Detection by PCR and Comparison of Sequences of VP28 Gene of White Spot Syndrome Virus Affecting *Penaeus monodon* in India

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ABSTRACT

White spot syndrome virus (WSSV) infection has resulted in severe production and economic losses in shrimp culture globally. Specific PCR primers were designed for the amplification of VP28 gene encoding for an envelope protein in WSSV. VP28 gene of SDDL 18/04-Indian isolate of WSSV was amplified by PCR and the PCR product was sequenced and submitted to GenBank (AY873785). The nucleotide sequence (AY873785) was compared with the VP28 gene sequences in the GenBank database (NCBI). Eleven sequences showed significant alignments with 100% homology which include the sequences from Korea (AY324881), Japan (AY249443), US south Carolina (AY249442), Indonesia (AY249441), China (AY249440, AF332093), Vietnam (AJ551447), Taiwan (AF 440570, AF272979) and The Netherlands (AF1739993, AF369029). However variations were observed in the sequences of five isolates, three from China (AY 249434, AY502435, AY682926) with a homology of 99%, one from Korea (AF380842) with a homology of 99% and one from India (AY 422228) with a homology of 98%.

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INTRODUCTION

White spot syndrome virus (WSSV) belonging to the family nimaviridae is an enveloped, non-occluded, rod-shaped DNA virus infecting penaeid shrimps and other crustaceans (Lightner, 1996). White spot syndrome has been reported in many shrimp growing countries of the world. Since WSSV has a wide geographic distribution and host range, studies on the comparison of morphology, virulence, genomic composition and protein composition among WSSV isolates have been carried out by various researchers (Wongteerasupaya *et al.,* 1996, Kasornchandra *et al*.,1998, Nadala Jr. *et al.,* 1998, Park *et al.,*1998, Lo *et al.,*1999, Wang *et al.*, 1999). VP28 envelope protein of WSSV is reported to play a key role in the systemic infection in shrimp (Van Hulten *et al.,* 2001). The present study was undertaken with an aim to know if there exists any variation in the nucleotide sequences of the VP28 gene of WSSV from different geographical regions.

MATERIALS AND METHODS

Sample collection

WSSV infected *Penaeus monodon* juveniles (6-7 g) collected from a shrimp farm located near Chennai, Tamilnadu, India were used for the study. The sample was fixed in 70% ethyl alcohol and used for polymerase chain reaction (PCR).

PCR Primer designing

The nucleotide sequences of VP28 gene encoding for the envelope protein in WSSV were collected from the GenBank database. Sequence alignments using BLAST A programme showed very few variations among the sequences of VP28 gene submitted from various countries and there is no variation at 5' and 3' end sequences. Based on the 5' and 3' end sequences of VP28 gene, the upper and lower specific PCR primers were designed so as to enable the amplification of complete VP28 gene.

PCR

DNA was extracted from the WSSV infected shrimp sample following the method described by Lo *et al*., (1996). Gills, pleopods or cephalothorax were separated and homogenized individually in NTE buffer. The homogenate was centrifuged at 3000 x g (4^0C) and the supernatant (200µl) was transferred to centrifuge tubes with 600 µl digestion buffer (100 mM Nacl, 10 mM Tris Hcl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulfate, 0.1 mg ml^{-1} proteinase K). After a 2 hr incubation at 65^0C the digest was deproteinised by successive phenol / chloroform / isoamyl alcohol extractions, the DNA were ethanol precipitated and dried. The dried DNA pellets were resuspended in 50µl of TE buffer (Tris-Hcl 100 mM, pH 8.0, 10mM EDTA, pH 8.0). The reaction mixture contained 2µl of template DNA, 1µM of each primer, 200µM of deoxynucleotide trisphosphate and 1.25U of Taq DNA polymerase in PCR buffer (Bangalore Genei Pvt Ltd). The PCR protocol comprised of initial denaturation for 3 min at 95^0C followed by 28 cycles of 30 sec at

95 ^{0}C; 30 sec at 58 ^{0}C; 30 sec at 72 ^{0}C with a final extension of 5 min at 72^{0}C. The PCR products were analysed by standard gel electrophoresis with 100 bp DNA marker using 1% agarose gels stained with ethidium bromide, visualized and documented in a gel documentation unit (Vilber Lourmet, France)

Sequencing of PCR products

The amplified PCR product of VP28 gene of WSSV (SDDL 18/04- Indian isolate) was purified using a silica membrane based column purification kit (Life technologies, USA). The purified PCR product was sequenced with an AB 13100 automated sequencer by a commercial company (Bangalore Genei Pvt., Ltd.).

