q-F/R	GGGTCGAGTGCCAAG-TATGC GCGATCTCGAGCA-CATTCTT	400	[29]
2.	β-actin Q-F/R	CGTGTGGATCGGTG-GTTCTA CCTCGGC-CAGACTCTTCGTA	150	[70]
3.	M13- F/R	GTAAAACGACGGC-CAGT AACAGCTATGACCATG	498	http://www.oligoengine.com

Table 2: Primers used for the confirmation of pSH-IRAK4 mediated silencing *in vitro.*

Inhibition ratio of pSh-IRAK4 construct expression = (1 - the expression intensity of PmIRAK-4 in the construct group/the expression intensity of IRAK4 in the control group) × 100% [30].

Relative quantification of gene expression using realtime PCR

The relative quantification of gene expression of the target genes was carried out using real-time PCR performed on ABI 7500 Real-Time PCR system (Applied Biosystem, USA). The primers (Table 3) and internal control gene β-actin were used for amplification of a sequence of 150 bp. 2 µL of cDNA equivalent to 25 ng RNA was used in the qPCR analysis using SYBR Green master mix (Fermentas, India) with the following programme: 95°C for 10 min followed by 40 cycles of 95°C for 15s, 60°C for 1 min. The qPCR was carried out in duplicates for each sample and 3 sample replicates were analysed for each time-point. The Comparative CT method was used for the calculation of fold-changes in gene expression. The threshold cycle (Ct) value was determined using the automatic setting on the real-time PCR system. The difference in the Ct value between target gene and the internal control β-actin gene, *ΔCt*, was calculated and the relative expression level of target gene to β-actin gene was determined using the equation $2^{-\Delta\Delta CT}$ [28].

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) using SPSS 16.1 software. The results were expressed as mean ±

S.E.M (standard error of the mean). P ≤ 0.05 was considered as statistically significant.

Results

Successful cloning of shRNA duplex into pSUPER Vector

DNA oligos of IRAK4 gene were designed and cloned into the BglII/ HindIII sites of the pSUPER vector followed by transformation and plasmid extraction. Plasmids were confirmed by colony PCR observing distinct bands in 1.2% agarose gel electrophoresis (Figure 2, 3). In colony PCR, a distinct band of 498 bp was observed in positive clones (Figure 2). Plasmid was isolated from positive colonies. The recombinant plasmid was double digested with EcoRI and HindIII. The shRNA sequence was found in correct order in recombinant plasmid. The two bands at 3176 bp and 281 bp were observed in positive clones as expected, while the empty vector was observed to have two bands at 3176 bp and 227 bp (Figure 4). Further, the insert was confirmed by sequencing using services of SciGenom Labs Private Ltd., India.

Figure 2: Colony PCR showing 498 bp amplicon using M13 primers on 1.2% agarose gel. M: Molecular weight marker; L1,L2,L3,L4,L5,L6: positive recombinant clones.

Figure 3: Isolated plasmid from positive clones shown on 1.2% agarose gel. M: Molecular weight marker; L1,L2,L3: positive recombinant clones, L4: pSUPER plasmid as control.

Figure 4: Identification of recombinant plasmids by restriction endonuclease digestion. M: marker; L1: Uncut plasmid, L2, L5: pSUPER Plasmid without insert, L3, L4: pSUPER Plasmid with insert cut with EcoR I/Hind III, respectively.

Silencing of IRAK-4 mRNA expression in transfected haemocytes

Relative quantitation of IRAK-4 gene expression in transfected haemocytes showed that the maximum suppression of IRAK-4 gene in cell culture was recorded as 96% at 12h and 93.5% at 24h post LPS induction in pSh-IRAK-4 group (Figure 5). Each level of mRNA expression of IRAK4 gene was quantified and normalized to the level of β-actin gene as the internal reference gene. The inhibitory ratio was calculated by comparing to the LPS group. The results were expressed as mean ± SEM from independent experiments followed by one-way ANOVA using SPSS16.0 software package. P ≤ 0.05 was accepted as the level of statistical significance.

Figure 5: Quantitation of the inhibitory percentage of IRAK4 mRNA in transfected HT cells. Each level of PCR product of IRAK4 gene was quantified and normalized to the level of β-actin. The inhibitory rate was calculated by comparing to the control cells. The results were expressed as mean ± SD from independent experiments.

