A New Serotype Confirmed by Partial Physical Mapping of cDNA clones from the Infectious Pancreatic Necrosis Virus (IPNV) isolated in Korea

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한국에서 분리된 전염성 췌장괴저 바이러스의 새로운 혈청형에 대한 유전자 분석

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ABSTRACT : The larger segment of double stranded RNA genome from a new serotype of Infectious Pancreatic Necrosis Virus(IPNV), DRT, has been partially cloned at Sma I site in pUC19 and compared with the restriction maps of VR-299 and Sp. Restrction sites found in DRT was distinct and hence a new serotype.

The cDNA clones of DRT were about 800, 850, and 1,400 bp long each and do not share any common restriction site. It is not clear yet if there exist any overlapping sequences among them. This partial cloning, however, was sufficient for the comparison of restriction maps with the other serotypes.

KEY WORDS : Serotype, pUC19, cDNA cloning, IPNV, DRT

Infectious Pancreatic Necrosis Virus (IPNV) had been first isolated from a brook trout in North America (Wolf *et al.*, 1960), with the characteristics of icosahedral symmetry of 55nm diameter without envelope and two segments of double stranded genomic RNA(Dobos, 1976 ; Macdonald and Yamamoto, 1977). More than 4 different viral proteins had been identified from IPNV (Dobos, 1977 ; Dobos and Rowe, 1977). IPNV, with oyster virus (OV) and infectious bursal disease virus (IBDV), belongs to the family of Birnaviridae established recently (Dobos *et al.*, 1979) for viruses with two segments of double stranded genomic RNA. Many different serotypes have been found in IPNV with different size of RNA segments and distinct ratio of viral proteins (Hedrick *et al.*, 1983). Among many different serotypes reported as yet, only three serogroups are referred to as standard serotypes;VR-299, Sp, and Ab (Macdonald and Gower, 1981 ; Okamoto *et al.*, 1983). They also show different geological distribution as VR-299 is found most frequently in North America area while Sp and Ab are mostly in Europe (Wolf and Quimby, 1971 ; Jørgensen and Kehlet, 1971).

Since 1983 when IPNV was isolated for the first time in Korea from returning adult chum salmon, our laboratory has isolated 7 different IPNV strains from several kinds of inland farming fish. Six of the isolates were identified as VR-299 by neutralization test(Hedrick *et al.*, 1985) and the other one, isolated from rainbow trout in Daechung Dam, showed no typical cross neutralization with any of antisera developed against standard reference serotypes. This isolate, named DRT, is not the only one with distinct serotype, and the number of such unidentified serotypes are even increasing worldwide. Since most fish for inland farming in Korea are usually imported from Japan in the forms of fertilized eggs or fries, they might have been previously to unknown pathogens which can possibly explain these serotypes found in Korea.

We have previously characterized this new serotype on the basis of cross neutralization and size of genomic RNAs in comparison with reference serotypes. In this study, we describe the distinct physical map of DRT. Even though it was constructed only partially, some of the restriction sites proved DRT to be a new serotype when compared with previously

reported physical maps for VR-299(Nagy *et al.*, 1987) and for Sp (Huang *et al.*, 1986).

MATERIALS AND METHODS

Purification of virus from tissue culture

The isolate, DRT, was multiplicated on chinook salmon embryo cells, CHSE-214 (originally provided by Dr. John L. Fryer, Oregon State Univ. OR, in 1982, and maintained thereafter in our laboratory), grown at 18℃ in Eagle's MEM (Flow Laboratories, North Ryde, N.S.W., Australia) supplemented with 10% fetal bovine serum and L-Glutamine. Antibiotics such as penicillin-streptomycin and gentamicin were added if necessary. All materials for tissue culture were purchased from Flow Laboratories unless otherwise mentioned. Cells and culture supernatant were harvested when severe cytopathic effect appeared on the monolyer cultures, and further disrupted with sonication (Lab-Line System Lab-Line Instruments, Melrose Park, IL). After centrifugation at 10,000rpm for 10min (Sorvall RC5C, Welmington, DE), virus was precipitated by addition of NaCl and PEG (M.W. 8,000, Sigma, St. Louis, MO), stirring gently on ice for 4hrs, at the final concentration of 0.5M and 9%, respectively. Viral particles were recovered from the pellet resuspended in TE buffer (pH7.6).

