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Original Article

Envelope Protein Gene *VP466*-a Target for PCR Detection of White Spot Syndrome Virus in Shrimp

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White spot syndrome virus (WSSV) is an enveloped and double-stranded DNA virus that belongs to the family *Nimaviridae* and genus *Whispovirus*, causing white spot disease (WSD) in shrimp. The virus is highly virulent and leads to 100% mortality within 10 days. Detection of WSSV and segregation of infected brood shrimp, post-larvae and cultured shrimp are currently considered as containment strategies to reduce the spread of WSD. This investigation describes a polymerase chain reaction method to detect WSSV in WSD infected cultured shrimp targeting *VP466* gene encoding the large structural protein in virus particle. *In silico* homology analysis of the primer pair designed in this work clearly identified WSSV *VP466* gene sequence with 100% specificity. A total of 16 shrimp samples from 16 farms were selected, where 6 shrimp samples were with characteristics WSD spot and 10 shrimp samples were asymptomatic. Among the 16 shrimp samples, 12 showed PCR positive amplifications for major envelope protein gene *VP466*. Sequencing of the amplicons followed by homology searching using BLAST further confirmed the presence of WSSV. Phylogenetic analysis of *VP466* gene sequences showed its close proximity to the WSSV strain of Indian origin. The present study demonstrates that the envelope protein *VP466* gene as a specific target for PCR detection and characterization of WSSV in WSD infected and carrier shrimps.

Keywords: Cultured shrimp, White spot syndrome virus (WSSV), *VP466* gene, PCR

Introduction

Shrimp aquaculture industry is growing very rapidly in Bangladesh and contributes nearly 5% to the national GDP (gross domestic product)[1]. However, the major obstacle for shrimp trade from Bangladesh to worldwide is the white spot disease (WSD). WSD is caused by white spot syndrome virus (WSSV), the only member of *Nimaviridae* family and *Whispovirus* genus[2]. WSD is the major devastating disease that cause the loss of millions of dollars in Bangladesh every year[3]. The virus has a wide host range and is highly virulent and leads to 100% mortality within 10 days of infection in case of farmed shrimp. In Bangladesh, WSSV was first detected in Cox's Bazar in 1994[4] and after that the shrimp aquaculture in Bangladesh has been seriously affected by WSSV resulting in extensive economic loss every year[5-6]. WSSV is considered as the most grave problem for shrimp aquaculture in Asia[6,7], where it surpassed all other disease agents as the leading cause of production losses[8]. WSD becomes endemic and regarded as one of the most important constraints to the shrimp aquaculture industry's sustainability and further expansion in Bangladesh[9]. Therefore, there is an urgent need to find out a way to minimize the incidence of WSD in Bangladesh. Molecular techniques based accurate and rapid detection of WSSV in WSD shrimps from endemic areas can be a potential solution to overcome the burden of WSSV transmission. Nowadays, there are several methods (western blot, single step, multiplex or nested PCR, hybridization assays, transmission electron microscopy, histopathological examinations, loop mediated isothermal detection etc.) for the detection of WSSV in cultured and captured shrimps and other crustaceans[7]. Many scientists have reported diagnostic protocols targeting *VP28* gene and ORF167 specific detection of WSSV by PCR technique[7,10-11] and our unpublished data. Previous study reported that VP466, the major structural protein of WSSV plays critical role in infection of virus and subsequent invasion in to host cell whereas suppression of this gene is directly involved in the inhibition of WSSV infection in shrimp[12]. Therefore, this study focused on the development of a PCR based detection technique targeting the major envelope protein gene *VP466* of WSSV in shrimps and implementation of this technique for the screening of WSSV infection in brood and post-larvae of shrimp which greatly assists shrimp farmers for the betterment of production.

Materials and Methods

Shrimp sample collection

A total of 16 shrimp samples from 16 different shrimp farms were collected from Satkhira district of Bangladesh in September 2013. The collected samples were immediately transported to the Microbial Genetics and Bioinformatics Laboratory (*www.microbialgen.du.ac.bd*) for further analysis by maintaining cold chain effectively.

DNA extraction

Total DNA was extracted from tissue samples of WSD suspected shrimps by automated DNA extraction system (MaxWell 16®

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Tissue DNA Purification kit; AS 1030, Promega, USA), according to manufacturer's instruction. The concentration and purity of the extracted DNA was measured by Nano-Drop 2000 (Thermo Scientific, USA).

PCR primer design

PCR primer pair (forward and reverse) was designed targeting the highly conserved region of *VP466* gene encoding envelope protein in WSSV using primer3 plus software to amplify 1032 bp amplicon. *In silico* cross match analysis of the selected primers (WSSV VP466F: 5´-CTCCAAAACCTTCAGCTTCG-3´ and WSSV VP466R: 5´-CCCAGGACTTTTCGAATGAAG-3´) were performed by BLAST search using blastn algorithm and its quality was further assessed by IDT OligoAnalyzer 3.1(at: *https://sg.idtdna.com/calc/analyzer*). The primers were synthesized at Integrated DNA Technologies, USA.