Post silencing analysis of upstream and downstream genes in IRAK-4 mediated pathway

After confirmation of silencing efficiency of construct, the expression of TLR genes of IRAK-4 mediated pathway was studied in the primary culture of haemocytes after LPS induction post 12h of transfection *in vitro* using real-time qRT-PCR with β-actin as the internal reference gene. For this, the transfected cells were harvested after 3, 12 and 24h by pooling cells from two wells for each group. The LPS control was also taken in account. Initially, the total RNA isolated and cDNA prepared from harvested cells and were analysed for the expression study. Quantitative real-time PCR revealed varied pattern of expression of genes of TLR pathway in the stimulated primary haemocyte culture. Relative expression of several genes viz., Toll, MyD88, IRAK-4, TRAF6, Dorsal, ALF, PEN, AST, Crustin were analysed by $2^{-\Delta\Delta CT}$ method, where *ΔCt* was calculated by subtracting Ct values of β-actin from Ct values of the corresponding target gene.

The relative % inhibition of IRAK-4 gene in various groups is summarised in table 4. As it is shown in figure 6 that Toll and MyD88 gene expression was significantly elevated to 14 and 10 fold change of Toll and at 17 and 33 fold change of MyD88, both in pSh-IRAK-4 group as well as in LPS group respectively post 24h of LPS induction (Figure 6 A and B). The expression level of and MYD88 and Toll increased dramatically post LPS induction.

SI. No.	Gene	pSh-IRAK-4 group	LPS group	Time	% Suppression
1.	Toll	↑ upregulation	↑ upregulation	NA	
2.	MyD88	↑ upregulation	↑ upregulation	NA	
3.	IRAK-4	↓ downregulation	↑ upregulation	3 h	81.6
				12 h	96
				24 h	93.5
4.	Tumor necrosis factor receptor associated factor 6 (TRAF6)	↓ downregulation	↑ upregulation	3 h	65.9
				12 h	92.1
				24 h	93.1
5.	Dorsal	↓ downregulation	↑ upregulation	3 h	93.7
				12 h	93.1
				24 h	89.6
6.	Crustin	↓ downregulation	↑ upregulation	3 h	92.2
				12 h	96.1
				24 h	89
7.	Anti-lipopolysaccharides factors (ALFs)	↓ downregulation	↑ upregulation	3 h	65.9
				12 h	92.1
				24 h	93.1
8.	Penaeidin	↓ downregulation	↑ upregulation	3 h	86.8
				12 h	90.8
				24 h	84.7
9.	Astakine	↓ downregulation	↑ upregulation	3 h	53.6
				12 h	81.7
				24 h	95.9

Table 4: Result of pSh-IRAK-4 silencing effect on TLR pathway genes in *P. monodon* haemocytes in *in-vitro* experiment

Figure 6: Relative mRNA transcription of TLR pathway genes in LPS and pSh-IRAK-4 group at 0, 3, 12, 24 h of post LPS induction. (A) TOLL (B) MyD88. mRNA levels were normalized with β-actin mRNA levels. Bars represent the mean ± SEM (n = 3). Significant differences between LPS and pSh-IRAK-4 group at each sampling point were indicated with alphabets. (P ≤ 0.05).

Overall, in all downstream molecules, more than 80% suppression was achieved in pSh-IRAK-4 group as compared to LPS group post 12 and 24h post LPS induction (Figure 7, 8 and Table 4). Downstream molecules of TLR pathway below IRAK-4 gene viz., TRAF6, Dorsal, and all AMPs (ALF, PEN, AST, Crustin) showed significantly lower expression as compared to LPS group. Hence, it was attributed to centralized role of IRAK-4 in TLR pathway. mRNA expression level of Toll and MyD88 post LPS induction remained unaffected by IRAK-4 silencing.

Figure 7: Relative mRNA transcription of TLR pathway genes in LPS and pSh-IRAK-4 group at 0, 3, 12, 24 h of post LPS induction. (A) TRAF6 (B) Dorsal. mRNA levels were normalized with β-actin mRNA levels. Bars represent the mean ± SEM (n = 3). Significant differences between LPS and pSh-IRAK-4 group at each sampling point were indicated with alphabets. (P ≤ 0.05).

Figure 8: Relative mRNA transcription of TLR pathway genes in LPS and pSh-IRAK-4 group at 0, 3, 12, 24 h of post LPS induction. (A) Anti-lipopolysaccharide factor (ALF) (B) Penaeidin (PEN) (C) Crustin (D) Astakine (AST). mRNA levels were normalized with β-actin mRNA levels. Bars represent the mean ± SEM (n = 3). Significant differences between LPS and pSh-IRAK-4 group at each sampling point were indicated with alphabets. (P ≤ 0.05).

