J. Fish Pathol., 10(2) : 87~95

Partial genomic sequence of baculovirus associated with white spot syndrome (WSBV) isolated from penaeid shrimp *P. chinensis*

Chong Kyung Kim, Sang Gyu Sohn*, Moon Soo Heo*, Tae Ho Lee, Hong Ki Jun and Kyung Lib Jang†

Department of Microbiology, Pusan National University, Pusan 609-735
**Pathology Division, NFRDA, Pusan 619-900, Korea*

Baculovirus associated with white spot syndrome (WSBV) is the causative agent of a disease with high mortalities and causes severe damage to shrimp cultures. In this study, we analyzed a recombinant clone (E3) obtained from a viral genomic library to characterize the causative agent in diseased shrimp *Penaeus chinensis* with white spot syndrome. According to the analysis of nucleotide sequence of E3, this clone did not showed considerable sequence homology with those of other known viruses, including baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV), indicating that WSBV is a novel virus causing a serious disease in *P. chinensis*. Based on the sequence of E3 clone, a pair of PCR primers was designed. After 30 cycles of amplification, a specific product of the expected size was detected only if the total nucleic acids extracted from the diseased shrimp was used as a template DNA, suggesting that this method can be used to diagnose the virus infection in diseased shrimp.

Key words : Genome analysis, *Penaeid chinensis*, PCR diagnosis, White spot syndrome

Recently, disease outbreaks caused mass mortality among cultured penaeid shrimps worldwide, especially in Asian countries. The disease is characterized by obvious white spots on the carapace, appendages and the inside surface of the body, and also signs of lethargy and red coloration on the hepatopancreas (Inouye *et al.*, 1994; Momoyama *et al.*, 1994; Nakano *et al.*, 1994; Takahashi *et al.*, 1994; Chou *et al.*, 1995). Most economically important shrimps such as giant tiger prawn *P. monodon*, redtail prawn *P. penicillatus*, and kuruma shrimp *P. japonicus* are affected by the virus.

The causative viral agent, baculovirus

† Corresponding author

associated with white spot syndrome (WSBV), was purified from diseased shrimp *Penaeus mondon* and histologically characterized (Chou *et al.*, 1995; Wang *et al.*, 1995). It is fusiform or rod-shaped with size of 70 to 150 nm at its broadest point and 250 to 380 nm long. The genome of WSBV is a double-stranded DNA longer than 150 kbp. Based on the morphological characteristics and genomic structures of the virus, WSBV was classified into a member of genus NOB (Non-Occluded Baculovirus) of the subfamily *Nudibaculovirinae* of *Baculoviridae* (Wang *et al.*, 1995).

Since 1993, massive damages on the production of cultured shrimp, *P. japonicus* and *P. chinensis* by WSBV also occurred in Korea. Considering increases of penaied shrimp culture and the economic impact of WSBV infections on shrimp farm industry, development of sensitive and rapid diagnostic methods are urgently required to prevent the disease or to control its spread.

Previously, we reported characterization of WSBV genomic DNA isolated from diseased shrimp *P. japonicus* cultured in Korea(Kim *et al.*, 1997). In this paper, we report our further studies on WSBV islated from penacid shrimp *P. chinensis* which is the major kind of shrimp cultivated in Korea in an attempt to analyze the partial genomic sequence of WSBV. This study was also aimed to provide the fundamental information for a rapid and sensitive diagnostic method, based on Southern hybridization and PCR amplification, to detect an early stage of viral infection.

Materials and Methods

WSBV genomic library construction

The purification of virions from the diseased shrimp was carried out as described by Wang *et al.* (1995). The extraction of viral genomic DNA from purified virions was performed by treating the virions with proteinase K and N-cetyl-N, N, N-trimethylammonium bromide (CTAB) followed by phenol-chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). For library construction, the WSBV DNA was digested with *Eco*RI (Promega), ligated to phagemid (pTZ18R) that had been digested with *Eco*RI, and then used to transform *E. coli* DH5α competent cells prepared as described by Cohen *et al.* (1989). Recombinant plasmid DNAs were isolated by alkaline lysis method of Birnboim and Doly (1979).