Isolation of purified Viral genomic RNA

Viral particles were digested with proteinase K (Sigma, St. Louis, MO) at 65℃ for 2hrs in the proteinase K reaction buffer (10mM Tris pH7.6, 5mM EDTA pH8.0, 1% SDS). Double stranded genomic RNA segments were purified by phenol : chloroform extraction. RNA segments were precipitated at -20℃ with two part volume of absolute ethanol containing 2.5M ammonium acetate. After further washing in 70% ethanol, RNAs were centrifuged at 12,000 rpm for 30 min at 4℃, and then dried in speedvac concentrator (Savant Instruments, Farmingdale, NY).

Double stranded genomic RNA segments extracted after proteinase K digestion were further digested with DNase I (200 μg/ml, Cooper Biomedical) and Vanadyl Ribonucleoside Complex (VRC, 10mM) at 37℃ for 1hr, in the reaction buffer for DNA digestion (0.1M HEPES pH7.0, 50mM $CaCl_2$, 0.1M $MgCl_2$, 10mM DTT). After digestion with DNase I, 100 mM EDTA (pH8.0) and TSE buffer (10mM Tris-HCl pH9.0, 1mM EDTA pH8.0, 0.5% SDS pH7.2) had been added in equal volume, and pure genomic RNAs were extracted by the phenol : chloroform method.

Separation of genomic RNA segments for cDNA synthesis

IPNV has two segments of double stranded RNA, and these segments were separated for cDNA synthesis by agarose gel electrophoresis in TBE buffer (0.089M Tris-borate, 0.089M Boric acid, 0 .008M EDTA) under 70V for 13hrs or under 50V for 18hrs. Each segment was adsorbed onto separate piece of NA45 membrane, presoaked in TBE buffer, with additional running under 150V for 10min. Washing twice with low salt NET buffer (0.15M NaCl, 0.1mM EDTA, 20mM Tris pH8.0), RNA segments were eluted with high salt NET buffer (1.0M NaCl) heating continuously at 66.5℃ for 1hr. After removing ethidium bromide with n-butanol, genomic RNAs were finally purified by phenol : chloroform extraction method. Precipitated RNA segments were evaluated its purity by scanning in the range of 220~320nm and by the estimation of OD ratio at 260 and 280nm, where the ratio for pure RNA preparation was referred to as $OD_{260}/OD_{280}=2.0$. The OD_{260} value was also used for quantitation, where one optical unit corresponds to 50μg/ml of dsRNA.

cDNA synthesis

Purified double stranded RNA segments were denaturated for first strand synthesis of cDNA at 52℃ for 1hr with dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) at the final concentration of 90%.

First strands were synthesized with 20U of reverse transcriptase (cDNA synthesis system plus, Amersham International) at 42℃ for 2hrs in the reaction mixture (50mM Tris-HCl pH8.3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT) where 20U of human placental ribonuclease inhibitor (HPRI), mixture of deoxynucleoside triphosphate (5mM dCTP, 10mM dATP, dGTP, and dTTP), random hexanucleotide primer, 20U of [α - ^{32}P] CTP, and 1μg of denatured RNA were added in order and mixed thoroughly. The whole volume of reaction mixture was adjusted to 50μl, and 1μl aliquot was used to confirm the successful synthesis of the first strands on TLC plates (0.1mm, cellulose MN300 polyethyleneimine impregnated).

Second strand synthesis was carried out in the total volume of 250μl reaction mixture with an aliquot of the first strand cDNA, 0.8U/μg RNA of ribonuclease H, and 23U/μg RNA of *E. coli* DNA polymerase I by continuous incubation at 12℃ for 60min, at 22℃ for 90min, and 70℃ for 10min in order. To make both sides of the newly synthesized double stranded cDNA blunt, T4 DNA polymerase at 2U/μg was added and incubated at 37℃ for 20 min. Complete cDNAs were purified by phenol : chlororom extraction method and precipitated with

anhydrous ethanol containing 2.5M ammonium acetate. cDNAs were analysed on 1.0% agarose gel electrophoretogram, and confirmed by autoradiography, exposed at R.T. for 2 days.