Figure 8

Discussion

RNAi is a post transcriptional gene silencing mechanism. It is broadly believed that this mechanism is naturally occurring and is conserved in vertebrates [31]. Being a simple and effective tool, it has replaced the gene knockout approach and is widely used in the study viral diseases, gene function assessment and gene therapy [32-35]. RNAi is currently considered as a very promising strategy for viral disease control in shrimp, however, the delivery of dsRNA/siRNA into target cells and tissues remains a big challenge, at present [32]. Even though most of the reports indicate that the delivery of dsRNA into shrimp by intramuscular injection [36-38] is possible. Since the effect of short interfering RNAs (siRNAs) is generally transient in transfected animal cells, small RNA expression vectors have been developed that express hairpin siRNA (also called short hairpin RNA or shRNA) to induce long-lasting RNA silencing in mammalian cells [39-41]. Posttranscriptional RNAi can significantly suppress gene expression due to degradation of the targeted mRNA by exogenously delivered dsRNAs into the mammalian cells *in-vitro.*

In order to understand the pathway of TLR system, the essential molecule involved in the transduction of Toll-pathway of *P. monodon*, Interleukin-1 (IL-1) receptor-associated kinase-4 (IRAK-4) was investigated by the mechanism of RNA interference (RNAi) by silencing of the mRNA expression of IRAK4 gene. In this study, a shRNA construct using pSUPER vector targeted to silence IRAK-4 gene TLR pathway in *P. monodon* has been developed. The IRAK-4 was selected as the target gene, which is known as an essential factor, which operates downstream of the TIR domain [42-44] modulating functions of both, innate and adaptive immunity [6,45]. It is the central mediator in NF-kB activation and innate immunity signaling and its kinase activity is necessary for activation of other signal transducing substrates of TLR pathway [9,46,47]. IRAK molecule plays an important role in the sequential formation and activation of IL-1-induced signaling complexes. IRAK-4 has been reported to be an essential component for the IL-1 signaling pathway and proposed to function as an IRAK kinase [6,9,48]. A bacterial defense function of this protein is well demonstrated by Suzuki., *et al*. 2002[b], where IRAK-4 deficient mice have increased mortality upon a bacterial infection [15]. The IRAK-4 is a key kinase in signal transduction by the lipoprotein receptor TLR 2 and the LPS receptor TLR 4 [16].

In this study, temporal relative expression of IRAK-4 gene was done to check the silencing efficiency of pSh-IRAK-4 construct *in-vitro*, primary haemocyte culture was maintained and transfected by pSh-IRAK-4 plasmid and followed by induction with LPS was carried out. Similar type of shRNA constructs were earlier used for silencing experiments *in vitro* [30,49]. As shown in figure 5, IRAK-4 mRNA expression was suppressed by 93.5% of that in the control cells at 24h of post LPS induction *in vitro*, while maximum suppression of 96% suppression was recorded at 12h post LPS induction *in vitro*. The result was in accordance with Gao., *et al*. 2008, who targeted VP1 gene of Infectious bursal disease virus (IBDV) by using shRNA, achieved 87.4% viral inhibition [50]. The experiment conducted by Zheng., *et al*. 2009, showed the gene silencing efficiencies *in vitro* using shRNA plasmid expression vectors targeting Cx43, had a better gene silencing effect (73.5%), for the protein level of Cx43, in the NIH/3T3 cells [51]. Similarly, more than 80% silencing was recorded in LvToll or LvIMD gene compared to dsEGFP injection [52]. Wang., *et al*. 2010, observed excellent silencing effects mediated by sequence specific dsRNA

and also reported that these silencing effects are time dependent [53]. In another study in which 5 shRNAs targeted on the sequence of the flgM gene were designed, and all of them significantly reduced the mRNA expression of the flgM gene with different efficiencies [54]. The similar results were noted down in case of the pcD-GIH-lh construct which effectively knocked down gih transcript both *in vitro* and *in vivo* and 73% gih silencing was observed in vitro [55], while Krishnan, 2008, obtained 90% silencing against a viral transcript in human fetal fibroblast cells [56].

