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# The Specific Detection of Infectious Pancreatic Necrosis Virus by Reverse Transcriptase-Polymerase Chain Reaction and Dot Blot Hybridization

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**ABSTRACT**

Based on reverse transcription--polymerase chain reaction (RT-PCR), a rapid, sensitive and specific detection method has been developed to detect virus in cultured fish cells. Fish viral RNA was extracted from cell cultures and used for cDNA synthesis. Specific primers of coding region of VP2 were used for ds cDNA synthesis. Using B, D primers and template #2 (ds cDNA from primer B), a single one 300 bp PCR product could be obtained, and the size of the product can be used as the indicator for serotype determination. By using the probe of this 300 bp PCR product, the DNA-RNA dot blot hybridization assay developed for IPNV detects 10 to 100 pg RNA of T42G, 10 ng RNA of SP, 1 to 10 ng RNA of EVE, and 10 ng RNA of AB, respectively. These methods could be improved by further studies.

**Key words:** IPNV, RT-PCR, dot blot hybridization

## Introduction

In the past decade, intensive cultures of marine and freshwater fish, shellfish and shrimps for food and sport have been developed dramatically fast. In the intensive aquaculture, disease has become a serious problem, since viral diseases in fish, shellfish and shrimps cannot be cured by therapeutic reagents, and environmental stressor(s) always speed up the mortalities following infections. Specific pathogen free (SPF) systems should be set up. The sensitive and effective diagnostic methods of important fish viruses are essential to control these diseases.

Infectious pancreatic necrosis virus (IPNV) is a serious pathogen of farm-reared salmonids and other non-salmonid fishes and shellfish (Hedrick *et al.*, 1983; Chen *et al.*, 1984, 1985; Hsu *et al.*, 1989a, b and 1993). The virus kills these fishes when they are fries or fingerlings. Even survivors become lifelong carriers, which preserve viruses in the population by continuously shedding and transmit them to progenies or other susceptible species. The virus is a serologically very heterogeneous (Leong and Fryer, 1993; Christie *et al.* 1988). IPNV that belongs to Birnaviridae which is an icosahedron with a two-segment dsRNA genome and four virion proteins (MacDonald and Dobos, 1981). It is distributed worldwide, and is the most extensively studied among all the fish viruses. The virion protein VP2, VP3 and VP4 are encoded by segment A RNA and are cotranslated as a polyprotein and cleaved into single proteins by VP4, protease (Duncan *et al*., 1987; Nagy *et al.*, 1987). Virion protein VP1, which is encoded by segment B RNA, have the function of RNA polymerase (MacDonald and Gower, 1981; MacDonald and Dobos, 1981) and VPg (genome linked virion protein) (Calvert *et al.*, 1991); whereas VP2 is a major capsid protein with the serotype specific and neutralization epitopes (Havarstein *et al.*, 1990; Dobos *et al.*, 1979; Tarrab *et al*, 1995; Frost *et al.*, 1995; Liao and Dobos, 1995). VP3 is a minor capsid protein, and VP4 is a protease, has only cis activity (Manning *et al.*, 1990).

Several quick diagnostic methods have been developed for IPNV detection. Among them, immunodot assay and polymerase chain reaction (PCR) are rapid, sensitive and specific. By the reciprocal cross neutralization tests of polyclonal antisera, three major serotypes designated. They are VR-299 ( which was changed to West Buxton (WB) by Hill and Way, 1983),

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Sp and Ab (MacDonald and Gower, 1981). In general, WB is the predominant serotype detected in the United States (MacKelvie and Artosob, 1969) and serotypes Sp and Ab found in Europe (Ball *et al.*, 1971; Jorgensen and Bregnballe, 1969). Most of the IPNV isolates found in Asia and Taiwan belong to the Ab and WB serotypes (Chen and Kou, 1985; Hsu *et al.*, 1989a and 1993). In this paper, the capillary polymerase chain reaction and dot blot hybridization methods were used to detect the IPNV infection.

## Materials and Methods

### Cell and viruses

Chinook salmon (Oncorhynchus tschawytscha) embryo cells (CHSE-214) were obtained from Dr. J.C. Leong, Oregon State University, Corvallis, USA. IPNV serotye Ab and Sp were a gift from professor Lo, National Taiwan University. EVE was obtained from Dr. T. Sano, Tokyo University of Fisheries, Japan. T42G was isolated from trout gill in our laboratory. Cells were grown in MEM with earle's salts (Sigma inc.) supplemented with 10% fetal calf serum (MEM-10), penicillin (100 IU/ml), streptomycin (100 mg/ml), gentamycin (25mg/ml) and fungizone (0.25mg/ml).

