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Genome sequence of a VP2/NS junction region of pillar cell necrosis virus (PCNV) in cultured Japanese eel *Anguilla japonica*

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ABSTRACT: Pillar cell necrosis virus (PCNV) is an aquatic birnavirus that was isolated from farmed Japanese eel experiencing mass mortality. In this study, a VP2/NS junction region in genome segment A of PCNV was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) and sequenced. The VP2/NS region in PCNV had the highest homology with that of a strain Ab of infectious pancreatic necrosis virus (IPNV). This result revealed that PCNV belongs to birnavirus genogroup II.

KEY WORDS: Genome sequence · VP2/NS junction region · Pillar cell necrosis virus · Birnavirus genogroup II

INTRODUCTION

In addition to infectious pancreatic necrosis virus (IPNV), many aquatic birnaviruses have been isolated from birds, insects, mollusks and teleosts, and have been reported to have many serotypes (Wolf et al. 1960, Cheville 1967, Dobost et al. 1979, Hill & Way 1988, Wolf 1988, Heppell et al. 1992).

Birnaviruses contain 2 segments of double-stranded RNA (dsRNA). Genome segment A (approximately 3100 bp) encodes 3 polypeptides (VP2, NS, and VP3) in the order 5′-VP2-NS-VP3-3′ (Nagy et al. 1987), and genome segment B (approximately 2900 bp) encodes VP1 only (Morgan et al. 1988). The junction region from VP2 to NS (VP2/NS) in genome segment A, having a length 310 bp, is a highly variable region that can be used to distinguish different strains. Heppell et al. (1993) compared the nucleotide sequence of 17 IPNVs, such as VR-299, Sp, Ab, N1, and He etc., and found 3 major groups, refered to as genogroups I, II, and III. Moreover, a genogroup of marine birnaviruses (MABV) was presented by Hosono et al. (1996), and a similar strain has recently been reported in Korea (Joh & Heo 1999). However, it is known that there are some discrepancies between the results of the serological assay and those of the nucleotide sequencing. Furthermore, Sp and Ab were classified in different serotypes in the serological assay (Hill & Way 1988), while results of the VP2/NS sequence revealed that both strains Sp and Ab should be in genogroup II (Heppell et al. 1993).

Pillar cell necrosis virus (PCNV) is a birnavirus that has been causing mass mortality in farmed Japanese eel *Anguilla japonica*. We reported that, serologically, PCNV is most similar to the strain Sp (Lee et al. 1999). In the present study, we compared the nucleotide sequence of the VP2/NS region of PCNV with the corresponding sequences of several other birnaviruses. These included IPNV isolates, strains VR-299, Sp, Ab, He and N1, and 1 MABV, strain SY. These regions were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) and then sequenced.

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MATERIAL AND METHODS

Virus purification and viral dsRNA extraction. PCNV was propagated in EK-1 cells in L-15 with 2% fetal bovine serum (FBS) at 25°C. When about 90% of the cells in the cell layer showed cytopathic effects, usually on about Day 5 post inoculation with PCNV, the cells were scraped off and centrifuged at 2000 × *g*. Subsequently, a 0.6 volume of 20% polyethylene glycol (PEG-6000) and 4.0 M NaCl were added, resulting in a final NaCl concentration of 2.5 M solution, and the mixture was chilled on ice overnight. After centrifugation (15 000 × *g*, 10 min, at 4°C), the obtained pellet was suspended in Proteinase K lysis buffer (500 mM Tris-HCl, 100 mM EDTA, 20% [w/v] SDS, 1 mg ml^{-1} Proteinase K). After lysis at 55°C for 2 h, nucleic acid was extracted with phenol/chloroform treatment, and precipitated with a 0.1 volume of 3 M sodium acetate and a 2.5 volume of ethanol. The dsRNA was resuspended in diethylpyrocarbonate (DEPC)-treated (RNase-free) water. The purity and concentration of the dsRNA was measured by a spectrophotometer (Amersham Pharmacia Biotec, USA) before it was stored at –80°C.