The molecular mechanisms underlying the majority of crustacean immune responses are very little documented but it is a subject of intense study. An increasing number of immune responsive genes/proteins involved in bacterial infection in shrimp and crayfish has been reported by various researchers [57-59]. By the molecular mechanism by analogy to other known systems, it is speculated that a TLR/MyD88/Tube/Pelle/TRAF6/NFkB cascade may exist in shrimps for immune gene regulation. Other components of TLR pathways have been reported from *L. vannamei* and *F. chinensis*, such as Dorsal (LvDorsal and FcDorsal) and putative Spätzle-like Toll ligands (FcSpz and LvSpz1e3). Furthermore, a homolog of the IRAK-4, a central signal transduction mediator of the TLR and TIR pathways, has been discovered in P. monodon (PmIRAK-4) [60]. In shrimps, the limited data available on the IRAK-4 mediated pathway has revealed that it plays a significant role in the shrimp antibacterial response. In accord, expression of genes of IRAK-4 mediated pathway was studied in the primary culture of haemocytes after LPS induction post 12h of transfection *in vitro* using real-time qRT-PCR with β-actin as the internal reference gene. Quantitative real-time PCR revealed varied pattern of expression of TLR genes in the stimulated primary haemocyte culture [56].

To further make clear whether bacterial infection could increase the expression of Toll and MYD88, we examined their expression level and found that Toll and MYD88 expression level was still in a significantly elevated level at 14 and 10 fold change of Toll and at 17 and 33 fold change of MyD88 both in pSh-IRAK-4 group as well as in LPS group respectively (Figure 6 A and B). One important question for the TLR-induced MyD88 dependent pathway is the requirement of the kinase activity of IRAK-4 in various signalling events. It was clear indication that IRAK-4 is a central molecule of TLR pathway, above which silencing of IRAK-4 has not affected mRNA expression level of Toll and MYD88 post LPS induction while it was completely opposite in the expression level seen in all downstream molecules below IRAK-4 molecule.

Significantly low expressions of IRAK-4 downstream molecules viz., TRAF6 and Dorsal was recorded (Figure 7 A and B). Percentage inhibition recorded was higher in TRAF6 at 24h while in Dorsal at 12h by 93% in pSh-IRAK-4 group. Here it was observed that in LPS group all downstream molecules were significantly upregulated. Pattern showed maximum expression of TRAF6 molecule at 12h whereas Dorsal molecule was expressed highest at 24h in LPS group indicated a sequential event in which IRAK-4 – TRAF6 – Dorsal play their role. This confirms the theory of TLR mediated pathway. Similar reports by Li., *et al*. 2002 in mice and Deepika., *et al*. (2014) supported that TLR-TRAF6 mediated signalling pathway in *P. monodon* was regarded as a vital line in defending against virus [6,17]. Another report supports the critical role of the TLR4 signalling pathway in the pathogenesis of ischemia/reperfusion injury has been documented in mammals [61]. Activation of TLR4 initiates the transmembrane signalling cascade and triggers intracellular signalling molecules including IRAK1 and IRAK-4 and TRAF6. These signalling molecules induce nuclear translocation of NF-κB and activator protein (AP)-1, resulting in the production of inflammatory cytokines such as TNF-α and IL-6 [62,63]. Previous studies have also reported that the downregulation of IRAK1 or TRAF6 affects ischemia/reperfusion injury [6,64].

Expressions levels of all AMPs (Crustin, ALF, PEN, AST) were also significantly lower as compared to LPS group (Figure 8 A, B, C and D). Further, significant upregulation of Crustin, ALF and PEN at 3 h in LPS group showed that they are immediate early AMPs whereas AST is a delayed AMP in response to bacterial infection. The functional analysis of potent immune genes such as AMPs, PRPs, toll receptors etc. have revealed the importance and role of these molecules in responses against major shrimp pathogens [65-67]. In this study, we support that the kinase activity of IRAK-4 is critical for the functions of IRAK-4 in TLR-mediated inflammatory and innate immune responses. Previous studies showed that IRAK-4 deficiency leads to severe impairment of TLR signalling, indicating an essential role of IRAK-4 in TLR-mediated pathways [15,68]. Our data is in agreement that inactivation of IRAK-4 kinase activity deals to reduced mRNA stability and diminished production of cytokines and chemokines in response to LPS stimulation as seen

by the downregulation of mRNA expression level in downstream molecules of TLR pathway below IRAK-4 gene viz., TRAF6, Dorsal, and all AMPs (ALF, PEN, AST, Crustin) expressions levels were significantly lower expression level as compared to LPS group. Similar reports were published earlier, expressing the critical role of IRAK-4 kinase activity for the maintenance of normal pro-inflammatory reactions *in vitro* [69].