Slot blot hybridization

Total genomic DNAs from either infected or uninfected shrimps were quantified spectrophotometrically spotted onto nitrocellulose membrane filters using slot blot manifolds (BRL) and bound to the membrane by UV crosslinking (Kafatos *et al.*, 1979). [^{32}P]dCTP-labelled probes were prepated by random-priming (Feinberg and Vogelstenin, 1983) and hybridization was performed as described by Sambrook *et al.* (1989).

DNA sequencing and sequence analysis

Nucleotide sequences were determined by the Sanger's dideoxy chain termination method

(Sanger *et al.*, 1977). For sequene homology comparison, nucleotide sequences of WSBV clones were submitted to European Bioinformatics Institute (EBI)'s world wide web site for Fasta homology search (Pearson and Lipman, 1988) with EMBL nucleotide sequence database.

PCR amplification

A pair of oligonucleotide primers for PCR amplification of WSBV gene were designed based on sequence data of the WSBV DNA. Sequences of E3F and E3R are 5' ATG CAC ACT GGT AGT AGG 3' and 5' TCA ATG TTA ATC CAC ACG C 3', respectively. PCR amplifications were performed out in a final volume of 20 μl of a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM of each dNTP, 15 pmol of each primer, approximately 10 ng of template if not otherwise stated, and 1 unit of Taq DNA polymerase. A drop of mineral oil was added to cover the reaction mixture which was amplified to 30 cycles (94℃ for 30 sec, 55℃ for 45 sec, and 72℃ for 45 sec) in a DNA thermal cycler (ThermoJeT).

Results

Partial cloning of WSBV genomic DNA

Several recombinants were obtained from a phagemid library constructed as described in Materials and Methods. To confirm one of the recombinants, named E3, contains a partial viral genomic sequence, slot blot hybridization was performed. When a probe prepared from the insert DNA of E3 was used, it strongly hybridized only with the genomic DNA from the diseased shrimp (Fig. 1C and D) but not with that of normal shrimp (Fig. 1A and B). The results suggest that E3 contains a fragment of the viral genomic DNA which was derived from the virus causing a fatal disease in shrimp. The probe could detect WSBV genomic DNA from 10 ng of total DNA extracted from the diseased shrimp (Fig. 1C and D). Interestingly, the signal for the genomic DNA from hepatopancreas (Fig. 1C) was stronger, especially at low concentrations, compared to that from the entire shrimp homogenate (Fig. 1D), suggesting that hepatopancreas may serve as host cells for WSBV.

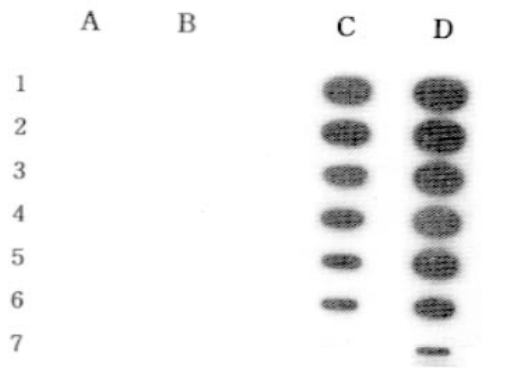

Fig. 1. Solt blotting analysis of the genomic DNA from either normal (A and B) or diseased (C and D) shrimps probed with the WSBV recombinant clone, E3. Total genomic DNA was prepared from either the entire shrimp homogenate (A and C) or shrimp hepatopancreas (B and D) and the indicated amounts of DNA was used for blotting. Key to slots: 1, 10μg; 2, 5μg; 3, 1μg; 4, 0.5μg; 5, 0.1μg 6, 50μg; 7, 10μg

DNA sequencing and sequence analysis.

