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Role of Thymidine Kinase Gene in White Spot Syndrome Virus

AK Soniyapriyadharishni[1]*, and PB Ramesh Babu[2]

[1]Research Scholar, Dept. of Bioinformatics, Bharath University, Chennai, Tamil Nadu, India.
[2]Professor, Head of the Dept., Dept. of Bioinformatics, Bharath University, Chennai, Tamil Nadu, India.

ABSTRACT

This paper explores the possibility of protecting *Penaeus monodon* against white spot syndrome virus (WSSV) infection via interference RNA technology by oral administration of bacterially expressed WSSV VP28dsRNA. Shrimp were given dsRNA orally by two methods. In the first method, pellet feed was coated with inactivated bacteria containing over expressed dsRNA of the WSSV VP28 gene, and in the second method, pellet feed was coated with VP28dsRNA chitosan complex nanoparticles. The treated shrimp were orally challenged with WSSV by feeding WSSV-infected tissue. The experiment was conducted for 30 days. The dsRNA-treated shrimp challenged with WSSV showed higher survival compared to control shrimp. Sixty-eight percent survivals is observed in shrimp fed coated with inactivated bacteria containing dsRNA of the WSSV VP28 gene whereas 37% survival is observed in shrimp fed with VP28dsRNA chitosan complex nanoparticle coated feed. The WSSV caused 100% mortality in shrimp fed with pellet feed coated with inactivated bacteria with empty LITMUS38i vector. At the end of the experiment, the tissue samples prepared from randomly selected shrimp that survived were analyzed via reverse transcriptase-polymerase chain reaction and Western blot analysis for WSSV. The samples were negative for WSSV. Based on the present data and the advantages of dsRNA, it was believed that oral administration of crude extract of bacterially expressed VP28dsRNA was a potential therapeutic agent against WSSV infection of shrimp.

Keywords: Thymidine kinase, gene, white spot syndrome, virus

Corresponding author

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INTRODUCTION

The recent discoveries of RNA interference and related RNA silencing pathways have revolutionized the understanding of gene regulation. RNA interference has been used as a research tool to control the expression of specific genes in numerous experimental organisms and has potential, as a therapeutic strategy to reduce the expression of problem genes. At the heart of RNA interference lies, a remarkable RNA processing mechanism that is now known to underlie many distinct biological phenomena. The selective and robust effect of RNA interference on gene expression makes, it a valuable research tool, both in cell culture and in living organisms because synthetic dsRNA introduced into cells induce suppression of specific genes of interest. RNA interference may also be used for large-scale screens that systematically shut down each gene in a cell, which can help to identify the components necessary for a particular cellular process or an event such as cell division. Exploitation of the pathway is also a promising tool in Biotechnology, Medicine and Genetics. RNA interference (RNAi) is a general name given to a gene-silencing process that is induced by double-stranded RNA (dsRNA).

White spot syndrome virus (WSSV) is the causative agent of white spot disease and is responsible for several economic losses in shrimp culture industry worldwide. This virus was first reported in Thailand as an accidental infection in laboratory reared shrimp in early 1994. This virus can survive and remain infective in sea water for 4-7 days without a host. WSSV is responsible for 100% mortality within a few days after onset of the infection and is a serious threat to the shrimp culture industry worldwide [1]. White spot syndrome (WSS) is a viral infection of penaeid shrimp. Since, White spot syndrome virus (WSSV), has a large DNA virus [2] with genome size ranging from 292 to 307 kb [3]. Its morphological characteristics [2], biological properties [4] and molecular data [5-6], strongly suggest that WSSV is a distinct virus , that does not belong to any of the currently recognized virus families. Given the uniqueness of WSSV, a better understanding of its molecular biology adequately interpret the nature of the virus and its molecular pathogenesis.