RT-PCR, cloning and sequencing. RT-PCR of the VP2/NS region in PCNV was performed using the forward primer (5′-AGAGATCACTGACTTCACAAGTGA-3′) and the reverse primer (5′-TGTGCACCACAGGAAAGATGACTC-3′) corresponding to IPNV Jasper

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PCNV-(eel)        1:CTGCCCACGTCAAAGGCATGGGGCTGGAGGGACATAGTCAGAGGGATCCGGAAAGTCGCC 60
IPNV-(Ab)         1:CTGCCCACGTCAAAGGCATGGGGCTGGAGGGACATAGTCAGAGGGATCCGGAAAGTCGCC 60
IPNV-(Sp)         1:CTGCCCACGTCAAAGGCATGGGGCTGGAGAGACATAGTCAGAGGAATTCGGAAAGTCGCA 60
IPNV-(N1)         1:CTGCCCACGTCAAAGGCATGGGGCTGGAGAGACATAGTCAGAGGAATTCGGAAAGTCGCA 60
IPNV-(VR-299)     1:CTACCAACCTCAAAGGCATGGGGATGGAGGGACCTGGTCAGAGGCATCAGAAAAGTGGCC 60
IPNV-(He)         1:CTCCCCACCTCCAAGGCCTGGGGCTGGAGGGACATTGTAAAGCAAATCCGGAGAATCGCC 60
MABV-(SY)         1:CTACCAACTTCTCAAGCCTGGGGGTGGAGAGACATTGTGAGAGGCATCCGGAAGGTGGCA 60
                    ** ** ** **  * ** ***** ***** *** * ** *     **  * *   * **

PCNV-(eel)       61:GCCCCAGTACTGTCAACGCTGTTTCCGATGGCAGCACCACTCATTGGGGTGGCAGACCAA 120
IPNV-(Ab)        61:GCCCCAGTACTGTCAACGCTGTTTCCGATGGCAGCACCACTCATTGGAGTGGCAGACCAA 120
IPNV-(Sp)        61:GCTCCTGTACTGTCAACGCTGTTTCCAATGGCAGCACCACTCATAGGAACGGCAGACCAA 120
IPNV-(N1)        61:GCTCCTGTACTGTCCACGCTGTTTCCAATGGCAGCACCACTCATAGGAATGGCAGACCAA 120
IPNV-(VR-299)    61:GCCCCCGTGCTGTCAACGCTCTTCCCAATGGCGGCTCCCCTTATAGGAGCTGCCGACCAA 120
IPNV-(He)        61:GCTCCTGTGCTATCAACAATGTTCCCCATGGCCGCCCCTCTCATTGGAATGGCCGACCAG 120
MABV-(SY)        61:GCACCAGTGCTGTCAACACTCTTCCCCATGGCAGCACCACTCATCGGAGCCGCCGACCAA 120
                    ** ** ** ** ** **  * ** ** ***** ** ** ** ** **    ** *****

PCNV-(eel)      121:CTCATCGGAGATCTCACCAACACCAACGCAGCAGGCGGAAGGTACCGCTCCATGGCCGCA 180
IPNV-(Ab)       121:CTCATCGGAGATCTCACCAACACCAACGCAGCAGGCGGAAGGTACCGCTCCATGGCCGCA 180
IPNV-(Sp)       121:TTCATTGGAGATCTCACCAAGACCAACGCAGCAGGCGGAAGGTACCACTCCATGGCCGCA 180
IPNV-(N1)       121:TTCATTGGAGATCTCACCAAGACCAACGCAGCAGGCGGAAGGTACCACTCCATGGCCGCA 180
IPNV-(VR-299)   121:TTCATTGGGGACCTCACCAAGACCAACTCAGCCGGGGGACGCTACCTGTCACACGCAGCC 180
IPNV-(He)       121:TTCATAGGCGACCTCACAAAAACCAACGCATCAGGCGGAAGATACAACTCACATGCCGCT 180
MABV-(SY)       121:TTCATCGGAGACCTGACCAAGACCAACGCAGCCGGAGGCCGCTACCTAACACATGCAGCA 180
                     **** ** ** ** ** ** ****** ** * ** **  * ***    *    ** **