To characterize the viral clone E3, 2,424 nt of the insert DNA was fully sequenced and shown in Fig 2. Two repeated sequences, composed of 84 and 56 nucleotides respectively, were observed in the middle of E3 sequences. The function of the repeats is not known as they do not show any sequence homology with other repeated or motif sequences from databases. According to the result of nucleotide sequence comparison, the sequence from this clone (and also other clones sequenced so far) showed no significant sequence homology with those of other known viruses or organisms (data not shown), indicating that WSBV is a new virus causing a serious disease in shrimp. Potential coding regions of E3 were searched by Fickett's methods using the PC/Gene program. As a result, two putative open reading frames from nucleotide 134 and 1523 encoding 192 and 103 amino acid residues, respectively, were assumed. It is not clear whether these regions are actually used to encode polypeptides during virus replication. Consistently with nucleotide sequence analysis of E3, no polypeptides either from viruses or organisms were identified to have a significant sequence homology with E3 (data not shown).

PCR amplification of viral DNA from infected shrimp

Total nucleic acids extracted from either diseased or normal shrimps were subjected to amplification by PCR using primers, E3F and E3R. The amplification using 10 ng of nucleic acids, as a template, from the diseased shrimp (Fig. 3A, lane 4) yielded a large amount of 504 bp PCR product corresponding to that obtained from a positive control DNA, E3 (Fig. 3A, lane 2). No proudct was detected from nucleic acid sample from a normal shrimp (Fig. 3A, lane 3) indicating that the product was derived from DNA of WSBV in the infected shrimp.

Sensitivity of PCR tests

The detection limit of WSBV DNA by PCR amplification was examined using 10-fold serially diluted samples of DNA ranging from 10 ng to 0.1 pg (Fig. 3B). After 30 cycles of amplification, PCR prouducts of the expected size were detected from samples containing more than 1 pg of WSBV DNA, but not from those with 100 fg or less (Fig. 2).

Discussion

Since 1993, massive damages by viral disease on the production of cultured shrimp have occurred all over the world especially in Asian countries including Korea. In this report, the causative viral agent purified from the diseased shrimp *Penaeus chinensis* with white spot syndrome was characterized on the molecular basis. According to the results of nucleotide sequence analysis, none of WSBV clones showed considerable sequence homology (over 50% sequence identity) with those of other known viruses, indicating that WSBV is a

```