### RNA preparation

The RNA extraction procedure described earlier by Hsu *et al.*,(1989) was used, with some modification. Monolayers of CHSE-214 cells in 25 $cm^2$ plastic tissue culture flasks were infected with the virus at a high multiplicity of infection (MOI) of 20 50% tissue culture infectious dose ($TCID_{50}$)/cell and incubated at 20 ℃ for five days. After completely observing cytopathic effect (CPE), the culture fluid was harvested and centrifuged. The supernatant was mixed with Sarkosyl to a final concentration of 0.1% and centrifuged at 130,000 g for 1 h. The pellet was mixed with 0.5% SDS and 200 ug/ml proteinase K in TNE buffer (0.1M Tris-HCl, 0.1M-NaCl, 1mM-EDTA, pH 7.3) at 37 ℃ for 4 hr. An equal volume of 2X SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% $\beta$ -mercaptoethanol) was added and boiled for 3 min. The RNA sample was electrophoresed in a 2% low melting agarose gel. After ethidium bromide staining, the RNA bands was cut from the gel and dissolved at 65 ℃ with TAE buffer (40mM Tris-acetate, 2mM EDTA). Then, the RNA was extracted with phenol/chloroform, and precipitated with alcohol, and stored at -70 ℃.

### cDNA synthesis

The viral dsRNA was mixed with dimethyl sulfoxide (DMSO) to a final concentration to 60% and heated to 60 ℃ - 100 ℃ for 10 min. It was cooled quickly by liquid N2 and DMSO diluted to 10% with diethylpyrocarbonate (DEPC)-treated water. The ssRNA was precipitated with alcohol, and used as a template in cDNA synthesis with 200 U of murine leukemia virus reverse transcriptase (BRL, Gaithersburg, Md.), rRNasin 25U and 40mM sodium pyrophosphate in a buffer consisting of 0.25M Tris hydrochloride (pH 8.3), 0.375 M KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol, and 0.5 mM deoxynucleoside triphosphates. The specific 20-mer oligonucleotides (identical to the plus strand of segment A of the IPNV genome of Jasper strain from nucleotide 25 to 1040 (Fig. 2) was used as primers indicated in the text. The reaction was performed at 42 ℃ for 3 hr. Then, the 2nd strand of cDNA was synthesized at 14 ℃ overnight with 2nd cDNA reaction buffer and E coli DNA polymerase I (23 U) and RNase H (0.8 U) ( Promega Inc.). For cDNA labelling, [ $\alpha$ -$^{32}$P]-dCTP was added into the reaction. After reaction, the synthesized dsDNA was extracted with phenol/chloroform, and precipitated with alcohol.

### Amplifition of target DNA

PCR was done by using 1X PCR buffer (10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% (W/V) gelatin) with 500μM each of deoxynucleotide triphosphate, 4 μM each of the primers, and 0.4 U of thermostable DNA polymerase (BRL). Reverse transcribed cDNA (50 ng) was used as a template. The amplification was performed in a DNA capillary thermal cycler (Idaho Technology, USA). The predenaturation was conducted at 94 ℃ for 5 min, and the cycle profile was 94 ℃ for 0 sec, then 64 ℃ for 0 sec, and final 70 ℃ for 25 sec. When both pairs of primers were applied sequentially, 40 cycles were performed.

### Electrophoresis

Electrophoresis was conducted in a horizontal, submarine 1 % agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA [pH 8.0]) at 7.1 V/cm for 40 min, and the gel stained with ethidium bromide.

### Analysis of PCR products

To test the specificity of the PCR product, two methods were used. One is the southern blot hybridization, and the other one is dot blot hybridization. For the southern blot hybridization, the DNA probe was prepared from pT72/A cDNA insert by random primer labeling. This probe contained full length of A segment. For dot blot hybridization, the probe was 300 bp labeled DNA from gel-eluted PCR product, synthesized from B,

