Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and Southern blot hybridization

Qiong Wang¹*, Linda M. Nunan**, Donald V. Lightner

Department of Veterinary Science and Microbiology, University of Arizona, Tucson, Arizona 85721, USA

ABSTRACT: White spot syndrome virus (WSSV) is widely distributed in most of the Asian countries where penaeid shrimp are cultured, as well as in some regions of the USA. Six geographic isolates of WSSV—1 each from penaeid shrimp from China, India, Thailand, and the US states of Texas and South Carolina, and 1 isolated from crayfish at the National Zoological Park in Washington, DC—were compared by combining the methods of restriction analysis and Southern blot hybridization. DNA was extracted from purified viruses and then digested with selected endonucleases: AccI, BglII, ClaI, BamHI, EcoRI, HindII, HaeI, SacI and XhoI. The blots were detected with digoxigenin-11-dUTP-labeled WSSV genomic probes: LN4, C42 and A6. No distinctive differences among the 5 WSSV isolates from penaeid shrimp were detected; however, differences in the WSSV isolate from crayfish were observed. A 2.8 kb DNA fragment originating from the crayfish isolate and encompassing the LN4 region was subcloned into pBluescript and sequenced for comparison with the LN4 fragment from the Thailand WSSV isolate. The results indicate that some genomic components of WSSV from different geographic regions share a high degree of homology. This method can be used to distinguish between the WSSV isolate from crayfish and the WSSV isolates from penaeid shrimp.

KEY WORDS: White spot syndrome virus · WSSV · Penaeid shrimp · Restriction analysis · Southern blot hybridization · Genomic variation

INTRODUCTION


Since WSSV has a wide geographic distribution and host range, efforts have been directed to compare morphology, virulence, genomic composition and protein composition among WSSV isolates (Wongteerasupaya et al. 1996, Kasornchandra et al. 1998, Nadala Jr et al. 1998a,b, Park et al. 1998, Lo et al. 1999, Wang et al. 1999). The results have demonstrated that WSSV isolates from different locations are almost identical, although slight differences may exist among some.

The objective of the present study was to compare the genomic composition of 6 geographic isolates of WSSV in order to develop genetic markers that could be used to distinguish different WSSV strains.

### MATERIALS AND METHODS

#### Viruses. The 6 WSSV isolates were derived from shrimp collected from China, India, Thailand, Texas and South Carolina and from crayfish maintained at the US National Zoological Park, Washington, DC (Table 1). The viral quantities were amplified in specific pathogen free (SPF) *Penaeus vannamei* (Wyban et al. 1992) (Oceanic Institute 'Kona' stock, population number UAZ 10-97). Viral extracts were prepared by centrifugation of tissue homogenate of infected shrimp at low speed (500 × *g*). The supernatant fluid was diluted 20 times with 2% saline before injection into SPF shrimp. WSSV infection in the shrimp and crayfish samples and in the experimentally infected SPF shrimp were confirmed by standard procedures using Davidson’s alcohol formalin acetic acid (AFA) fixed tissue followed by routine histological processing, and hematoxylin and eosin-phloxine (H & E) staining (Bell & Lightner 1988, Lightner 1996). The severity of infection was rated according to Lightner (1996).

#### Virus purification. A gradient purification procedure was modified from the method of Bonami et al. (1990). WSSV-infected tissue was collected from the epidermis, stomach, appendages and gills of severely infected shrimp. A Tris-NaCl buffer (TN; 20 mM Tris-Cl, 400 mM NaCl, pH 7.4) was used throughout the purification procedure. The tissue homogenate was first clarified at 500 × *g* and 3000 × *g* sequentially. The viruses in the resultant supernatant fluids were pelleted for 1 h, at 70 000 × *g* (4°C), and then loaded carefully onto the top of 15 to 45% (w/w, in 1 TN) linear Renograin-76® (Solvay Animal Health, Inc., Mendota Heights, MN) gradients. The gradients were centrifuged at 153 200 × *g* for 1.5 h at 4°C. The visible bands were drawn out with syringes, and then centrifuged at 124 100 × *g* for 1.5 h at 4°C to pellet the viruses. Each viral pellet was then resuspended in 500 µl distilled H₂O.

#### DNA extraction. The DNA extraction procedure was modified from the method of Lo et al. (1996a,b). Digestion buffer, 500 µl (100 mM NaCl, 10 mM Tris-HCl, pH 8; 25 mM EDTA, pH 8; 0.5% N-lauryl sarcosine; 124100 × *g* ·etermined for 1.5 h at 4°C to pellet the viruses. Each viral pellet was then resuspended in 500 µl distilled H₂O.