   1 gaattccaat caatgggaac aaataccaac cccatagaga cttcatcagt gggtgtgaat accaacccac ttccaacccc cccccaagat tggtaattac
 101 tcctttaacg aatgatgtac cagaattgga catgatgtgg ctttattcgc cttccagagg aggtggaaat tctagaatga gtgcaaatac aggaacatct
 201 cccctgtcta acacccaat tcctacctgc ttcacaggag gtgcgaatgt agtagtgcct aatggatttg tccctcccac gttcccctta gaatgtgacg
 301 aagatgatcc aagtatttccc aattcttaca attacgaaca ggataaagtc tttcatccat tttatgagta tatggccaaa tatctatccc ctcttgttcc
 401 atcatataac aagggacgga cttgtaatgt tgtccaggag tggttcaagg gatcccttcc tcttgcaaag cgtagaggaa cagtcccccaa attctgtagt
 501 aacatttccc acgcttttctt ttgtaatatg gatgtatgta ctgccatgtg caaatgggcg aagactgtaa ttagacatgg acaatattgt aatagatgta
 601 tcgtaaggag gtcatgtaca tccatgcttg catatcacta cattgtttgc agagacgctt catgtgatgt tcccaagtgc agggaaaggg ttcgcaacga
 701 catggatgac tgattgattg gttgatatgt gacattttct gtatattgtg taaataagat accaataaac taatgtttta tatatgattc tatttttta
 801 aaaaccttta aaaatatata tataaaatga tgtatttttg aaactacact ctggcagaat cagaccagac ccctgaccta aagcagacca cagggagtc
 901 ttagaggagc cacacttaga ccgatcccct tcatcccica atgttaatca cacgcaagta aaaacaccac ttcctagaaa gggagggaag gggtgacttg
1001 gtgatatcat aactgggga tttcctctcc agatatctgg ctgtacacgt gtagcgctt ctgggcgcga caagaaaaa attagtgata tcataactgg
1101 ggaatttcct ctccagatat ctgggctgtac acgtgtaagc gcttctgggc gcgacaagaa aaaaattagt gatatcataa ctgggggaatt tcctctccag
1201 atatctgggt aagaaaaaaa attagtgata tcataactgg ggaatttcct ctccagatat ctgggtaaga aaaaaaatta gtgatatcat aactgggaa
1301 ttcctctccc agatatctgg ctgtacacat gtgagtgttc taatctcatt ttttatatag aaaatataac tgaattagct ctctaaacct ttcctcattt
1401 cttactcctc ttgtttgtta tgtcctacta ccagtgtgca tataagaact ggagagatgg gagttttaaa taacaaccct tcttaatccc tttcatgttt
1501 atatttaata aatataatca ccatggatat ttcaaataag acattatttt tagtagtcgg tacctttttt ctgaccactt gtgcatcctg cagtccaact
1601 caaatcgtct ggaacctcat ggtagcttca tttgttggat ttctaggaca taaactactt aaaaacatta cacctgtcaa tctggatctt gtcggaaaaa
1701 gcttcgtatt ctctgcaagt ttaaccatct cagaagaagc ccgtttattg aggattggaa acgtattaag ggactataat ggcaacaatt ttgaagagta
1801 tgaagaagaa gaagatagtg gtattgaaga ataataataa taataatggt ttgaataaaa tatagagata catttatatt gttttatttg cattatatat
1901 aaaaaagcac tacaaaattt gtacacatac attgaggaga aaaattgata caatttcttc ctttttttta ctggtatctg atttcttgat attcgagaga
2001 gttagtagta gcagaagaag tagcaccaac tccggctgca gaagttgtag gatagggcgg tggcaccggt ggcagcggca gcggcagcag cttcacggcg
2101 ttgttgttgt tccaggataa tctttgcgcg tttattcatt tcagtggtgc gtgtaaagat gcctctgttg tattggacca ccttattcct atattcccg
2201 atgtccttat cgcgcttcta aaaaaacttg gccatttcctc cacaacgttg acgggctgcc catgcagtca tagccataaa atcaaattgg tcagggtga
2301 tattatggtt gtttagaagg tccacagcgt aggccataca gctagcggca cgttgaatat tggagtactt gctgtttctt ggtttaacgg tatggatcac
2401 cttacttcca tcattcaaga attc
```