Conclusions

The present study demonstrated the first-time successful silencing of IRAK-4 gene in *P. monodon* through shRNA mediated approach. Our study suggests that shRNA mediated silencing could be achieved in *P. monodon* for only a short duration but efficient silencing was achieved at 24h post transfection. The mRNA expression study of TLR genes after stimulating with LPS helps to provide useful information for understanding host-pathogen interaction and pathogenesis of shrimp pathogens. IRAK-4 is known to be highly important gene of TLR pathway, and silencing of it lead to understand the downstream molecules of TLR which is the integral immune component of invertebrates. The observations made in the study indicate that TLR-induced MyD88 dependent pathway is the requirement of the kinase activity of IRAK-4 in various signaling events, the upstream molecules above IRAK-4 gene (Toll and MyD88) were analysed for this purpose. Due to knockdown of IRAK-4, its kinase activity was reduced and its ability to induce the production of cytokines and chemokines in response to LPS stimulation was also diminished to a significant level. As seen by the downregulation of mRNA expression level in downstream molecules of TLR pathway below IRAK-4 gene viz., TRAF6, Dorsal, and 4 AMPs (ALF, PEN, AST, Crustin) compared to LPS group. In downstream molecules of IRAK-4, pattern of expression showed maximum expression of TRAF6 molecule at 12h whereas Dorsal molecule was expressed highest at 24h in LPS group indicated a sequential event in which IRAK-4 – TRAF6 – Dorsal play their role. This confirms the theory of TLR mediated pathway. Again, expressions levels of all AMPs (Crustin, ALF, PEN, AST) were also significantly lower in pSh-IRAK-4 plasmid as compared to LPS group as these AMPs are guided by Dorsal gene. Further, significant upregulation of Crustin, ALF and PEN at 3 h in LPS group showed that they are immediate early AMPs whereas AST is a delayed AMP. Taking all these results together, we are now able to deduce the pathway of a TLR induced by LPS ligand which could activate NF-kB activities and the downstream cascades of AMPs of the Toll pathway in *P. monodon*. Thus, this study could be projected to set benchmark on role of IRAK4 in shrimp immune system and could also help in developing strategy for disease defence in shrimp. Selection of an effective target sequence is the key point of RNA interference. shRNA construct mediated silencing needs to be assessed in *in vivo* experimental infection as well as the effect of knock down on the expression of several other downstream effector molecules of the immune system needs to be elucidated. Once the construct is validated, it may be used for several basic studies related to *P. monodon* defence mechanism which will help in developing defence strategies against diseases for which species is vulnerable, with ultimate goal of encouraging the monodon culture in future.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Bibliography

1. Nie Li., *et al*. "Toll-like receptors, associated biological roles, and signaling networks in non-mammals". *Frontiers in Immunology* 9 (2018): 1523.

2. Roy Craig R and Edward S Mocarski. "Pathogen subversion of cell-intrinsic innate immunity". *Nature Immunology* 8.11 (2007): 1179-1187.

3. Hemmrich Georg., *et al*. "The evolution of immunity: a low-life perspective". *Trends in Immunology* 28.10 (2007): 449-454.

4. Ge Hui., *et al*. "Molecular cloning and expression of interleukin-1 receptor-associated kinase 4, an important mediator of Toll-like receptor signal pathway, from small abalone Haliotis diversicolor". *Fish and Shellfish Immunology* 30.4-5 (2011): 1138-1146.

5. Janssens Sophie and Rudi Beyaert. "Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members". *Molecular Cell* 11.2 (2003): 293-302.

6. Li Shyun., *et al*. "IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase". *Proceedings of the National Academy of Sciences* 99.8 (2002): 5567-5572.

7. Wang Zhulun., *et al*. "Crystal structures of IRAK-4 kinase in complex with inhibitors: a serine/threonine kinase with tyrosine as a gatekeeper". *Structure* 14.12 (2006): 1835-1844.

8. Towb Par., *et al*. "Tube Is an IRAK-4 homolog in a Toll pathway adapted for development and immunity". *Journal of Innate Immunity* 1.4 (2009): 309-321.

9. Suzuki Nobutaka., *et al*. "IRAK-4 as the central TIR signaling mediator in innate immunity". *Trends in Immunology* 23.10 (2002a): 503-506.

10. Swantek JL., *et al*. "IL-1 receptor-associated kinase modulates host responsiveness to endotoxin". *Journal of Immunology* 164 (2000): 4301-4306.