Sequence submission, comparison and alignment

The sequence information of VP28 gene of SDDL 18/04-Indian isolate was submitted to GenBank (NCBI, USA) and the Accession number is AY873785. The sequence information was compared with the sixteen other sequences in the GenBank database submitted from India and elsewhere using BLAST A and FAST N programmes (NCBI, USA). The multiple nucleotide sequence alignment were carried out using CLUSTAL W programme (MEGA software).

RESULTS

PCR amplification of VP28 gene encoding for an envelope protein in WSSV resulted in a 615 bp product as shown in Figure 1. The details of VP28 gene sequences used for comparison, their GenBank accession numbers and their homology with SDDL

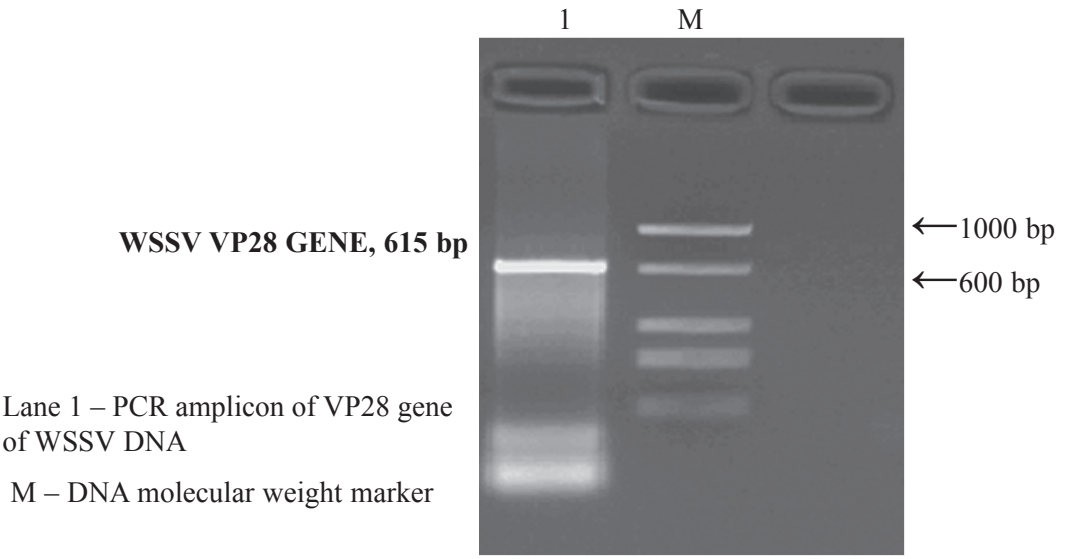

Figure 1. PCR amplification of VP28 gene of WSSV isolate from India.

18/04- Indian isolate are presented in Table.1. Sequence comparison and alignment result using CLUSTAL W programme (MEGA software) is presented in Figure 2. VP28 gene sequences showing variation on comparison with the sequence of SDDL 18/04- Indian isolate are presented in Figure 3.

Table 1. VP28 gene sequences used for comparison, their GenBank Accession numbers and their homology with SDDL 18/04- Indian isolate.

COUNTRY OF ORIGIN	GENBANK ACCESSION NO.	HOMOLOGY %
1. CHINA	AY249440	100
2. CHINA	AY249434	99
3. CHINA	AY502435	99
4. CHINA	AF332093	100
5. CHINA	AY682926	99
6. INDIA	AY422228	98
7. INDONESIA	AY249441	100
8. JAPAN	AY249443	100
9. KOREA	AF 380842	99
10. KOREA	AY324881	100
11. TAIWAN	AF272979	100
12. TAIWAN	AF440570	100
13. THE NETHERLANDS (WSSVTH-1 isolate of Thailand)	AF173993	100
14. THE NETHERLANDS (WSSVTH-1 isolate of Thailand)	AF369029	100
15. USA	AY249442	100
16. VIETNAM	AJ551447	100

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AY873785:61   gctgtatttattgtgatttttaggtatcacaacactgtgaccaagaccatcgaaacccac 120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
AF249434:85   gctgtatttattgtgatttttaggtatcacaacactgtgaccaagaccatcgaaacccgc 144

AY873785:61   gctgtatttattgtgatttttaggtatcacaacactgtgaccaagaccatcgaaacccac 120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
AF502435:61   gctgtatttattgtgatttttaggtatcacaacactgtgaccaagaccatcgaaacccgc 120

AY873785:181  ggatcaggctacttcaagatgactgatgtgtcctttgacagcgacaccttgggcaaaatc 240
               |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
AY682926:181  ggatcaggctacttcaagatgactgatgtgtcctttgacagcgacaccttgggtaaaatc 240

AY873785:421  ccaaagattaacccatcaaaggcctttgtcggtagctccaacacctcctccttcaccccc 480
               ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
AF380842:421  ccaaagattaacccatcaaaggcttttgtcggtagctccaacacctcctccttcaccccc 480

AY873785:481  gtctctattgatgaggatgaagttggcacctttgtgtgtggtaccacctttggcgcacca 540
               ||  | ||||||||||||||||||||||||||||||||||||||||||||||||||||
AY422228:481  gtttttattgatgaggatgaagttggcacctttgtgtgtggtaccacctttggcgcacca 540
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Figure 3. VP28 gene sequences showing variation on comparison with the VP28 sequence of SDDL 18/04- Indian isolate (AY873785).