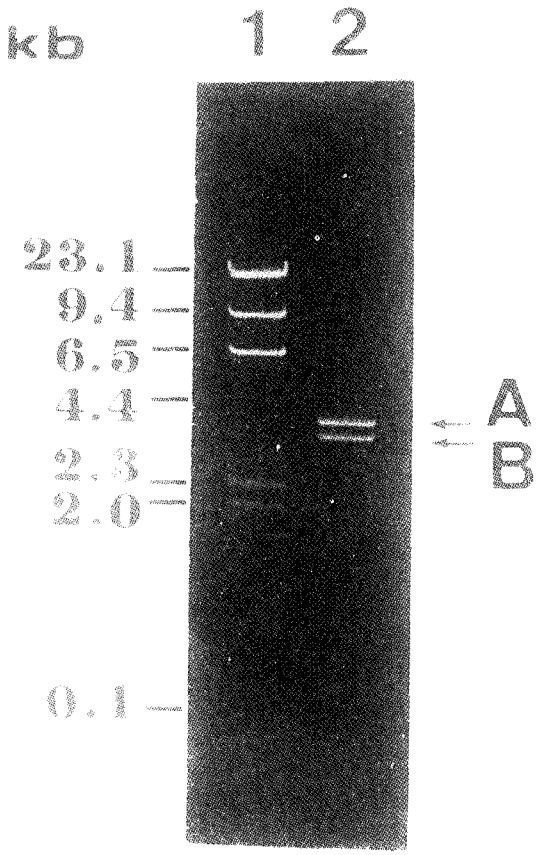


**Figure 1.** Electrophoregram of dsRNA of IPNV. Purified dsRNA of IPNV was analyzed on ethidium bromide-stained gel electrophoresis. (1) molecular weight marker, *Hind III* -digested λ DNA. (2) purified T42G dsRNA.

D primers. The hybridization procedure described earlier by Christie *et al.* (1988) was used, with some modification. In short, 60% DMSO heat-denaturated dsRNAs from four strains of IPNV (T42G, SP, EVE, and AB) were blotted onto a nylon membrane (Hybond -C-extra NC paper, Amersham) and fixed to the membrane with a UV-Fluo-Link (Vilber Lourmat, France). After prehybridization of the membrane in a mixture of 6X SSC (1X SSC is 150 mM NaCl and 15 mM sodium citrate) -5X Denhardt solution-100 μg of boiled salmon sperm DNA per ml-0.08% sodium phophate-0.5% sodium dodecyl sulfate for 1 h at 42 ℃, radio labeled probe in 6X SSC, 1X Denhardt solution-0.08% sodium phosphate mixture was added and hybridized at 42 ℃ for 2.5 h. Then the membrane was washed with 2X SSC + 0.1% SDS for three times, and 0.2X SSC + 0.5% SDS for three times. Kodak X-Omat AR X-ray film was exposed to the dried membrane for overnight or 2 days at -70 ℃.

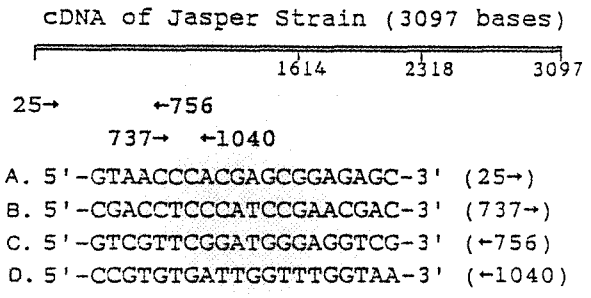


**Figure 2.** The locations of the specific primers (A, B, C, and D) on the genome segment A (cDNA of Jasper strain with 3097 bases).

## Results

### Viral RNA purification

For RT-PCR optimal condition testing, T42G (VR-299 type) strain of IPNV was used. T42G isolated from the rainbow trout gill was cultured and purified. Two hundred nanograms of purified dsRNA of it was electrophoresed in 1% agarose gel and stained with ethidium bromide. The length of A and B segments are 3.8 kb, and 3.2 kb, respectively (Fig. 1).

### Reverse transcription

Based on the conserved cDNA sequence of the VP2 of N1 and Jasper strain , two sets primers for IPNV RT-PCR were designed (Fig. 2.) and synthesized. For the RT-PCR, the RNA of IPNV was extracted as described in Material and Methods. For optimal reverse transcription, the random hexamers (5 μg) was used as primers in the cDNA synthesis. The optimal condition for the first strand cDNA synthesis was 42 ℃ for 3 h and for the 2nd strand cDNA synthesis 14 ℃ for 16 h. Then, the cDNA synthesis of VP2 with specific primer was conducted and compared. The yield of the first strand and ds cDNA synthesis with different primers was compared by $\alpha$ -$^{32}$P-labeling (Fig. 3). The optimal yield of cDNA synthesis was from C primer, then from the D primer, the A and B primer, respectively.