Blunt end repairing of cDNA and ligation into pUC19 vector

Blunt ends of cDNA were repaired at 37℃ for 30min using Klenow fragment (Boeringer Manheim) in polymerase reaction buffer in the presence of dNTP. Reaction continued further at 75℃ for 15min, and precipitated with ethanol. Repaired cDNA fragments were inserted into Sma I cut pUC19 vector with ligase (BRL) in 20μl final volume of reaction mixture at R. T. overnight.

Transformation

A competent bacterial strain *E. coli* K12 JM109 was kept on ice for 45min with ligate preparation before 90sec heat shock at 42℃. After heat shock, the aliquot was kept on ice again for 5min, then 1hr incubation followed at 37℃ with prewarmed SOC media. McConkey (Ampicillin) agar plates were used for selection of transformed colonies.

Analysis of inserted cDNAs

White colonies on McConkey agar plates with ampicillin incubated at 37℃ overnight were selected as positive transformants. They were coded individually and transferred onto new plates. Each colony inoculated into 5ml medium of LB was incubated at 37℃ overnight and plasmid was isolated by alkaline lysis method (Birnboim and Doly, 1979). Plasmids were identified if they contain cDNA inserts with BamH I and Hind III (Promega), and size of the inserts were estimated on 0.8% agarose gel comparing with size marker of Lambda-EcoRI -Hind III.

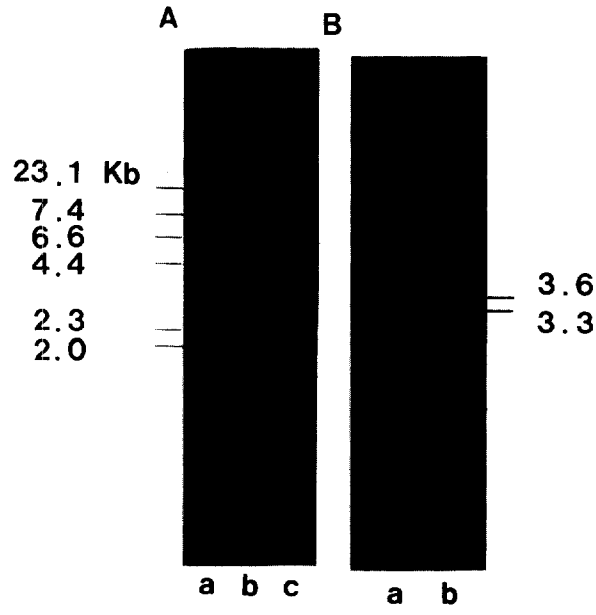

Fig. 1. *Separation of genomic RNA segments and cDNA synthesis.*
A. Two segments of double stranded genomic RNA have been purified separately by adsorption onto NA45 membrane. The larger segment (b) was approximately 3.6 kbp long and the smaller one (c) 3.3 kbp. Hind III digested lambda DNA size marker (a) was compared with. **B.** The autoradiogram of cDNA fragments with various length synthesized along each segment as template, large and small RNA segment, respectively.

RESULTS AND DISCUSSION

Cloning of cDNAs from the genomic RNA segments of the isolate, DRT

Two segments of double stranded genomic RNA of the IPNV strain DRT from rainbow trout in Korea were isolated separately using NA45 membrane. They were approximately estimated 3.6 and 3.3 kb long, respectively (Figure 1A), which are smaller, as each segment was previously compared (Park *et al.*, 1989), than those of other reference serogroups. Nevertheless, the overall length of clones segments of VR-299 and Sp are shorter than estimated on agarose gel electrophoretogram (Nagy *et al.*, 1987; Huang *et al.*, 1986). The cDNA fragments synthesized from each of these RNA segments were confirmed on the agarose gel and also by autoradiography (Figure 1B). The final yield of cDNA synthesis should have been much higher, inasmuch as we have used cDNA synthesis kit with random primers. Low efficiency of cDNA synthesis, which is possibly due to poor denaturation of genomic RNA, might be responsible for low plating efficiency of transformant selection on McConkey plates with ampicillin. We have tried different methods for denaturation of RNA segment for cDNA synthesis. The RNA segments of DRT, however, were not easily denatured in the presence of 90% DMSO, or renatured very quickly when precipitated with ethanol. Even when we finally obtained enough amount of cDNA for cloning, most of the genomic RNA segments appeared renatured, or not denaturated ever, on the agarose gel electrophoretogram.

