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SHORT COMMUNICATION

Variations of structural protein sequences among geographical isolates of white spot syndrome virus

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Abstract White spot syndrome virus (WSSV) is the causative agent of a disease that causes severe mortalities in cultured shrimp worldwide. The sequence of five structural genes of an Indian isolate of WSSV was compared with sequences from other WSSV isolates deposited in GenBank. Among the structural genes analyzed, the sequences of vp28 and vp19 had maximum divergence with nucleotide changes at ten different positions from twenty eight submissions for vp28 and six changes from seventeen submissions for vp19. The vp68, vp26, and vp281 genes were found to be highly conserved between isolates.

Keywords WSSV · Structural protein · vp28 · vp26 · vp19 · vp68 · vp281 · vp466

Introduction

White spot syndrome virus, a major pathogen of cultured shrimp first appeared in 1990s in Taiwan and China (Zhang et al. 1994; Wongteerasupaya et al. 1995; Nadala et al. 1998). Since then WSSV has spread rapidly to shrimp farming areas around the world causing heavy economic losses. White spot syndrome virus not only infects shrimp but also has a much wider host range which includes other invertebrate aquatic organisms such as crab, crayfish, and fresh water prawn, *Macrobrachium* sp. (Ramasamy 1995; Lo et al. 1996; Flegel 1997; Wang et al. 1998; Mohan et al. 1998).

WSSV is an enveloped ovoid virus with rod-shaped nucleocapsid with flat ends (Wang et al. 1995). WSSV is a major shrimp pathogen that is highly virulent in penaeid shrimp (Lo et al. 1996; Chen et al. 1997; Flegel 1997) and can result in of up to 90–100 % mortality within 3 to 7 days (Zhan et al. 1998). The virus belongs to the genus *Whispovirus* (Van Hulten et al. 2000; Tsai et al. 2000) under the family Nimiviridae. The complete genome sequence of WSSV has been determined for three different isolates with GenBank Accession numbers AF369029, AF440570, and AF332093 (Van Hulten et al. 2001, Yang et al. 2001, Lo and Kou 2001) for

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viruses isolated from Thailand, Taiwan, and China, respectively. The genome of WSSV is approximately 300 kb in length with 180 putative open reading frames. Most of the open reading frames of WSSV encode certain structural genes. Structural proteins of viruses are classified as envelope and non-envelope proteins. Envelope proteins play a vital role in virus entry assembly and release (Hsiao et al. 1999; Lin et al. 2000; Chiu and Chang 2002). Neutralization experiments of antibodies against six WSSV envelope proteins (vp28, vp26/P22, vp466, vp281, vp68, and vp292) revealed that four envelope proteins (vp28, vp466, vp68, and vp281) play major roles in the initial steps of WSSV infection in shrimp (Wu et al. 2005). There is little genetic variation in the structural genes of WSSV from different geographical locations. (Lo et al. 1999; Moon et al. 2003; Satish et al. 2004; You et al. 2004). In this study, sequences of the genes encoding for six major structural proteins of WSSV from different geographical locations were compared with a WSSV isolate from south India.

Materials and methods

Total DNA extraction

Black tiger shrimp (*Penaeus monodon*) naturally infected with WSSV were obtained from a shrimp farm in Cochin, India. The samples were immediately transferred to sterile polythene bags and transported to the laboratory in ice. On arrival in the laboratory, the total DNA was extracted from pleopods as described previously (Moon et al. 2003).

PCR amplification of structural genes

The viral structural protein genes: vp28, vp26, vp19, vp68, vp281, and vp466 were amplified using six primer pairs (Table 1) designed based on the sequences of a Taiwanese isolate of WSSV (Yang et al. 2001). The primers were designed so as to include both the upstream and down stream sequences of the genes to be amplified. The PCR reaction was carried out in a final reaction volume of 50 µl containing 0.5 µM each of forward and reverse primer, 100 ng of DNA, 200 µM dNTP (MBI Fermentas, USA), 2.5 mM $MgSO_4$, 1× *Pfu* buffer, 1.25 U of *Pfu* DNA polymerase (MBI Fermentas), and remaining autoclaved Millipore water. The amplification was performed in a PTC-150 Mini cycler (MJ Research, USA). The gene amplification conditions were as follows: primary denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, and final extension at 72 °C for 5 min. The PCR amplified products were analyzed on 1.5 % agarose gel along with DNA molecular weight marker and documented using a gel documentation system (Alpha Imager 1220, Alpha Innotech, USA).