### RT-PCR and southern blot hybridization

For the RT-PCR, three major serotypes of IPNV-WB (T42G), AB , SP and EVE (AB like) strains were used. The ds cDNA products from A, B, C or D primer designated as template #1, #2, #3, or #4, respectively,

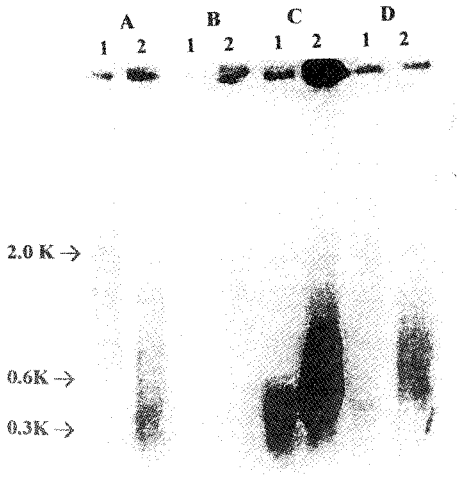


**Figure 3.** The cDNA synthesis with different specific primer. The specific primers, A, B, C, and D (described in Fig. 2) were designed and synthesized according to the cDNA sequence of Jasper and N1. The first strand cDNA product (lane 1) and the ds cDNA product (lane 2) were analyzed on 1% agarose gel.

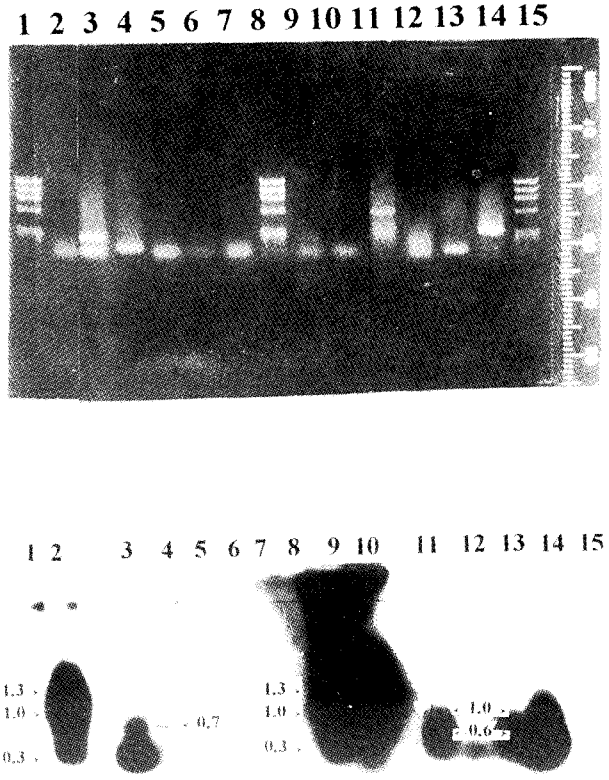


**Figure 4.** RT-PCR products using different pairs of VP2 coding region primers and different IPNV strains. (A) product visualization on ethidium bromide-stained 1% agarose gel electrophoresis. (B) the autoradiogram of the gel (A) hybridized with the probe of $^{32}$P-labeled pT72/A insert. (1, 8, 15) $\phi$ X174 HaeIII-digested DNA marker. The molecular weight size are 1.35 kb, 1.07kb, 0.87kb, 0.6kb, 0.31kb. (2) PCR products of pT72/A with A, C primers. RT-PCR products of T42G : (3) with template #1 and A, C primers. (4) with template #3 and A, C primers. (5) RT-PCR products of AB serotype with template #3 and A, C primers. (6) RT-PCR products of EVE , with template #3 and A, C primers. (7) RT-PCR products of SP, with template #3 and A, C primers. PCR roducts of pT72/A: (9) with A, D primers. (10) with B, D primers. RT-PCR products of T42G: (11) with template #1 and A, D primers. (12) with template #2 and B, D primers. (13) with template #4 and A, D primers. (14) with template #4 and B, D primers.