#### Table 1. Description of the WSSV samples collected directly from the original geographic locations

<table>
<thead>
<tr>
<th>WSSV isolate</th>
<th>Identification number</th>
<th>Collection location</th>
<th>Host species</th>
<th>Life stage of host</th>
<th>Wild or cultured</th>
<th>Collection time</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSSV China</td>
<td>UAZ95-116A</td>
<td>Shandong, China</td>
<td><em>Penaeus chinensis</em></td>
<td>Adult</td>
<td>Cultured</td>
<td>May 1995</td>
</tr>
<tr>
<td>WSSV India</td>
<td>UAZ95-314</td>
<td>Visak, India</td>
<td><em>Penaeus monodon</em></td>
<td>Juvenile</td>
<td>Cultured</td>
<td>28 Aug 1995</td>
</tr>
<tr>
<td>WSSV Thailand</td>
<td>UAZ95-38A</td>
<td>Thailand</td>
<td><em>Penaeus monodon</em></td>
<td>Adult</td>
<td>Cultured</td>
<td>27 Feb 1995</td>
</tr>
<tr>
<td>WSSV Texas</td>
<td>UAZ97-85</td>
<td>Texas, Gulf of Mexico</td>
<td><em>Penaeus monodon</em></td>
<td>Adult</td>
<td>Wild</td>
<td>13 May 1997</td>
</tr>
<tr>
<td>WSSV South Carolina</td>
<td>UAZ97-5</td>
<td>South Carolina</td>
<td><em>Penaeus setiferus</em></td>
<td>Adult</td>
<td>Captive wild</td>
<td>8 Jan 1997</td>
</tr>
<tr>
<td>From crayfish</td>
<td>UAZ96-52</td>
<td>National Zoological Park, Washington, DC</td>
<td><em>Orconectes punctimanus</em></td>
<td>Subadult</td>
<td>Captive wild</td>
<td>8 Mar 1996</td>
</tr>
</tbody>
</table>
0.5% mg ml⁻¹ Proteinase K), was added to a 100 µl
viral suspension and incubated for 1 h at 65°C. Then
100 µl of 5 M NaCl and 70 µl of CTAB/NaCl solution
(10% N-cetyl-N, N, N-trimethylammonium bromide in
0.7 M NaCl) was added and incubated at 65°C for
10 min. Afterwards, DNA was extracted with phenol-
chloroform, precipitated with ethanol, and dissolved
into 30 µl H₂O.

Endonuclease digestion of DNA. Nine endonucleo-
ases—AccI, BamHI, BglII, Clal, EcoRI, HaeIII, HindII,
SacI and XhoI—were used to digest the extracted viral
DNA according to the manufacturer’s protocol (Boehr-
ringer Mannheim, now Roche Molecular Biochemicals,
Indianapolis, IN). To ensure complete digestion, each
reaction was performed in a 100 µl volume for more
than 10 h.

Gel electrophoresis. After endonuclease digestion,
DNA was precipitated with ethanol and dissolved in
15 µl H₂O. One µl of 10× RNase Plus™ gel loading
buffer (5 Prime - 3 Prime, Inc., Boulder, CO) was added
to digest RNA in the samples. Electrophoresis was per-
fomed in a 1% agarose gel (containing 0.5 µg ml⁻¹
ethidium bromide) in 0.5×TBE (Tris-Borate-EDTA)
buffer (Sambrook et al. 1989). A digoxigenin-labeled
DNA molecular Marker III (Roche Molecular Bio-
chemicals) was co-electrophoresed to indicate the molecular
weight of the DNA samples.