11. Takeda Kiyoshi and Shizuo Akira. "Toll-like receptors in innate immunity". *International Immunology* 17.1 (2005): 1-14.

12. Umasuthan Navaneethaiyer., *et al*. "Insights into molecular profiles and genomic evolution of an IRAK4 homolog from rock bream (*Oplegnathus fasciatus*): immunogen-and pathogen-induced transcriptional expression". *Fish and Shellfish Immunology* 43.2 (2015): 436-448.

13. Liu Yingying., *et al*. "Lipopolysaccharide-induced gene expression of interleukin-1 receptor-associated kinase 4 and interleukin-1β in rough skin sculpin (*Trachidermus fasciatus*)". *Fish and Shellfish Immunology* 33.4 (2012): 690-698.

14. Wang Guo-Dong., *et al*. "Molecular cloning and responsive expression of macrophage expressed gene from small abalone Haliotis diversicolor supertexta". *Fish and Shellfish Immunology* 24.3 (2008): 346-359.

15. Suzuki Nobutaka., *et al*. "Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4". *Nature* 416.6882 (2002[b]): 750-754.

16. Dunne Aisling., *et al*. "IRAK1 and IRAK4 promote phosphorylation, ubiquitination, and degradation of MyD88 adaptor-like (Mal)". *The Journal of Biological Chemistry* 291.47 (2016): 24802.

17. Deepika A., *et al*. "Toll-pathway in tiger shrimp (*Penaeus monodon*) responds to white spot syndrome virus infection: evidence through molecular characterisation and expression profiles of MyD88, TRAF6 and TLR genes". *Fish and Shellfish Immunology* 41.2 (2014): 441-454.

18. Hannon Gregory J and John J Rossi. "Unlocking the potential of the human genome with RNA interference". *Nature* 431.7006 (2004): 371-378.

19. Tomari Yukihide and Phillip D Zamore. "Perspective: machines for RNAi". *Genes and Development* 19.5 (2005): 517-529.

20. Kwang Jimmy. "Oral vaccination of baculovirus-expressed VP28 displays enhanced protection against white spot syndrome virus in *Penaeus monodon*". *PloS one* 6.11 (2011): e26428.

21. Rajeshkumar S., *et al*. "Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV)". *Fish and Shellfish Immunology* 26.3 (2009): 429-437.

22. Sun Piera S., *et al*. "Evaluation of methods for DNA delivery into shrimp zygotes of *Penaeus* (Litopenaeus) vannamei". *Aquaculture* 243.1-4 (2005): 19-26.

23. Taxman Debra J., *et al*. "Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown". *RNA Therapeutics.* Humana Press, (2010): 139-156.

24. Shekhar Mudagandur S and Yuanan Lu. "Application of nucleic-acid-based therapeutics for viral infections in shrimp aquaculture". *Marine Biotechnology* 11.1 (2009): 1-9.

25. Su Jianguo., *et al*. "A key gene of the RNA interference pathway in the black tiger shrimp, *Penaeus monodon*: identification and functional characterisation of Dicer-1". *Fish and Shellfish Immunology* 24.2 (2008): 223-233.

26. de Fougerolles A. "VORNLOCHER HP MARAGANORE J". *Lieberman Journal* (2007): 443-453.

27. Sambrook J and D W Russell. "Molecular cloning: a laboratory manual". New York, Cold Spring Harbor Lab. Press (2001).

28. Schmittgen Thomas D and Kenneth J Livak. "Analyzing real-time PCR data by the comparative CT method". *Nature Protocols* 3.6 (2008): 1101-1108.

29. Sreedharan K., *et al*. "Ontogeny and expression analysis of tube (interleukin-1 receptor-associated kinase-4 homolog) from *Penaeus monodon* in response to white spot syndrome virus infection and on exposure to ligands". *Agriculture Gene* 3 (2017): 21-31.

30. Yu Dongmei., *et al*. "Construction and identification of a vector expressing RNA interference aimed at the human cyclinD1 gene and its expression *in vitro*". *Chinese Journal of Clinical Oncology* 4.5 (2007): 338-342.

31. Chen Weizao., *et al*. "RNA silencing: A remarkable parallel to protein-based immune systems in vertebrates?". *FEBS Letters* 579.11 (2005): 2267-2272.

32. Chen Xiuhui., *et al*. "RNA interference-based therapy and its delivery systems". *Cancer and Metastasis Reviews* 37.1 (2018): 107-124.