MATERIALS AND METHODS

Shrimp, *Penaeus monodon* (10-15g body weight), were collected from grown-out ponds or sea and were maintained in 1000 litre fibre glass tanks with air lift biological filters at room temperature (27-30ºC) with salinity between 20 and 25 parts per thousand (ppt). Natural seawater was used in all the experiments. It was pumped from Bay of Bengal near Chennai and allowed to sediment to remove the sand and other suspended particles. The seawater was first chlorinated by treating the seawater with sodium hypochlorite at concentration of 25 ppm and then dechlorinated by vigorous aeration, before being passed through a sand filter and used for the experiments. The animals were fed with aritifical pellet feed (CP feed, Thailand). Temperature and pH were recorded; salinity was measured with a salinometer and dissolved oxygen was estimated Winkler method. The animals were kept in this tank for five days for accumulation prior to experiments. From the experimental animals, five from a group of thirty were randomly selected and screened for the WSSV by PCR using the primer designed by Sahul Hameed et al. [7]. Only healthy animals were used for the experiments.

WSSV infected shrimp, Penaeus monodon with prominent white spots were collected from shrimp farms located near Nellore, Andhra Pradesh. And the hemolymph was drawn directly from the heart of infected shrimps using sterile syringes. The pooled hemolymph was centrifuged at 3000rpm for 20 minutes at 4°C. The supernatant fluid was then re-centrifuged at 8000rpm for 30 minutes at 4° C and the final supernatant fluid was filtered through a 0.4 µm filter. The filtrate was then stored at -20°C for infectivity experiments. Before, storage the total protein contents was determined.

The WSSV was propagated and purified from experimentally infected marine shrimp following the procedures adopted by Sahul Hameed et al. [7]. Fifty adult shrimp (*Penaeus monodon*) of 15-20 g body weight were placed into five tanks for WSSV propagation and isolation. Each shrimp was injected intramuscularly with the hemolymph of WSSV infected shrimps (300µg of total protein per animal). The shrimps were examined at the interval of 8 hrs for signs of morbidity and mortality. Moribund shrimps were removed and hemolymph was collected. The pooled hemolymph were frozen and thawed thrice and centrifuged at 8000 × g for 30min at 4°C. The supernatant was recentrifuged at 8000 × g for 30 min at 4ºC and the final supernatant fluid was filtered through 0.4 µm filter. The presence of WSSV in the hemolymph was checked by PCR using published primers of Sahul Hameed et al. [7] and the filtrate was then stored at -20ºC for infectivity studies.

Extraction of WSSV-DNA

WSSV-DNA from the purified viral particles of WSSV was extracted by Proteinase K method described by Wang et al. [8] with some modifications. A viral suspension of 200 µl was transferred to 1.5 ml polypropylene centrifuge tubes. Triton X-100 was added to a final concentration of 0.02% and the tube was incubated at room temperature for 10 minutes to remove the viral envelopes. Protinase K and EDTA were added to give final concentration of 100 µg/ ml and 5mM, respectively. After digestion at 65ºC for 1 hr, 0.05 ml of 10% sodium lauryl sarcosine (SLS) was added and incubation was continued at 65ºC for an addition one hour. Finally the DNA was extracted with phenol/chloroform/isoamyl alcohol mixed in the ratio of 25:24:1, precipitated with ethanol and dissolved in TE buffer (10 mM Tris HCL, 1 mM EDTA, pH 7.6). The viral DNA obtained was quantified by measuring optical absorbance at 260 nm using UV spectrophotometer (Hitachi). The DNA was used as the template for PCR amplification of structural gene of WSSV.

PCR Amplification of structural gene of WSSV

The viral structural gene TK was amplified using primers designed based on already published sequences [9]. To carry out cloning and sequencing the primers were designed with two restriction sites i.e. *BamHI in* forward and *EcoR1* in reverse primers. The sequences of the primers with the restriction sites and the corresponding annealing temperatures are given below in Table 1**.** The amplification of the TK gene was performed in thermal mini-cycler (Eppendrof, Germany) as given in Table 2 for WSSV.