DNA probe labeling. Three DNA inserts—LN4
(Nunan & Lightner 1997), C42 (GenBank accession
number AF295124) and A6 (GenBank accession num-
ber AF295123)—were labeled with digoxigenin DIG-
11-dUTP by PCR using a Genius™ non-radioactive
DNA labeling and detection kit (Roche Molecular Bio-
chemicals). The C42 clone was developed using a
China WSSV isolate (Table 1), and the LN4 and A6
cloned were from a Thailand WSSV isolate (Table 1).
The C42 and A6 clones were generated from purified
viral DNA of the respective strains by using the standard
cloning procedure for endonuclease digestion,
ligation to a cloning vector and transformation into
competent E. coli bacterial strains (Sambrook et al.
1989). The cloning of the LN4 fragment was accom-
plished by using PCR primers that were designed from
sequence information from another penaeid virus of
shrimp, Baculovirus penaei (BP). This cloning method
is discussed in detail in Nunan & Lightner (1997). Three
pairs of primers, derived from sequence data of the
DNA viral inserts—5’ CTT GTG TCC AGA TGT GTG
3’ & 5’ GGA GAT CCT TCG ACG AAT 3’, and 5’ AGG
TAT AGT GGC TGT TGC 3’ & 5’ CTG GAG AGG
ACA AGA CAT 3’, and 5’ TGT AGC AGC AGA GAA
GAG 3’ & 5’ ACT GCA CCA AAT TGT CCA CC 3’—
were used to generate probes for C42, A6 and LN4
respectively. For each insert, a 100 µl labeling reaction
solution containing H₂O, 10 µl 10× PCR Buffer II,
10 pmol of each dNTP, 10 µl DIG label mix, 200 pmol
MgCl₂ and 1 µl AmpliTaq Gold DNA polymerase (PE
Applied Biosystem, Foster City, CA), as well as 250 ng
DNA template and 250 ng of each primer for C42 and
LN4 or 200 ng DNA template and 100 ng of each
primer for A6, was reacted in a thermal cycler (PE
Applied Biosystem). PCR initiation with a hot start at
94°C for 10 min; continued with 50 cycles of 94°C for
1 min, 55°C for 1 min, and 72°C for 2 min; and ended
with an extension at 72°C for 7 min. The probes were
precipitated with 10 µl 200 mM EDTA (PH 8.0), 11 µl
4 M LiCl, 1 µl 20 mg ml⁻¹ glycogen, and 360 µl absolute
ethanol and then resuspended in 100 µl distilled H₂O.
Using the low molecular mass ladder (Gibco BRL,
Grand Island, NY) as a standard, the concentration of
probes was adjusted to 50 ng µl⁻¹ in H₂O. The speci-
ficity of these probes was tested by in situ hybridiza-
tion according to Bruce et al. (1993) and dot blot
hybridization according to the application manual
from Roche Molecular Biochemicals.

Southern blot hybridization. The Southern blot
hybridization procedure was modified from Sambrook
et al. (1989). DNA was blotted onto a positively
charged nylon membrane (Roche Molecular Bio-
chemicals), then hybridized with DIG-labeled DNA probe
(50 ng ml⁻¹) under stringent conditions (wash with
0.1 x SSC/0.1% SDS at 65°C for 15 min, 2 times).
A chemiluminescent kit CSPD® (Roche Molecular Bio-
chemicals) was applied to visualize the probe signal on
X-ray film. To rehybridize the same membrane with
different probes, the probe was denatured from the
membrane with alkaline solution (0.2 N NaOH, 1% SDS)
at 37°C for 30 min.

Gene cloning and sequencing. The endonuclease
Clal was used to digest the viral DNA of the crayfish
isolate and the vector pBluescript II SK+ according to
the manufacturer’s protocol (Roche Molecular Bio-
chemicals). The digested pBluescript plasmids were
dephosphorylated using alkaline phosphatase (Roche
Molecular Biochemicals) and then ligated with viral
restriction fragments using T4 DNA ligase (Roche
Molecular Biochemicals). Transformation was per-
formed using JM109 High Efficiency Competent Cells
(1 x 10⁸ cfu µg⁻¹ DNA, Promega, Madison, WI) fol-
lowing the manufacturer’s protocol. Resulting white
bacterial clones were streaked for isolation and then
grown to a large quantity in Terrific Broth (12 g Bacto-
trypton, 24 g yeast extract, 4 ml glycerol, 100 ml 0.17 M
KH₂PO₄/0.72 M K₂HPO₄ in 1 H₂O). Plasmids were
quickly extracted by using Insta-Mini-Prep™ (5 Prime
- 3 Prime, Inc.) for initial screening. For preparing plas-
mids for sequencing, a modified mini alkaline-lysis/
polyethylene (PEG) precipitation procedure was adapted
from the PRISM™ Ready Reaction DyeDeoxy™ Termi-
nator Cycle Sequencing kit protocol of Applied Bio-
systems, Inc. DNA sequencing was performed on an Applied Biosystem (ABI) 373 DNA sequencer using fluorescently labeled dideoxynucleotides and Taq polymerase at the Division of Biotechnology at the University of Arizona.

RESULTS AND DISCUSSION

All shrimp samples used for viral extraction were confirmed to be heavily infected with WSSV by histological examination and in situ hybridization (data not shown). The sizes of labeled probes LN4, C42, and A6 were 750, 450 and 1600 bp, respectively (Fig. 1). All these probes at similar concentration showed similar high specificity in the dot blot and in situ hybridization assays (data not shown).