Cloning of PCR products in pTZ57R/T

An A-overhang was added to the PCR product for cloning into TA vector. The reaction was carried at 72 °C for 45 min in a reaction volume of 30 µl containing 26 µM dATP, 1× *Taq* buffer, 1.25 units of *Taq* DNA polymerase (MBI Fermentas), and the PCR product. The A-overhang added PCR product was purified using MinElute Gel purification kit (Qiagen, Germany). InsTA clone PCR cloning kit (MBI Fermentas) was used to clone the PCR product to pTZ57R/T vector. Ligation was set at 4 °C overnight in a 30 µl reaction volume using 5 U of T4 DNA ligase (MBI Fermentas). Competent *E. coli* JM109 cells were used as host system for transformation. The transformed cells were incubated at 37 °C overnight on LB agar plates containing 40 µl of 0.1 M IPTG and 4 µl of 20 mg/ml X-gal in order to enhance blue white selection by α-complementation. Plasmids were isolated from the overnight culture by alkaline lysis method (Sambrook et al. 1989).

The recombinant clones having the specific insert in the forward orientation was then sequenced from both directions to get a contiguous sequence in an automated sequencer (ABI prism, model 377, Version 3.0). The nucleotide sequences obtained and the deduced amino acid sequences were analyzed using bioinformatics tools. Sequences were compared with database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) blastn and blastp (Altschul et al. 1990).

Table 1 Primers used for amplification of WSSV structural genes

Sl. No.	Gene	Sequence (5′–3′)	Product size (bp)
1	vp28	**F**-5′TTCACGAGGTTGTCATCACC3′	799
		R-5′TGGTATAAATTTCCTCAATTGTTTT3′	
2	vp26	**F**-5′TGGATCCAACCAACACGTAA3′	713
		R-5′CTTGTATTTTTATTCAAACAAAACCTT3′	
3	vp19	**F**-5′GGTGTCCTGACAAAAAACCGTA3′	477
		R-5′TTGTCCCTGATGTTGTGTTTTC3′	
4	vp68	**F**-5′AACACTTCTGGGTGAAACCTA3′	323
		R-5′TCGGACAAATAAAAGAATTGGAA3′	
5	vp281	**F**-5′AGAAACCCAAGGAAGGGTTG3′	927
		R-5′TTTGTTTGCAACACCCTTTT3′	
6	vp466	**F**-5′TCAAGACCAGTACACGTAATTTGAT3′	1471
		R-5′ TGATGTCTGAGCCATTTTTATTATG3′	

Results and discussion

Six envelope protein genes of WSSV: vp28, vp26, vp19 vp68, vp281, and vp466 on PCR amplification and sequence analysis revealed genes of expected size 615, 615, 366, 207, 846, and 1401 bp, respectively. These sequences were submitted to the GenBank with the assigned accession numbers EF534254, EF534253, EU012447, EF534252, EF534251, and EF534255, respectively. The gene sequences were compared with reported nucleotide and amino acid sequences (Tables 2, 3, 4, 5, 6, 7). The nucleotide sequence of vp28 was compared with twenty eight GenBank sequences from various geographical locations (Table 2). Only one sequence reported from India (Accession No.DQ013883) had 100 % homology with the WSSV isolate reported in this study. There was an A → G nucleotide substitution at position 125 for all the other reported sequences, which translated into a substitution of aspartic acid for glycine. The nucleotide change at position 125 was present only in two WSSV isolates from India including the isolate reported in this paper indicating that this mutation is restricted to India. A Chinese isolate (Accession No.AY682926) and a Korean isolate (Accession No. AF380842) had a nucleotide substitution at position 234 and 444, respectively, from T → C both of which did not translate to change of amino acid. Two sequences from China (AF502435 and AY249434) had an additional mutation at nucleotide position 119 from G → A which translated into an amino acid substitution from arginine to histidine. A Thailand isolate (Accession No. EF194079), a Chinese isolate (Accession No. DQ979320), and an Indian isolate (Accession No. AY422228) had three nucleotide substitutions including that at position 125. The Thailand isolate had nucleotide substitution at position 113 from T → A and at 434 from T → C which translated into change of amino acid from valine to glutamic acid and leucine to proline, respectively. The Chinese isolate had T → C nucleotide substitutions at positions 306 and 536 with change of amino acid from valine at both positions to proline and alanine, respectively. The Indian isolate had substitutions from T → C at positions 483 and 485 with change of amino acid from phenylalanine to serine in the first position, while the second position was conserved. There was nucleotide substitutions at ten different positions with the isolates studied, seven of which translated to a change of amino acid.