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Table 1 Sequence of the primers

Primer	Product size	Sequence(5'-3')	Annealing temperature
WSSV-F	634 bp	CGCGGATCCGATGGCTGGTCGTGTAGAGCT	68ºC
WSSV-R	634 bp	CGCGAATTCCTAACCGCACCTGTCACCTCC	66ºC

Table 2 Optimized PCR parameters for amplification of TK gene of WSSV

PCR Parameter	Temperature(ºC)	Duration
i)Initial denaturation	95	40 Seconds
ii)35 Cycles of	95	40Seconds
Denaturation	57	1 Minute
Annealing	72	50 Seconds
Extension	72	50 Seconds
iii)Final extension	72	10 Minutes

Cloning of WSSV structural gene

The WSSV structural genes were cloned into LITMUS38i vector as shown in Fig. 1.

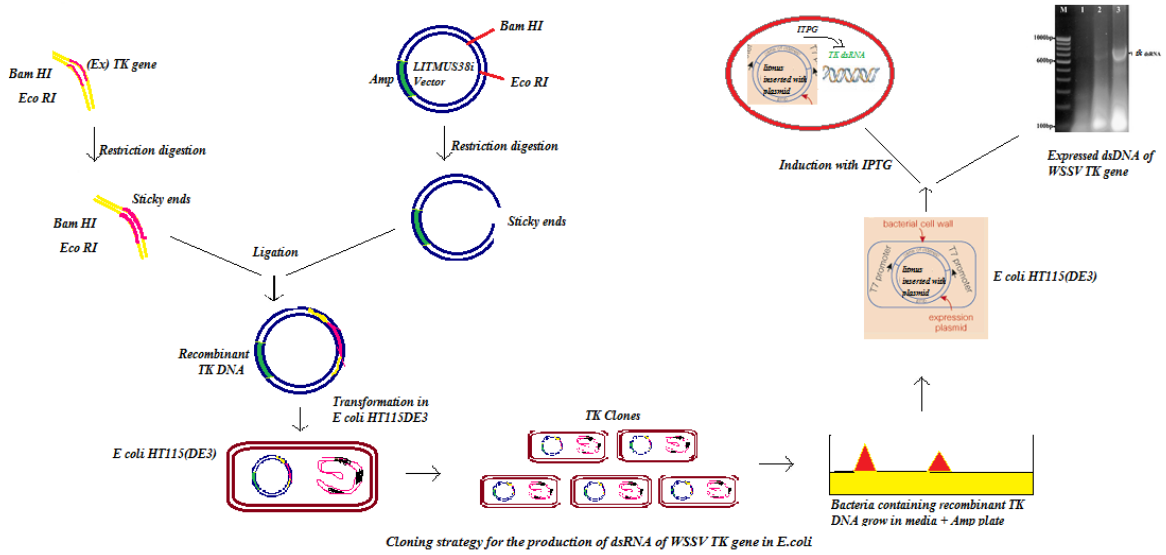

Fig. 1: Cloning strategy for the production of dsRNA of WSSV TK gene in E.coli

Preparation of structural genes for cloning

The amplified PCR products containing TK gene was eluted from agarose gel. The PCR product was purified from agarose gels using a commercially available DNA purification kit (Bangalore Genei, India). Reagents used in elution procedure were: binding buffer, glass concentrate, wash buffer, salt reduction buffer and distilled deionized water. Synthesis of double-stranded RNA using LITMUS38i (Logical in vitro Transcription, Multiple Unique Sites) vector by both *in vivo* and *in vitro* methods is accomplished by simultaneous transcription of both strands of template DNA with T7 RNA Polymerase. The LITMUS38i vectors use the consensus wildtype T7 promoter sequence, ensuring maximum yield of transcript. Both