The 5 shrimp isolates and 1 crayfish isolate of WSSV gave strong DNA hybridization signals for the areas of the genome studied. All 3 probes, LN4, C42, and A6, reacted with each of the 6 isolates of WSSV. No apparent differences were detected among the 5 geographic isolates originally obtained from shrimp. These 5 WSSV isolates presented very similar band patterns when their DNA was digested with endonucleases and hybridized with the 3 specific genomic probes. Our findings are consistent with the results reported in a previous publication (Lo et al. 1999) in which several PCR products were analyzed using the restriction fragment length polymorphism (RFLP) assay, with little or no band differences among WSSV infected shrimp from various geographic locations. However, the genomic fragments studied in this paper account for only about 1% of the total viral genome (200–300 kb), and it is possible that genomic differences may occur in other parts of the genome.

Differences in DNA composition between the crayfish isolate of WSSV and other WSSV isolates were detected through several combinations of restriction analysis and Southern blot hybridization, as summarized in Table 2. Of these combinations, ClaI for DNA digestion, and LN4 or C42 for hybridization, best dis-

<table>
<thead>
<tr>
<th>Genomic probe</th>
<th>LN4</th>
<th>C42</th>
<th>A6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crayfish WSSV</td>
<td>6</td>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
<td>Other WSSV</td>
<td>15</td>
<td>0.1</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2. Different lengths of viral DNA fragments detected in the crayfish WSSV and other geographic isolates of WSSV by Southern blot analysis when using the following combinations of endonuclease digested DNA (BglII, ClaI, EcoRI, SacI, and XhoI) and genomic probes (LN4, C42 and A6)
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tonguished the crayfish isolate of WSSV (Fig. 2 & 3).
Since differences occurred with several endonucle
ases, it is not likely that the difference was due to a
point mutation randomly arising in a viral population.
However, it is possible that a host species may play a
role in the selection of a mutant within a viral popula-
tion. Further studies are needed to conclude whether
the differences observed resulted from geographic
isolation or from host selection. That the crayfish iso-
late of WSSV is slightly different from other WSSV iso-
lates is also supported by previous virulence and pro-
tein composition studies (Wang et al. 1999, 2000). More
research is needed to correlate information on the
genomic sequence, protein data and virulence. The
method used in this paper, however, can be employed
to distinguish crayfish WSSV from the other WSSV iso-
lates. The technique may also be useful for examina-
tion of new WSSV isolates.

The Southern blot hybridization results indicate that
the regions flanking the C42 and A6 fragments have
fewer variations than the region flanking the LN4 frag-
ment. The 2.8 kb Clal fragment from the crayfish iso-
late of WSSV (GenBank AF178573), which overlaps a
portion of the LN4 region, was compared with the LN4
sequence data from the Thailand isolate (Fig. 4). The
LN4 fragment itself was almost identical in the 2 iso-
lates, except in the primer region, which was not unex-
pected since the LN4 primer was originally developed
using the sequence data from a different penaeid virus
of shrimp, BP (Nunan & Lightner 1997). Mismatched
base pairs between the 2 fragments are not unex-
pected. The LN4 fragment was sequenced from a
PCR product, and single base pair substitutions and
frame shift errors are inherent in the PCR process (Bell
1989). The Southern blot results (Figs. 2 & 3) and
the sequence information from the 2.8 kb Clal and LN4
fragments (Fig. 4) (Nunan & Lightner 1997) de-

Fig. 3. Southern blot hybridization with the C42 probe.
Lanes 1, 3, 5, 7, 9, and 11 show DNA of WSSV digested with
Clal from the following geographic regions: 1, China; 3, India;
5, Thailand; 7, South Carolina; 9, crayfish from Washington,
DC; 11, Texas. Lanes 2, 4, 6, 8, and 10 show undigested DNA
of WSSV from the following regions: 2, India; 4, Thailand;
6, South Carolina; 8, crayfish from Washington, DC; 10, Texas

Fig. 4. Sequence comparison of the overlapping region of the LN4 PCR fragment from the Thailand WSSV isolate (upper
sequence) and the 2.8 kb Clal fragment from the crayfish WSSV isolate (lower sequence in bold). The underlined region in the
LN4 sequence denotes the forward primer. Homologous base pairs are shaded
monstrate that the variation in the crayfish WSSV genome is located about 3.5 kb from the Clal site of the 2.8 kb fragment. The significance of this region to pathogenicity has not yet been determined. When the full sequence of the flanking region of the LN4 fragment is known, a PCR assay may be developed to differentiate different WSSV strains.

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