The nucleotide sequence of the gene encoding for envelope protein vp26 was compared with 16 reported sequences and it was revealed that there was 100 % similarity with fourteen of the reported sequences (Table 3). The G → A nucleotide substitution at the nucleotide position 575 for the Chinese isolate (Accession No. AY220746) resulted in a change of amino acid from arginine to lysine, while a nucleotide change for the Vietnamese isolate (Accession No. AJ551446) at nucleotide position 345 from T → C was conserved and did not translate to change of amino acid. The vp26 gene was found to be highly conserved among isolates from different geographical locations with just two nucleotide variations from the sixteen isolates compared.

The vp19 sequence was compared with seventeen reported sequences, and it was found that there was 100 % sequence similarity with four WSSV isolates reported from India (DQ681071), Vietnam (AY160771),

Table 2 Nucleotide and amino acid substitution in vp28 gene

Accession nos.	Position of mutation	Base substitution	Change in amino acid
DQ013883	Nil	Nil	Nil
DQ902658, DQ681069, DQ098011, AY324881, AF332093[a], AF272979, AY249443, AY249442, AY249441, AY249440, AF227911, AJ551447, DQ013882, DQ013881, DQ007315, AF369029[a], AY168644, AF440570[a], AF173993, AY873785	125	A → G	D → G
AF380842	125	A → G	D → G
	444	T → C	Nil
AF502435, AY249434	125	A → G	D → G
	119	G → A	R → H
AY682926	125	A → G	D → G
	234	T → C	Nil
EF194079	125	A → G	D → G
	113	T → A	V → E
	434	T → C	L → P
DQ979320	125	A → G	D → G
	306	T → C	V → P
	536	T → C	V → A
AY422228	125	A → G	D → G
	483	T → C	F → S
	485	T → C	Nil

[a] Complete genome sequence of WSSV

Table 3 Changes in vp26 gene sequence in GenBank

Accession nos.	Position of mutation	Base substitution	Change in amino acid
AF308164, AF332093[a], AF272980, AY249439, AY249438, AY249437, AY249436, AY249435, AY422230, AF380841, AF369029[a], AF440570[a], AF173992, DQ681070	Nil	Nil	Nil
AY220746	575	G → A	R → K
AJ551446	345	T → C	Nil

[a] Complete genome sequence of WSSV

Table 4 Changes in vp19 gene sequence in GenBank

Accession nos.	Position of mutation	Base substitution	Change in amino acid
DQ681071, AY160771, AF440570[a], AJ937860	Nil	Nil	Nil
AY316119, AY873786	57	T → C	Nil
AF332093[a], AF402997	196	C → T	P → S
AY249448, AY249447, Ay249446, AY248445, AY249444, AY220744, AF369029[a]	218	T → A	V → D
AY422227	25	T → C	S → P
	44	T → C	V → A
DQ902655	25	T → C	S → P
	44	T → C	V → A
	287	C → T	T → M

[a] Complete genome sequence of WSSV

Table 5 Changes in vp68 gene sequence in GenBank

Accession nos.	Position of mutation	Base substitution	Change in amino acid
AF332093[a], AF369029[a], AF440570[a], AF4114664	Nil	Nil	Nil