Optimization of PCR reaction targeting VP466 gene

PCR reaction with newly designed primers, WSSV VP466F and WSSV VP466R was optimized in this study. 25µl PCR reaction mix was prepared using 2X GoTaq® Hot Start Colourless Master Mix (Promega, USA), 400 nm of each of the primers and about 100 ng of the total extracted DNA as template. PCR reaction was initiated by preheating at 94ºC for 5 min followed by 30 cycles of 60s at 94ºC, 60s at 53ºC, 90s at 72ºC and final extension of 7 min at 72ºC.

PCR amplification and sequencing

Extracted DNA from samples was screened for the presence of WSSV by conventional PCR using the newly designed primer pair WSSV VP466F and WSSV VP466R for major envelope protein gene, *VP466*. The PCR amplicon was analyzed by 0.8% agarose gel containing ethidium bromide and visualized under UV-illuminator (AlphaImager, USA). The amplified PCR product was purified using Wizard® SV Gel and PCR Clean Up System (Promega, USA). The purified PCR product was subjected to automated cycle sequencing using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystem®, USA) according to the manufacturer's instruction and those sequences were analyzed in ABI genetic Analyzer (Applied Biosystem®, USA). The sequences obtained in this study have been deposited in NCBI GenBank database under the accession numbers KJ817421 and KJ817422.

Phylogenetic study of WSSV based on VP466 gene

The *VP466* gene sequences of Bangladesh origin were searched for homology using NCBI Blast, and other homologous prototypic WSSV *VP466* gene sequences were retrieved from the database. Multiple sequence alignment of the subjects and prototypic sequences was performed by MEGA 5.2[13] and aligned session was analyzed to assess phylogenetic relatedness. The structural protein of another shrimp virus *Hepatopancreatic parvovirus* (EU290601) was used as out-group of the phylogenetic tree. The best statistical model for the phylogenetic tree was evaluated according to BIC (Bayesian Information Criterion) scoring model followed by analysis for model selection[14]. Neighbour-joining tree was constructed based on the best nucleotide substitution model with 1000 bootstrap replication value to evaluate the robustness of the constructed tree.

Results and Discussion

WSSV is considered as a rapidly replicating and extremely virulent shrimp pathogen. In the present investigation, we have sampled 16 shrimp samples from Satkhira district of which 6 samples showed characteristic spots (Figure 1) of WSD and the others 10 were asymptomatic of WSD. However, these spots are not always present, and since similar spots can be produced by some bacteria in high alkalinity and stressed condition, they are not considered a reliable sign for preliminary diagnosis of this disease[2].

Designed primer pair amplifies VP466 gene of WSSV

In this study, a new primer pair targeting *VP466* gene was developed and primer pair showed 100% specificity with reference WSSV *VP466* gene sequences during *in silico* analysis. Data from wet laboratory based experiment (conventional PCR) accessing the specificity of introducing primer pair well corroborated with the *in silico* data. Newly designed primer pair was able to amplify specific target (partial *VP466* gene) from crude DNA (shrimp + viral DNA) extracted from WSD suspected shrimp samples (Figure 2).

Figure 1. *Characteristics WSD specific spot appeared on exoskeleton of shrimp (indicated by arrow head). The photograph of the WSD suspected shrimp sample (SH-14) was taken during sample collection from Satkhira district in Bangladesh.*

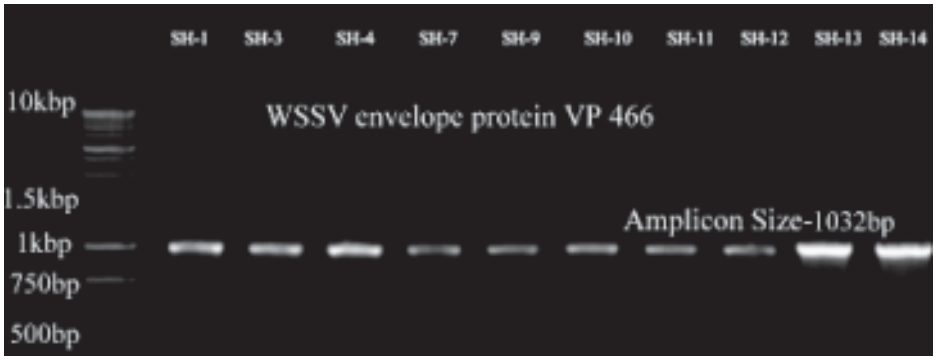

Figure 2. *PCR amplification (representative) of the partial VP466 gene of WSSV confirms the presence of WSSV in the cultured shrimp of coastal region in Bangladesh. Here ~1032 bp amplicon of VP466 gene was amplified and visualized in 0.8% agarose gel where 1kb ladder (Promega, USA) was used.*

Detection of WSSV in symptomatic and asymptomatic cultured shrimp

PCR with the designed primer targeting the *VP466* gene of WSSV was able to amplify the target gene in 12 shrimp samples out of 16 studied samples with a desired amplicon of 1032 bp (Figure 2, Table 1). Positive PCR amplicons were detected in 6 symptomatic WSD shrimp samples and in 6 asymptomatic shrimp samples implicating the efficiency of the primers in detection of WSSV.