PCNV-(eel)      181:GGAGGACGCTACAAAGACGTAATGGACTCCTGGGCCAGCGGCGGACCCGACGGGAAGTTC 240
IPNV-(Ab)       181:GGAGGACGCTACAAGGATGTAATGGACTCCTGGGCCAGCGGCGGACCCGACGGGAAGTTC 240
IPNV-(Sp)       181:GGAGGGCGCCACAAAGACGTGCTCGAGTCCTGGGCAAGCGGAGGGCCCGACGGAAAATTC 240
IPNV-(N1)       181:GGAGGGCGCCACAAAGACGTGCTCGAGTCCTGGGCAAGCGGAGGGCCCGACGGAAAATTC 240
IPNV-(VR-299)   181:GGAGGCCGCTACCATGATGTCATGGACTCATGGGCCAGCGGGTCCGAGGCAGGAAGCTAT 240
IPNV-(He)       181:GGAGGGCGGTACAAGGACGTTCTGGAGACATGGGCAAGCGGATCCAACACTGGCCGCTTC 240
MABV-(SY)       181:GGAGGACGCTACACTGATGTAATGGACTCCTGGGCCAGCGGCACAGACACTGGGAGGTTC 240
                    ***** **  **   ** **  * **  * ***** *****         **    *

PCNV-(eel)      241:TCCCAGGCTCTAAAGAACAGACTGGAGTCTGCCAACTACGAGGAAGTCGAGCTTCCTCCC 300
IPNV-(Ab)       241:TCCCAGGCTCTAAAGAACAGACTGGAGTCTGCCAACTACGAGGAAGTCGAGCTTCCTCCC 300
IPNV-(Sp)       241:TCCCGAGCCCTCAAGAACAGGCTGGAGTCCGCGAACTACGAGGAAGTCGAGCTTCCACCC 300
IPNV-(N1)       241:TCCCGAGCCCTCAAGAACAGGCTGGAGTCCGCCAACTACGAGGAAGTCGAGCTTCCACCC 300
IPNV-(VR-299)   241:TCAAAGCACCTCAAGACCCGGCTTGAGTCCAATAACTATGAGGAAGTGGAGCTTCCAAAG 300
IPNV-(He)       241:TCAATGAGCCTCAAGAAACGCCTAGAGTCAACAAACTATGAGGAAGTGGAACTTCCACGC 300
MABV-(SY)       241:TCACGCAACCTCAAAGACCGGCTGGAGTCAAACAACTATGAGGAGATGGAACTTCCTCCA 300
                    **       ** **     * ** *****    ***** *****  * ** *****

PCNV-(eel)      301:CCTTCAAAAG                                                   310
IPNV-(Ab)       301:CCTTCAAAAG                                                   310
IPNV-(Sp)       301:CCCTCAAAAG                                                   310
IPNV-(N1)       301:CCCTCAAAAG                                                   310
IPNV-(VR-299)   301:CCAACAAAGG                                                   310
IPNV-(He)       301:CCCGAACGTG                                                   310
MABV-(SY)       301:CCAACGAAAG                                                   310
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Fig. 1. Nucleotide sequence alignment of VP2/NS region in PCNV and other birnaviridae. All the sequences were displayed from the 5′ to 3′ direction. The conserved nucleotides in all the sequences are indicated by an asterisk (*). The different nucleotides between PCNV and IPNV-Ab are boxed

segment A according to Heppell et al. (1992). The obtained RT-PCR product was connected with the pGEM-T vector (Promega, USA) following the supplier's recommendations. The recombinant plasmid was transformed into *Escherichia coli* strain XLI-Blue MRF′ (Stratagene, USA) using the calcium chloride method (Sambrook et al. 1989), and recombinant clones were extracted using the alkali-sodium dodecyl sulfate (alkali-SDS) method (Sambrook et al. 1989). Nucleotide sequences were determined with the thermo sequences fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotec, USA) with a DNA sequencer DNA-4000L (Li-Cor, USA).

RESULTS

An approximately 0.3 kb long RT-PCR product was obtained. As this was the expected length of the VP2/NS region, it was inserted in the plasmid vector and the recombinant plasmid was used to transform *Escherichia coli* cells. Three clones that contained the RT-PCR product were sequenced. The nucleotide sequences were determined for both the sense and antisense strands. The sequences of all 3 distinct clones were identical. The obtained sequence was homologous to the previously reported sequences of the VP2/NS regions of the major IPNVs (strains Ab, Sp, He, VR-299, N1) and MABV (strain SY) (GenBank accession codes are IPNLWVR, IPNSEGAA, IPNSPAA, IPNSEGA, IPNHEAA and BIVVP2NS2, respectively). The sequence of the VP2/NS region in PCNV was highly homologous with strains Ab (99.0%), N1 (89.0%) and Sp (88.7%) (Fig. 1, Table 1), but less homologous with strains VR-299 (74.3%), He (72.2%) and SY (75.2%). The phylogenetic tree was constructed based on the predicted amino acids sequence alignment of the VP2/NS region in PCNV and other birnaviruses (data not shown). PCNV was in the same branch as strains Sp, Ab, and N1 (genogroup II). The PCNV branch was different from the branches of strain VR299 (genogroup I) and He (genogroup III) of IPNV as well as from the strain SY of MABV (Fig. 2).