Fig. 2. Nucleotide sequence of the WSBV recombinant clone E3. Bold letters indicate potential initiation sites of polypeptide coded by E3, as analyzed by Fickett's method. Repeated sequences are underlinedLocations of the primers used for PCR are shown by italic letters. The NCBI/GenBank Data Libraries accession number of this sequence is U92007.

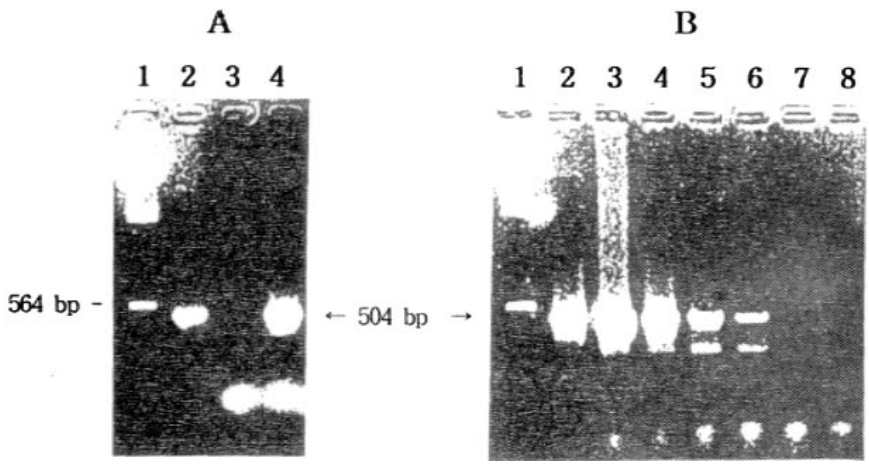

Fig. 3. PCR amplification of WSBV DNA. The amplified products with the expected size were indicated by arrows. (A) Detection of WSBV DNA from total genomic DNA of infected shrimp. Lanes (1) λ phage DNA Hind Ⅲ fragment marker, (2) a positive control plasmid pS1, (3) total genomic DNA from uninfected *P. chinensis*, (4) total genomic DNA from infected *P. chinensis*. (B) Detection limit of the purified WSBV DNA with 30 cycles of PCR incubation. Lanes: (1) λ phage DNA Hind Ⅲ fragment marker, (2) a positive control plasmid pE3, (3) 10 ng of WSBV DNA, (4) 1 ng, (5) 0.1 ng, (6) 10 pg, (7) 1 pg, (8) 100 fg.

novel virus causing a serious disease in shrimp. Since the nucleotide sequence of WSBV described in this manuscript may not be enough to identify WSBV, we are trying to analyse DNA sequences from other clones to obtain further information on WSBV.

It is usually impossible to treat virally infected individuals, especially when massive numbers are involved as in the case of cultured shrimp. To prevent the outbreak of viral disease early, it is necessary to develop a proper diagnostic method. Slot blot hybridization using a probe made of WSBV genomic DNA was sensitive to detect viral genome in 10 pg or lower amounts of DNA from diseased shrimps. Interestingly we obtained better results when using genomic DNA derived from the hepatopancreas instead of the entire shrimp homogenate, suggesting that hepatopancreas serves as host cells for WSBV. This result is supported by the observation of a severe atrophy at the hepatopancreas in the diseased shrimp (Chou *et al.*, 1995). Furthermore, PCR amplification method using primers from the WSBV DNA sequence showed the possibility of specific detection of the infected virus in diseased shrimp. This method was as sensitive enough to specifically amplify the target region of WSBV in 1 pg of purified viral DNA. In addition, this method is very convenient because a total genomic DNA of shrimp can be directly used, without purification of virions from the infected tissues. Therefore, this method could be used as a rapid and sensitive diagnostic method to detect WSBV infection in shrimp.

Recently, similar diagnostic methods for the detection of WSBV from shrimp, based on the sequences of WSBV isolated from *P. monodon* (Lo *et al.*, 1996) and *P. japonicus* (Kimura *et al.*, 1996; Kim *et al.*, 1997) were reported. Since our clone described in the manuscript does not show any nucleotide sequence homology with their isolates, they might represent different fragments of the same virus. Also, it cannot be excluded that they derived from different viruses with no significant sequence homology, but with similar effects on shrimp each other.

Lastly, WSBV DNA was detected from a particular species of crab by a similar procedure used in this study (manuscript in preparation). Considering this species of crab was one of the most common residents in shrimp farm, it could transmit virus onto shrimp by acting as a vector of WSBV. Therefore, it might be possible to minimize economic loss in shrimp farms by starting shrimp culture with uninfected hatchery shrimp after diagnosis with PCR and removing a potential vector(s) such as crab during cultivation.

Acknowledgement

This work was supported by a grant from the Department of Agriculture, Forestry, and Fishery of Korea and partly by a grant from Pusan National University.

References

Birnboim, H. C. and Doly, J. : A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res., 7 : 1513, 1979.

Chou, H. Y., Huang, C. Y., Wang, C. H., Chiang, H. C. and Lo, C. F. : Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis. Aquat. Org., 23 : 165-173, 1995.

Cohen, S. N., Chang, A. C. Y. and Hs, L. : Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Esicherichia coli* by R-facfor DNA. Proc. Natl. Acad. Sci. USA, 69: 2110, 1989.

Feinberg, A. F. and Vogelstein, B. : A technique for radiolabeling DNa restriction endonuclease fragments to high specific activity. Anal. Chem., 132: 6-13, 1983.

Inouye, K., Miwa, S., Oseko, N., Nakano, H. and Kimura, T. : Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: electron microscopic evidence of the causative virus. Fish Pathol., 29 : 149-158, 1994.

Kafatos, F. C., Jone, C. W. and Efstratiadis, A. : Determination of nucleic acid sequence homology and relative concentrations by dot blot hybridization procedure. Nucleic Acid Res., 7 : 1541-1542, 1979.