Table 6 Changes in vp281 gene sequence in GenBank

Accession nos.	Position of mutation	Base substitution	Change in amino acid
AF332093[a], AF369029[a], AF440570[a], AF411634	807	A → T	Nil
DQ979321	807	A → T	Nil
	841	C → A	P → T
AY517490	807	A → T	Nil
	843	G → A	Nil

Table 7 Changes in vp466 gene sequence in GenBank

Accession nos.	Position of mutation	Base substitution	Change in amino acid
AF332093[a], AF440570[a], AF395545	Nil	Nil	Nil
AF369029[a]	1212	A → T	K → N

[a] Complete genome sequence of WSSV

Taiwan (AF440570), and Mexico (AJ937860) (Table 4). Point mutations: T → C, T → A and C → T were found at six different nucleotide positions with 13 reported sequences. To ascertain whether the variation at nucleotide level resulted in change in at amino acid level, the protein sequences of vp19 gene were also compared. The T → C point mutation at position 57 (Accession No. AY316119 and AY873786) did not result in change in amino acid while a C → T mutation at position 196 (Accession No. AF332093 and AF402997) resulted in change amino acid from proline to serine. A T → A mutation at position 218 (Accession No. AY249444 to AY249448 and AF369029) had a change in amino acid from valine to aspartic acid. Two reported sequences from India, (Accession Nos. AY422227 and DQ902655), gave a T → C mutation at nucleotide positions 25 and 44 that translated into change of amino acids; serine to proline and valine to alanine, respectively. The WSSV isolate with Accession No. DQ902655 had a C → T extra substitution at position 287 other than at nucleotide positions 25 and 44 that translated into methionine, a change in the amino acid from threonine.

The vp68 sequence was 100 % similar with the four reported sequences (Table 5). Comparison of vp281 sequence with six published sequences revealed A → T, C → A, and G → A mutations at three different nucleotide positions (Table 6). The A → T mutation at nucleotide position 807 present in all the reported sequences did not translate into change of amino acid. While the C → T mutation at position 841 (Accession No. DQ979321) translated into change in amino acid from proline to threonine. The G → A mutation at position 843 (Accession No. AY517490) did not translate into change of amino acid. Three reported nucleotide sequence of vp466 had 100 % homology with the sequence reported in the present study (Table 7). There was an A → T nucleotide substitution at position 1212 for a Thailand isolate, which translated into a substitution of lysine to arginine.

In the present study, the sequences of six envelope proteins were compared with reported sequences of WSSV isolates. A common ancestor for the virus isolate could not be designated as there was variation in sequence with one envelope protein or other. The vp26 gene of the Taiwanese, Thailand, Chinese, and Korean isolates were identical at both nucleotide and amino acid levels, while the vp28 gene was 99 % identical at nucleotide level and 100 % identical at amino acid level (Moon et al. 2003). Sequence of five WSSV structural proteins (vp15, 19, 24, 26, and 28) from 5 different geographical WSSV isolates when compared with four WSSV isolates reported showed that the sequence of each gene were identical among the 9 isolates with the exception of vp28 gene of a Korean isolate with 1 base change from that of other WSSV isolates. However, this base change did not result in change of amino acid sequence (You et al. 2004). The extent of

sequence variation among the isolates suggests that newer strains of WSSV are evolving that may have variations in their virulence potential. Chang et al. 2002 attributed the point mutations in the envelope proteins to the non-infectious nature of the virus isolate.

Highly conserved DNA and amino acid sequence of the structural proteins of WSSV provide the basis for the development of sensitive nucleic acid and antibody-based detection methodology for WSSV (You et al. 2004). Many PCR and protein-based diagnostic tests target the envelop proteins. However, variations in sequence of envelop proteins may result in false negative results. Recombinant vp28 and vp19 were found to have potential for use as injected vaccine or oral vaccine (Witteveldt et al. 2004). The variations at nucleotide and amino acid in the structural genes can result in difference in the protection offered by these proteins when used as vaccines. Hence further studies need to be done to ascertain the efficacy of these proteins in providing protection against the virus.

Author contribution Research concept and the experiments were performed by TCJ, LAR and RJ. KVL and PKS analyzed the data and reviewed the manuscript. All authors approved the final manuscript.

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Conflict of interest The authors declare that they have no conflict of interest in the publication.

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