Both ends of the synthesized cDNA fragments are usually not blunt, having flanking sequences which make ligation difficult, Klenow fragment was used to repair the both ends, which increased the

possibility to obtain positive colonies on selection media.

Three positive colonies with cDNAs synthesized were analyzed with restriction enzymes, EcoRI, BamHI, and Hind III, in combination (Figure 2).

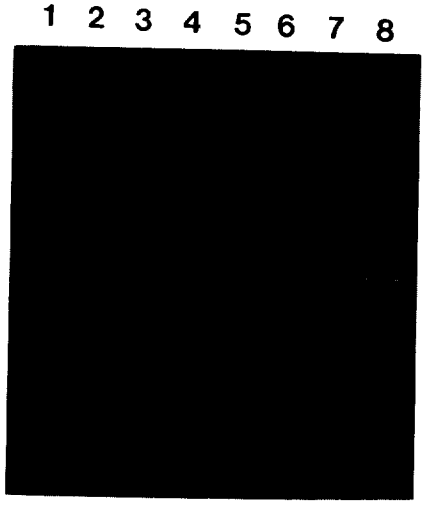

Fig. 2. *Analysis of cDNA clones selected on McConkey agar plate containing ampicillin.*
Clones in pUC19 were analyzed by 0.8% agarose gel electrophoresis. Those inserts were approximately 800, 850, and 1400 bp. lane 1, pUC19; lanes 2-4, pA15, pA40, pA70, in order; lane 5, EcoR I-Hind III digested lambda DNA size marker; lanes 6-8, inserts from pA15, pA40, and pA70, in order, cut out with BamH I-Hind III.

using the A segment of DRT genomic RNAs as the template were selected and the size of the inserts The inserts were known to be 800, 850, and 1400 bp long, and the vectors with these inserts were previously coded as pA70, pA15, and pA40, respectively, when selected on McConkey media. The sequences of these cDNA inserts were confirmed by DNA-RNA hybridization for the correct sequences of the DRT genomic RNA.

Physical mapping of cDNA clones

The inserts from pA15, pA70, and pA40 were digested with several restriction enzymes for physical mapping. As previously reported (Nagy *et al.*, 1987; Huang *et al.*, 1986), physical mapping of the other serotypes like VR-299 and Sp have showed distinct patterns of restriction sites. In the hope that we may have mostly the complete sequence of segment A, or overlapping sequences at least, restiction enzymes were carefully selected so that we could compare the physical map of DRT with those of VR-299 and Sp to understand the uniqueness of the serotype expressed by DRT.

Restriction enzymes known to have at least one site for either VR-299 or Sp have been used for the enzymes Pst I and Pvu II were found in the sequences of VR-299, and both Acc I and Sma I sites were in Sp, while EcoR I and Xho I were both in VR-299 and Sp. Partial restriction mapping with these enzymes was shown in Figure 3. The insert from pA15 was cut only by Pst I producing 150 and 700 bp long fragments, while Pvu II has the restriction site on the insert of pA40 with 550 and 850 bp products. No enzyme cut the insert from pA70. The order and polarity of these inserts are not clear. The segment A of VR-299 is 3,097 bp long, while that of Sp has the size of 2.9 Kbp, approximately. Most of the restriction enzymes used for restriction mapping were of only one site through the whole sequences. It is interesting that VR-299 and Sp have sequences longer than 1.0 Kbp without any sites for commonly used restriction enzymes for physical mapping, which may explain the insert in pA70 representing such long sequence without any common restriction enzyme sites of DRT.