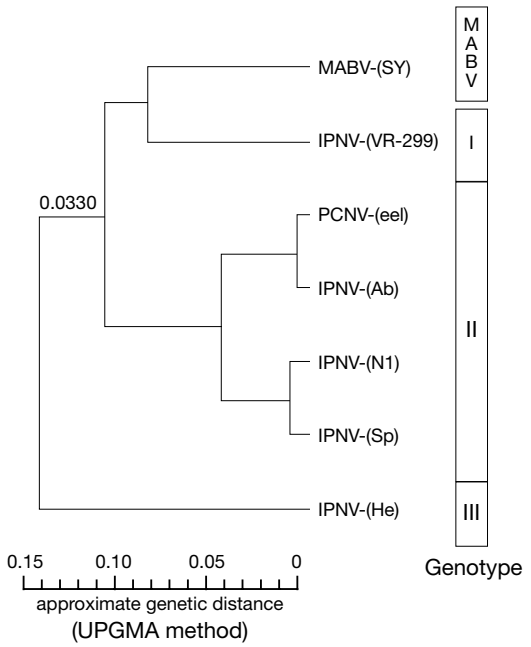

Fig 2. Phylogenetic tree (UPGMA) represents the relationship between the PCNV and the other birnaviruses. Horizontal bar indicates the genetic distance. Genogroups proposed by Heppell et al. (1993) and Hosono et al. (1996)

DISCUSSION

In this study, we determined the nucleotide sequence of the reverse-transcribed VP2/NS region in PCNV. Current evidence indicates that VP2 of birnaviruses carries the serotype-specific epitopes that are responsible for the induction of neutralizing protective antibodies. The conformation of these epitopes is highly dependent on the sequence of this region (Heppell et al. 1995). Heppell et al. (1993) established a PCR assay to amplify and characterize the highly variable VP2/NS junction region. This region could be prepared easily, and more than 20 sequences were deposited in the genome database for the genogrouping of aquatic birnaviruses. Therefore, this region was characterized in the present study.

Table 1. Homologies between nucleotide sequences deduced from the cDNA fragment of PCNV and the corresponding portion on 6 published sequences of aquatic birnaviruses (at the 310 bp of the VP2/NS junction region) (similarities, in %)

IPNV-(Ab)	99.0					
IPNV-(He)	72.2	72.5				
IPNV-(N1)	89.0	88.7	74.6			
IPNV-(Sp)	88.7	88.4	74.6	99.0		
IPNV-(VR-299)	74.3	74.9	72.5	73.6	74.3	
MABV-(SY)	75.2	75.8	72.5	72.6	72.9	78.1
Strain	PCNV-(eel)	IPNV-(Ab)	IPNV-(He)	IPNV-(N1)	IPNV-(Sp)	IPNV-(VR-299)

An amplified RT-PCR product of PCNV had exactly the expected length: 310 bp. The sequence homology values among previously known IPNVs, MABV and PCNV ranged from 99.0 to 72.2% as shown in Table 1. PCNV was most homologous with strain Ab (99.0%), although it was serologically closer to Sp than Ab (Lee et al. 1999). A similar state was found in the strain N1, which was recognized as a new serotype in 1988 (Christie et al. 1988). However, the VP2/NS regions of strains N1 and Sp differed by only 3 bases (Havarstein et al. 1990). Furthermore, some birnaviruses that were reported to belong to different serotypes were found to be closely related to each other at the genetic level and were categorized into 3 genogroups (Heppell et al. 1993).

The nucleotide sequence of the reverse-transcribed VP2/NS region determined in this study indicates that the PCNV belongs to genogroup II of Heppell et al. (1993) and Hosono et al. (1996), which includes both strains Sp and Ab.

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