feature the pUC origin for high plasmid DNA yields, the M13 origin for production of single-stranded template for sequencing or mutagenesis, and ampicillin resistance. Additionally, the polylinkers are inserted in-frame with the lacZ α-fragment, allowing screening for cloned inserts by α-complementation. A fragment corresponding to the gene of interest was cloned into a LITMUS38i vector between two T7 promoters and was transformed into a bacterial strain (HT115[DE3]) carrying IPTG-inducible expression of T7 RNA Polymerase, which lacks the double-strand-specific RNase III. LITMUS 38i are specifically designed for the purpose of in vivo dsRNA synthesis.

Restriction digestion and ligation of DNA

Restriction enzyme digestions were performed using *BamHI* and *EcoRI* enzymes in the buffers recommended by the manufacturer (New England Biolabs, MA, USA). Restriction enzyme digestions were performed as follows:

DNA (1-3µg) (vector and insert)	5 µl
Buffer (10X)	2 µl
Restriction Enzyme (2-3 units/µg of DNA)	1 µl
Deionized water	12µl

The above reaction mixture was incubated at 37°C for 2-3 hours. The completion of digestion of DNA was monitored by agarose gel electrophoresis. The digested DNA was purified on QIAquick PCR columns and used for ligation with the appropriate digested vector.

Ligation of digested vector and insert DNA was performed as follows:

Vector DNA (50 ng)	1 µl
Insert DNA (20-50ng)	1 µl
Ligase buffer (10X)	1 µl
10 mM ATP	1 µl
T4 DNA ligase (10 Weiss units)	1 µl
Deionized water	5 µl

Ligations were performed at 16°C for 15 hours. Molar ratios of 4:1 or 6:1 of insert to vector were used in the ligation reactions.

Ligation mixture was transformed into DH5α according to the method of Hanahan [10] using $CaCl_2$ for the preparation of competent cells. The resultant transformants were selected on LB agar plates supplemented with ampicillin. The transformants of the respective cloned genes were subjected to plasmid extraction for the transformation into the HT115(DE3) expression host.

The plasmid containing a target gene insert is transformed into *E. coli* HT115(DE3) by standard techniques [11]. The genotype of E. coli HT115 is F^-, mcrA, mcrB, IN(rrnD-rrnE)1, lambda-, rnc14::Tn10 (DE3 lysogen: lacUV5 promoter-T7 polymerase). T7 polymerase gene expression is driven by the lacUV5 promoter, which is IPTG inducible. rnc14 encodes RNase

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III. On disruption by Tn10, rnc14 is unable to degrade the dsRNA expressed *in vivo*. Tn10 carries a Tetracycline resistant gene; therefore, two antibiotics were selected, Ampicillin for the plasmid and Tetracycline for the transposon.

RESULTS

The major WSSV structural gene TK (Thymidine Kinase) was selected for the production of corresponding dsRNA. The amplified WSSV structural gene was cloned into LITMUS38i vector (Fig. 2). The recombinant plasmid restricted with *BamHI* and *EcoRI* revealed the presence of viral gene in the LITMUS38i vector as shown in Fig. 3. The resulting plasmid was transformed into the HT115(DE3) RNase III-deficient *E. coli* strain. HT115(DE3) was chosen to take advantage of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase gene contained within a stable insertion of a modified lambda prophage λ DE3. Here the result reports the use of an RNase III-deficient and IPTG induction of HT115(DE3) to produce virus-derived dsRNA and its application to protect shrimp against WSSV. *E. coli* HT115(DE3) transformed with recombinant plasmid, was induced with 1 mM IPTG to produce dsRNA. After induction with IPTG at 37°C, dsRNA was isolated from induced and non-induced LITMUS38i-cloned with WSSV structural gene in *E. coli* HT115(DE3) (containing TK gene) and LITMUS38i *E. coli* HT115(DE3) (containing empty vector). The isolated dsRNAs' corresponding to structural gene was analyzed by agarose gel electrophoresis (Fig. 4). A band of dsRNA corresponding to TK gene was observed in 1.2% agarose gel electrophoresis in the IPTG-induced *E. coli* HT115(DE3) LITMUS38i cloned with structural gene (Fig. 5. lane 3), whereas no dsRNA band was found at the same position in both the non-induced *E. coli* HT115(DE3) LITMUS38i cloned with structural gene and induced empty LITMUS38i *E. coli* HT115DE3 (Fig. 5. lanes 1 and 2). This result showed that TK dsRNAs was expressed in *E. coli*. (Fig. 5). This technology enabled to produce uniform quality of the dsRNA of WSSV structural genes and was used to protect the shrimp against WSSV.