When three cDNA clones of DRT were compared, it is not clear as yet whether there exist any overlapping sequences among them. If there is no overlapping sequences, then around 3.0 Kbp has been cloned out of 3.6 Kbp sequences. Even if some sequences are overlapped, the cloned area offers sufficient evidences of different serotype since any two clones do not match any sequences found in either of restriction maps.

The inserts from pA15 and pA40 with distinct patterns of restriction site can clearly prove that the genomic structure of DRT should be different from two other reference serotypes. Three out of five Pst I sites in VR-299 sequences are found within 100 bp range, while the Pst I site of DRT is unique within 150 bp in both direction (Figure 3). Other enzyme sites also do not imply any common sequences between VR-299 and DRT. In the case of Sp, no restriction enzyme shares any site in common with DRT sequences. Comparison of the restriction maps for segment B is not required at present only if to prove the new serotype of DRT.

With partially constructed restriction map of DRT, the new serotype of IPNV isolated from rainbow trout in Daechung Dam has been proved its uniqueness. Obtaining of more cDNA fragments with partially overlapping sequences will be necessary to complete the genomic RNA sequences and to verify the structural genes for viral proteins. Sub-

sequent cloning of cDNAs for this purpose is now in progress, and this will provide a good body of information for the different nature of this new serotype.

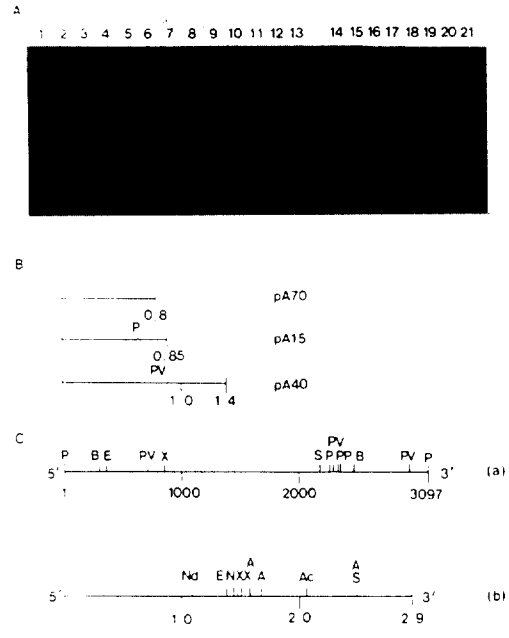

Fig. 3. *Physical mapping with restriction enzymes.* **A.** Inserts from pA15, pA40, and pA70 were digested with various restriction enzymens. lane 1, pA15 ; lanes 2-6, insert of pA15 digested with Acc I, Pvu II, Xho I, Sma I, in order ; lane 8, pA70 ; lanes 9-13, insert of pA70 digested as in lanes 2-6 ; lane 14, pA40 ; lanes 15-20, insert of pA40 digested with Acc I, Pst I, Pvu II, Xho I, Pvu II −Xho I, Sma I, in order ; lanes 7 and 21, EcoR I −Hind III digested lambda DNA size marker. **B.** Partially constructed physical map for the large segment of DRT RNA genome. Polarity of the large segment of DRT RNA genome. Polarity of the clones in not clear at present. One site each of Pst I and Pvu II were determined on inserts of pA15 and pA40, respectively. C. Physical maps for VR-200(a) and Sp(b), constructed by Nagy *et al.*(1987) and Huang *et al.*(1986), respectively.

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적 요

우리나라에서 양식하고 있는 송어로부터 분리된 한 주의 전염성췌장괴저 바이러스(IPNV)가 독특한 혈청형을 가지고 있음을 확인하고, 이를 DRT라 이름한 뒤, 이 바이러스로부터 분리한 RNA유전자를 cDNA이다.바이러스의 3.6 Kbp 정도의 크기를 가지는 A segment에서 cDNA를 합성하고 pUC19에 클론하였다. 대략 3 Kbp 정도를 망라하는 세가지 클론을 선택하여 분석하여 본 결과, 각각 800, 850, 1400bp 정도의 유전자가 포함하여 있었고, 이들로부터 부분적으로 만들어진 유전자 지도에서 제한효소 절단부위의 양상으로 미루어 새로운 혈청형임을 확인하였다.

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