Kim, J. S., Kim, P. K., Sohn, S. G., Sim, D. S., Park, M. A., Heo, M. S., Lee, T. H., Lee, J. D., Jun, H. K. and Jang, K. L. : Development of a PCR procedure for the detection of baculovirus associated with white spot syndrome (WSBV) in shrimp. J.

Fish Dis., 20:101-107, 1997.

Kimura, T., Yamano, K., Nakano, H., Momoyama, K., Hiraoka, M. and Inouye, K. : Detection of Penaeid Rod-shaped DNA (PRDV) by PCR. Fish Pathol., 31 : 93-98, 1996.

Lo, C.-F., Leu, J.-H., Ho, C.-H., Chen, C.-H., Peng, S.-E., Chen, Y-T., Chou, C.-M., Yeh, P.-Y., Huang, C.-J., Chou, H.-Y., Wang, C.-H. and Kou, G-H. : Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis. Aquat. Org., 25 : 133-141, 1996.

Momoyama, K., Hiraoka, M., Nakano, H., Koube, H., Inouye, K. and Oseka, N. : Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: histopathological studies. Fish Pathol., 29: 141-148, 1994.

Nakano, H., Koube, H., Umezaea, S., Momoyama, K., Hiraoka, M., Inouye, K. and Oseko, N : Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: epzootiological survey and infection trials. Fish Pathol., 29: 135-139, 1994.

Pearson, W. R. and Lipman, D. J. : Improved tools for biological sequence comparison. Proc. Natl. Aca. Sci. USA, 85: 2444-2448, 1988.

Sambrook, J., Fritsch, E. F., and Maniatis, T. : Molecular cloning: a laboratory manual (2nd ed). Cold Spring Harbor Laboratory Press, 1989.

Sanger, F., Nicklen, S., and Coulson, A. R. : DNA sequencing with chain-terminating inhibitors. Proc. Natl. Aca. Sci. USA, 74 : 5463, 1977.

Takahashi, Y., Itami, T., Kondo, M., Maeda, M., Fujii, R., Tomonaga S., Supamattaya K. and Boonyaratpalin, S. : electron microscopic evidence of bacilliform virus infection in Kuruma shrimp (*Penaeus japonicus*). Fish Pathol., 29: 121-125,1994.

Wang, C. H., Lo, C. F., Leu, J. H., Chou, C. M., Yeh, P. Y., Chou, H. Y., Tung, M. C., Chang, C. F., Su, M. S. and Kou, G.H. : Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis. Aquat. Org., 23: 239-242, 1995.

대하새우로부터 분리한 WSBV의 게놈서열 분석

김종경 · 손상규* · 허문수* · 이태호 · 전홍기 · 장경립

부산대학교 자연과학대학 미생물학과, *국립수산진흥원 병리과

새우의 갑각에 흰점을 유발하는 특징을 가진 WSBV는 Baculovirus의 일종으로 여러 종류의 새우에 높은 치사율을 보이는 병원체로서 새우양식에 막대한 피해를 주고 있다. 본 연구에서는 국내에서 양식중인 대하에 질병을 유발한 WSBV의 특성을 알아내고자 치사한 새우로부터 바이러스의 게놈을 클로닝하여 재조합클론(E3)을 분자생물학적으로 분석하였다. E3의 염기서열을 분석한 결과, 이 클론은 AcNPV를 포함한 지금까지 알려진 어떠한 바이러스와도 뚜렷한 상동성(60%)을 보이지 않아 WSBV가 기존의 바이러스와 구분되는 새로운 바이러스임을 알 수 있다. E3의 염기서열에 기초하여 한쌍의 PCR 프라이머를 작성하였다. 병든 새우로부터 분리한 DNA를 30회 증폭한 결과, 예상크기의 산물을 얻을 수 있어 이 방법은 바이러스의 감염여부를 알아낼 수 있는 진단법으로도 활용가능하다.

Key words : Genome analysis, *Penaeid chinensis*, PCR diagnosis, White spot syndrome