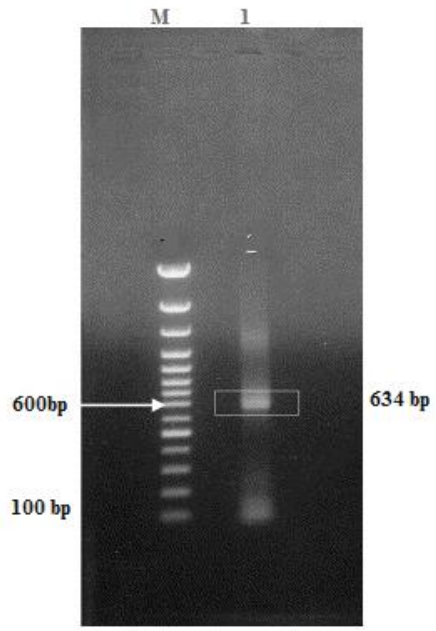

Fig. 2 Agarose gel showing the amplification of WSSV structural gene using gene specific primers. M-100bp Marker; 1- TK gene.

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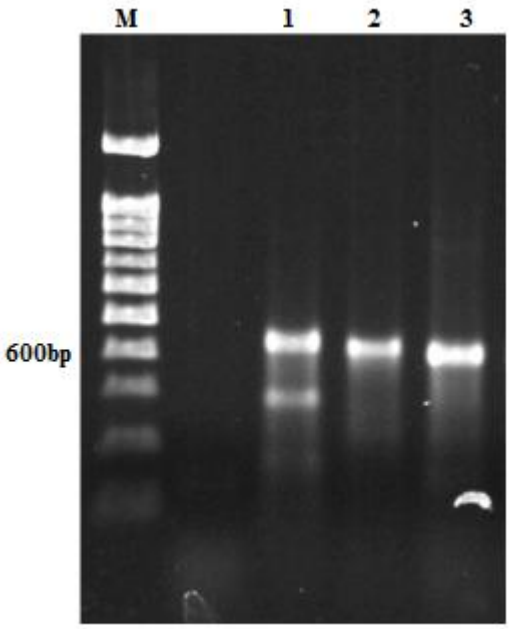

Fig. 3 Restriction profile of TK recombinant LITMUS38i plasmid with *BamHI* and *EcoRI* showing inserts of WSSV structural gene. M-1bp marker; 1-Uncut LITMUS38i; 2- restricted LITMUS38i vector; 3- TK recombinant plasmid.