Table 1. *Samples profile indexing the PCR amplification status of VP466 gene of WSSV and appearance of characteristics White Spot Disease (WSD) specific spot*

Sample ID	Appearance of characteristics WSD specific spot	PCR amplification status of the *VP466* gene of WSSV
SH-1	Yes	Yes
SH-2	No	No
SH-3	Yes	Yes
SH-4	No	Yes
SH-5	No	No
SH-6	Yes	Yes
SH-7	No	Yes
SH-8	No	No
SH-9	No	Yes
SH-10	No	Yes
SH-11	Yes	Yes
SH-12	No	Yes
SH-13	No	Yes
SH-14	Yes	Yes
SH-15	No	No
SH-16	Yes	Yes

Circulatory WSSV is related to WSSV isolated from India

At the onset of the research presented in this research article, only limited molecular information was available to WSSV. Sequencing of the amplified amplicon 1,032 bp followed by homology searching using BLAST (blastn algorithm) further confirmed the presence of WSSV in studied samples. Phylogenetic analysis using the *VP466* gene sequences clearly indicated the close phylogenetic affiliation of local circulatory WSSV strain from Bangladesh is homologous to the WSSV strains isolated from India (Figure 3)[15]. Hossain *et al*. reported the presence of WSSV (34%) by PCR in *Penaeus monodon* broods taken from hatcheries on the east coast of India and its first incidence was recorded in 2001[6]. As the phylogenetic evidence of our isolated WSSV strain shared 99% sequence homology with other isolates from Indian origin in Blast analysis. So, based on the data of isolate's ancestral genetic route, it can be predicted that the possible intrusion of this virus from our neighbour countries.

PCR-based rapid detection of WSSV: A strategy for WSD control

PCR targeting the *VP466* gene of the WSSV is very efficient and reliable to detect the WSSV circulating in the coastal region shrimp farms of Bangladesh. The present study confirms the WSSV circulation in the Satkhira district, extensive shrimp culture zone of the southwest part in Bangladesh. To overcome this burden, WSSV in brood shrimp and post-larvae must be detected prior culturing. It was previously proposed that a PCR-based monitoring strategy for detecting WSSV infection in cultured shrimp can be an efficient management practice that would allow for the detection of viral infection in early stage[10]. As *VP466* gene encodes a major structural protein which is responsible for the infection and invasion of the virus to its host, so a method of WSSV detection targeting *VP466* gene might be an effective strategy for rapid detection of infectious viral particles. Thus, PCR technique based rapid identification of WSSV, segregation and rejection of infected stocks (Brood and PL) is a demand of time in the management of this disease for sustainable shrimp farming in Bangladesh.

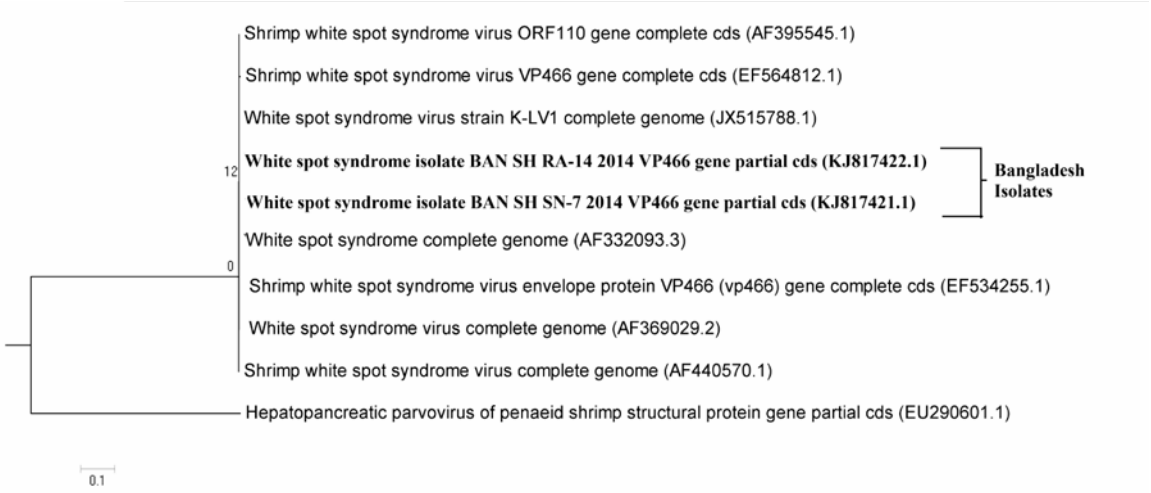

Figure 3. *Neighbor-joining tree based on VP466 sequences of WSSV showing close agreement among WSSV VP466 gene sequences from Bangladesh and other countries. Here WSSV VP466 sequences of local origin (shown as bold) clustered within monophyletic clade of other WSSV VP466 sequencing flanked by VP466 sequences of Indian origin while structural protein of other shrimp associated virus like hepatopancreatic parvovirus branched out earlier. This phylogeny was re-constructed in MEGA 5.2.*

Acknowledgements

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