33. Downward, Julian. "RNA interference". *Bmj* 328.7450 (2004): 1245-1248.

34. Cottrell Tricia R and Tamara L Doering. "Silence of the strands: RNA interference in eukaryotic pathogens". *TRENDS in Microbiology* 11.1 (2003): 37-43.

35. Lee Y., *et al*. "microRNA maturation: Stepwise processing and subcellular localization". *EMBO Journal* 21 (2002): 4663-4670.

36. Robalino Javier., *et al*. "Induction of antiviral immunity by double-stranded RNA in a marine invertebrate". *Journal of Virology* 78.19 (2004): 10442-10448.

37. Wu Wenlin., *et al*. "Antiviral phagocytosis is regulated by a novel Rab-dependent complex in shrimp *Penaeus japonicus*". *The Journal of Proteome Research* 7.01 (2008): 424-431.

38. Xu Jianyang., *et al*. "Silencing shrimp white spot syndrome virus (WSSV) genes by siRNA". *Antiviral Research* 73.2 (2007): 126-131.

39. Elbashir Sayda M., *et al*. "RNA interference is mediated by 21- and 22-nucleotide RNAs". *Genes and Development* 15.2 (2001): 188-200.

40. Miyagishi Makoto., *et al*. "Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells". *The Journal of Gene Medicine: A Cross-disciplinary Journal for Research on the Science of Gene Transfer and its Clinical Applications* 6.7 (2004): 715-723.

41. Rubinson Douglas A., *et al*. "Corrigendum: A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference". *Nature Genetics* 39.6 (2007): 803-804.

42. Mu Chuang., *et al*. "Long non-coding RNAs (lncRNAs) of sea cucumber: large-scale prediction, expression profiling, non-coding network construction, and lncRNA-microRNA-gene interaction analysis of lncRNAs in *Apostichopus japonicus* and *Holothuria glaberrima* during LPS challenge and radial organ complex regeneration". *Marine Biotechnology* 18.4 (2016): 485-499.

43. Yu JY., *et al*. "RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells". *Proceedings of the National Academy of Sciences of the United States of America* 99 (2002): 6047-6052.

44. Brummelkamp T R., *et al*. "A system for stable expression of short interfering RNAs in mammalian cells". *Science* 296 (2002): 550-553.

45. Lye Elizabeth., *et al*. "IRAK-4 kinase activity is required for IRAK-4-dependent innate and adaptive immune responses". *European Journal of Immunology* 38.3 (2008): 870-876.

46. Zhang Linlin., *et al*. "Transcriptome analysis reveals a rich gene set related to innate immunity in the Eastern oyster (Crassostrea virginica)". *Marine Biotechnology* 16.1 (2014): 17-33.

47. Suzuki Nobutaka., *et al*. "IL-1 receptor-associated kinase 4 is essential for IL-18-mediated NK and Th1 cell responses". *The Journal of Immunology* 170.8 (2003): 4031-4035.

48. Bennett Joshua and Daniel T Starczynowski. "IRAK1 and IRAK4 as emerging therapeutic targets in hematologic malignancies". *Current Opinion in Hematology* 29.1 (2022): 8.

49. Einav Yulia., *et al*. "shRNA-mediated RNA interference as a tool for genetic synthetic lethality screening in mouse embryo fibroblasts". *FEBS Letters* 579.1 (2005): 199-202.

50. Gao Yulong., *et al*. "Effective inhibition of infectious bursal disease virus replication *in vitro* by DNA vector-based RNA interference". *Antiviral Research* 79.2 (2008): 87-94.

51. Zheng Cuihong., *et al*. "Gene silencing efficiency of shRNA expression vectors targeting Cx43 *in vitro*". *Frontiers of Medicine in China* 3.2 (2009): 130-135.

52. Hou Fujun., *et al*. "RNAi knock-down of shrimp *Litopenaeus vannamei* Toll gene and immune deficiency gene reveals their difference in regulating antimicrobial peptides transcription". *Developmental and Comparative Immunology* 44.2 (2014): 255-260.

53. Wang KH., *et al*. "RNAi knock-down of the *Litopenaeus vannamei* Toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with Vibrio harveyi". *Developmental and Comparative Immunology* 34.1 (2010): 49-58.

54. Sun Yujia., *et al*. "Dual RNA-seq reveals the effect of the flgM gene of *Pseudomonas plecoglossicida* on the immune response of *Epinephelus coioides*". *Fish and Shellfish Immunology* 87 (2019): 515-523.