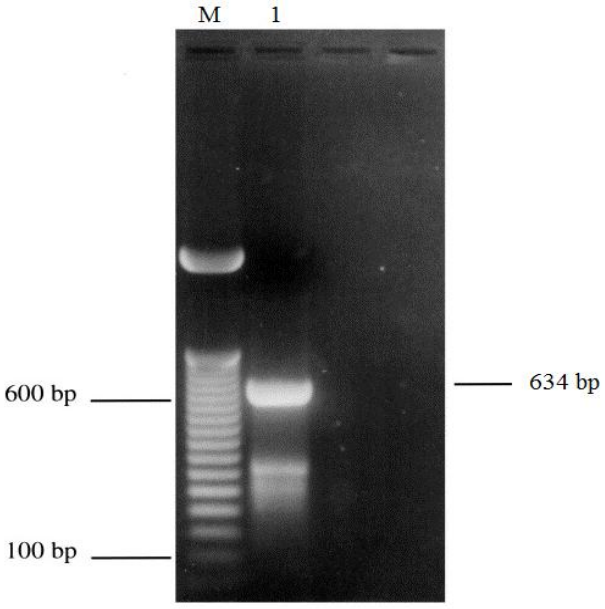

Fig. 4 PCR confirmation of recombinant plasmid

Fig. 5 Bacterially expressed dsRNA of WSSV TK gene. Lanes M-100bp Marker; 1- control dsRNA from HT115(DE3) transformed with LITMUS38i vector without any insert; 2- uninduced dsRNA of TK from HT115(DE3) with LITMUS38i encoding TK gene; 3- induced dsRNA of TK from HT115(DE3) with LITMUS38i encoding TK gene.

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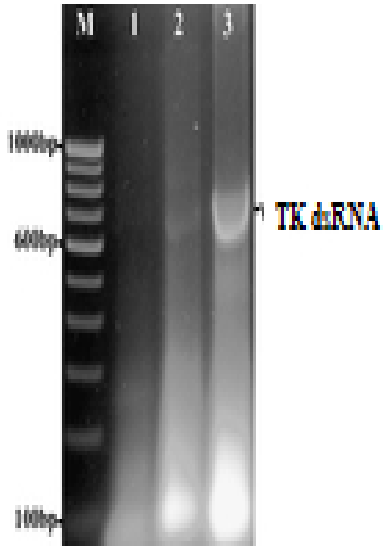

Fig. 5 Bacterially expressed dsRNA of WSSV TK gene.

DISCUSSION

RNAi is a process of sequence specific, posttranscriptional gene silencing found naturally in plants, yeast and animal cells. This gene silencing mechanism is suggested as an extremely useful antiviral therapy with many advantages. In this paper, it is aimed at the major WSSV structural gene TK to produce corresponding dsRNA because the proteins expressed by them are abundantly present in the WSSV. The silencing efficiency of the three bacterially expressed dsRNA corresponding to WSSV structural genes (VP28, VP24, VP19) was investigated and it was found that all of them except VP26dsRNA could effectively reduce the mortality of shrimp against WSSV. This is the first *in vivo* analysis of WSSV structural genes function in WSSV infection by RNAi using bacterially expressed long dsRNA. This work presents evidence that the use of bacterially expressed long dsRNA corresponding to WSSV structural gene TK sequences could act as an effective mechanism against WSSV in *P. monodon*. The results establish that no interference with VP26dsRNA and control bacterial dsRNA extracted from HT115(DE3) transformed with LITMUS38i vector without any insert. LITMUS38i vector without any insert yielding 163 bp long dsRNA was used and considered as irrelevant dsRNA control. In the present study, the results showed that the degree of protection afforded by specific dsRNAs varies between different viral genes targeted. Although the reason for these differences was not addressed in the present study, it seems likely that gene silencing effect of dsRNA is known to vary strongly with the targeted position of the mRNA.

From this study, it is apparent that dsRNA inhibition of viral infection is a very powerful tool for inhibition of WSSV and has a high therapeutic potential. A simple, fast, safe and inexpensive procedure is proposed for an efficient production of viral derived dsRNA using RNase III deficient bacteria, with a view to providing a practical control of virus diseases. When applied on shrimps, the dsRNAs specific for viral genes induce a potent and virus specific antiviral response that results in highly effective control of viral disease. The dsRNAs caused specific degradation of the viral mRNA and resultant protection against virus

infections. Although RNAi is an extremely powerful tool for gene silencing as antiviral therapy against WSSV, its mechanism of action still remains unknown.

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