DISCUSSION

Five major virion proteins have been reported to be present in WSSV. VP26, VP24 and VP15 are present in the nucleocapsid and VP28 and VP19 are located in the envelope. VP28 gene, which encodes for an envelope protein in WSSV, plays a key role in systemic infection in shrimp with WSSV (Van Hulten *et al.,* 2001). Studies on WSSV gene structure and their functions would help to evolve novel diagnostic techniques and new strategies for the control of the virus infection. As PCR is the widely accepted diagnostic method for screening of shrimp for viruses, specific primers were designed to amplify the VP28 gene of WSSV to study the nucleotide sequence information and variations in the VP28 gene of WSSV from different countries. Genetic variation in viruses has significant implication for diagnosis and epidemiology (Morse, 1994). Mutation in the nucleotide sequence can prevent binding of PCR primers to target sequences (Kwok *et al.,* 1990). This leads to false negative PCR results and non-specific PCR products, restricting the use of PCR based diagnostic kits to some strains of the viruses. Although WSSV has a wide geographic distribution, Lo *et al.,* (1999) showed the similarity of WSSV isolates from different geographical regions by amplifying ten different DNA fragments of the entire WSSV genome using ten different PCR primer sets. However, the significance of the amplified region has not been studied. Similarly, PCR amplification of a portion of WSSV gene from China, Japan, Indonesia, Thailand, Malaysia and India has revealed their close relatedness (Kasornchandra *et al.,* 1998). Nucleotide sequence comparison of VP28 gene of Korean isolate with Taiwanese, Thai and Chinese isolates suggested that they have originated from the same ancestor (Moon, 2003). The results of this study, which compared the VP28 gene sequence of an Indian isolate with sixteen other isolates

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WSSV-SDDL18/04India ATG GAT CTT TCT TTC ACT CTT TCG GTC GTG TCG GCC ATC CTC GCC
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ATC ACT GCT GTG ATT GCT GTA TTT ATT GTG ATT TTT AGG TAT CAC
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      AAC ACT GTG ACC AAG ACC ATC GAA ACC CAC ACA GAC AAT ATC GAG
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ACA AAC ATG GAT GAA AAC CTC CGC ATT CCT GTG ACT GCT GAG GTT
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      GGA TCA GGC TAC TTC AAG ATG ACT GAT GTG TCC TTT GAC AGC GAC
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ACC TTG GGC AAA ATC AAG ATC CGC AAT GGA AAG TCT GAT GCA CAG
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ATG AAG GAA GAA GAT GCG GAT CTT GTC ATC ACT CCC GTG GAG GGC
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
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Figure 2. Nucleotide sequence alignment results of VP28 gene of WSSV isolates from different countries.

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WSSV-SDDL18/04      CGA GCA CTC GAA GTG ACT GTG GGG CAG AAT CTC ACC TTT GAG GGA
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ACA TTC AAG GTG TGG AAC AAC ACA TCA AGA AAG ATC AAC ATC ACT
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      GGT ATG CAG ATG GTG CCA AAG ATT AAC CCA TCA AAG GCC TTT GTC
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      GGT AGC TCC AAC ACC TCC TCC TTC ACC CCC GTC TCT ATT GAT GAG
WSSV-India           ... ... ... ... ... ... ... ... ... ... ..T .T. ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      GAT GAA GTT GGC ACC TTT GTG TGT GGT ACC ACC TTT GGC GCA CCA
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ATT GCA GCT ACC GCC GGT GGA AAT CTT TTC GAC ATG TAC GTG CAC
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04       GTC ACC TAC TCT GGC ACT GAG ACC GAG TAA
WSSV-India           ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ...
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Figure 2. (continued)

from different geographical regions also showed that there is not very high variation as the homology ranged from 98%-100%. Unlike earlier studies, the present study has compared the nucleotide sequence of VP 28 gene, which plays significant role in causing WSSV infection, with all the available VP 28 sequences in the Genbank database. It is interesting to note that among the VP28 sequences which showed variation in nucleotide sequence comparison, an Indian isolate (AY 422228) showed lesser homology (98%) compared to the other isolates from distant geographical locations viz., three isolates (AY 249434, AY 502435, AY 682926) from China and one isolate from Korea (AF 380842) with a homology of 99%.

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