55. Das Rekha., *et al*. "Captive maturation studies in *Penaeus monodon* by GIH silencing using constitutively expressed long hairpin RNA". *Aquaculture* 448 (2015): 512-520.

56. Krishnan P. "Silencing Wssv Genes in *Penaeus monodon* Using DNA Vector Based RNAi Constructs: Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Ph. D. (Fish Biotechnology)". Diss. Central Institute of Fisheries Education, (2008).

57. Somboonwiwat Kunlaya., *et al*. "Differentially expressed genes in hemocytes of Vibrio harveyi-challenged shrimp *Penaeus monodon*". *BMB Reports* 39.1 (2006): 26-36.

58. De Lorgeril Julien., *et al*. "A relationship between antimicrobial peptide gene expression and capacity of a selected shrimp line to survive a Vibrio infection". *Molecular Immunology* 45.12 (2008): 3438-3445.

59. Wang Pei-Hui., *et al*. "The shrimp NF-κB pathway is activated by white spot syndrome virus (WSSV) 449 to facilitate the expression of WSSV069 (ie1), WSSV303 and WSSV371". *PloS one* 6.9 (2011): e24773.

60. Watthanasurorot Apiruck., *et al*. "A mammalian like interleukin-1 receptor-associated kinase 4 (IRAK-4), a TIR signaling mediator in intestinal innate immunity of black tiger shrimp (*Penaeus monodon*)". *Biochemical and Biophysical Research Communications* 417.1 (2012): 623-629.

61. Ellett Justin D., *et al*. "Toll-like receptor 4 is a key mediator of murine steatotic liver warm ischemia/reperfusion injury". *Liver Transplantation* 15.9 (2009): 1101-1109.

62. Takeda Kiyoshi and Shizuo Akira. "TLR signaling pathways". *Seminars in Immunology* 16.1 (2004).

63. Seki Ekihiro and David A Brenner. "Toll-like receptors and adaptor molecules in liver disease: update". *Hepatology* 48.1 (2008): 322-335.

64. Medvedev Andrei E., *et al*. "Cutting edge: expression of IL-1 receptor-associated kinase-4 (IRAK-4) proteins with mutations identified in a patient with recurrent bacterial infections alters normal IRAK-4 interaction with components of the IL-1 receptor complex". *The Journal of Immunology* 174.11 (2005): 6587-6591.

65. Somboonwiwat Kunlaya., *et al*. "Localization of anti-lipopolysaccharide factor (ALFPm3) in tissues of the black tiger shrimp, *Penaeus monodon*, and characterization of its binding properties". *Developmental and Comparative Immunology* 32.10 (2008): 1170-1176.

66. Ponprateep Sirikwan., *et al*. "Gene silencing reveals a crucial role for anti-lipopolysaccharide factors from *Penaeus monodon* in the protection against microbial infections". *Fish and Shellfish Immunology* 32.1 (2012): 26-34.

67. Shanthi S and B Vaseeharan. "cDNA cloning, characterization and expression analysis of a novel antimicrobial peptide gene penaeidin-3 (Fi-Pen3) from the haemocytes of Indian white shrimp Fenneropenaeus indicus". *Microbiological Research* 167.3 (2012): 127-134.

68. Picard Capucine., *et al*. "Pyogenic bacterial infections in humans with IRAK-4 deficiency". *Science* 299.5615 (2003): 2076-2079.

69. Koziczak-Holbro Magdalena., *et al*. "IRAK-4 kinase activity is required for interleukin-1 (IL-1) receptor-and toll-like receptor 7-mediated signaling and gene expression". *Journal of Biological Chemistry* 282.18 (2007): 13552-13560.

70. Dechamma Mundanda Muthappa., *et al*. "Expression of Toll-like receptors (TLR), in lymphoid organ of black tiger shrimp (*Penaeus monodon*) in response to Vibrio harveyi infection". *Aquaculture Reports* 1 (2015): 1-4.

71. Amparyup Piti., *et al*. "Two prophenoloxidases are important for the survival of Vibrio harveyi challenged shrimp *Penaeus monodon*". *Developmental and Comparative Immunology* 33.2 (2009): 247-256.

72. Söderhäll Irene., *et al*. "An ancient role for a prokineticin domain in invertebrate hematopoiesis". *The Journal of Immunology* 174.10 (2005): 6153-6160.

73. Natt Francois. "siRNAs in drug discovery: target validation and beyond". *Current Opinion in Molecular Therapeutics* 9.3 (2007): 242-247.