were used as the template for PCR. The PCR reactions with different templates from different strains (T42G, SP, EVE, and AB) and different sets primers were carried out in the capillary thermal cycler. From A, C primers and template #1 or #3, the presumed PCR product should be 0.73 kb DNA fragment (Fig. 4, lane 2 to 7). However, the presumed size DNA fragment was not shown in AB, EVE, or SP strain (Fig. 4A, lane 5, 6, and 7), even in the southern blot (Fig. 4 B, lane 5, 6, and 7). It only appeared in the southern blot from T42G (Fig. 4B, lane 3 and 4) , but not in the agarose gel. In agarose gel, the only one single 0.3 kb PCR band appeared only in template #1 of T42G (Fig. 4A, lane 3), but also showed in the southern blot from pT72/A and T42G RT-PCR product (Fig. 4B, lane 2, 3 and 4). The strong 1.0 and 1.3 kb hybridization bands appeared only in the pT72/A autoradiogram (Fig. 4B, lane 2). From A, D primers and template #1, #2, or #4, the presumed PCR product should be 1.04 kb. In agarose gel, there was only 0.6 kb PCR product from template #1 of T42G (Fig. 4A, lane 11), but there were strong 0.6 and 1.0 kb hybridization bands in pT72/A, or T42G from template #1 or #4 (Fig. 4B, lane 9, 11, and 13) and an extra strong 1.3 kb and weak 0.3 hybridization bands in pT72/A (Fig. 4B, lane 9). While using B, D primers and template #2 or #4, the presumed PCR product should be 0.3 kb. The single 0.3 kb PCR product only appeared in agarose gel from

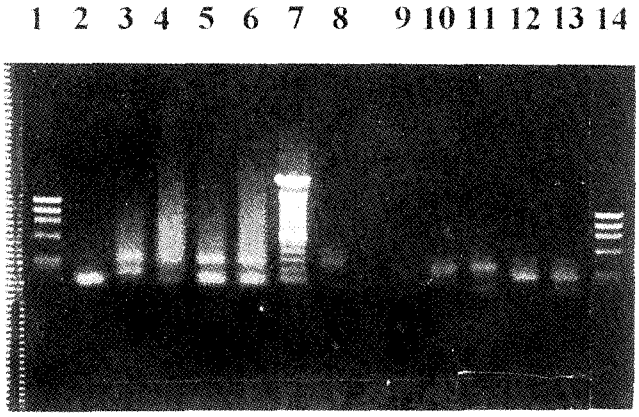


**Figure 5.** RT-PCR products using B, D primers of VP2 and template #2 of different serotype IPNV. Products were visualized on ethidium bromide-stained 1% agarose gel electrophoresis. (1 and 14) $\phi$X174 HaeIII-digested DNA marker as described in Fig. 4. (7) 100 bp DNA marker. RT-PCR products of T42G : (2 and 5) with 20 mM $Mg^{++}$. (4 and 6) with 30 mM $Mg^{++}$. RT-PCR products of AB: (8) 20 mM $Mg^{++}$. (9) 30 mM $Mg^{++}$. RT-PCR products of EVE: (10) 10 mM $Mg^{++}$. (11) 20 mM $Mg^{++}$. RT-PCR products of SP: (12) 10 mM $Mg^{++}$. (13) 20 mM $Mg^{++}$.

template #4 of T42G (Fig. 4A, lane 14), but two extra 0.6 and 1.0 kb hybridization bands also showed (Fig. 4B, lane 14). The PCR product of the pT72/A from B, D primers appeared weak 0.3, strong 0.6, 1.0 and 1.3 kb hybridization bands (Fig. 4B, lane 10). The only one single 0.3kb hybridization band appeared in the PCR product from template #2 of T42G and B, D primers (Fig. 4B, lane 12).

The above results indicated that the presumed size of 300 bp DNA from B, D primers could be seen the only one band in the hybridization autoradiogram in the RT-PCR (Fig. 4B, lane 12). Therefore, the B, D primers and template #2 were used in the following PCR experiments. Different strains of IPNV and various concentration of $Mg^{++}$ were tested for the optimal condition of IPNV RT-PCR. As shown in Fig. 5, all three serotype of IPNV could be obtained the 300 bp DNA fragment in the RT-PCR, the denaturation temperature was 60℃, the optimal $Mg^{++}$ concentration was 20 mM for AB strain, 20 mM for EVE , 10 mM for SP , and 20 mM for T42G (VR-299) . The size of PCR DNA obtained from these RT-PCR was 300 bp for AB strain, 300-350 bp for EVE, 250-300 bp for SP, and 300 bp for T42G.

**DNA-RNA dot blot hybridization**

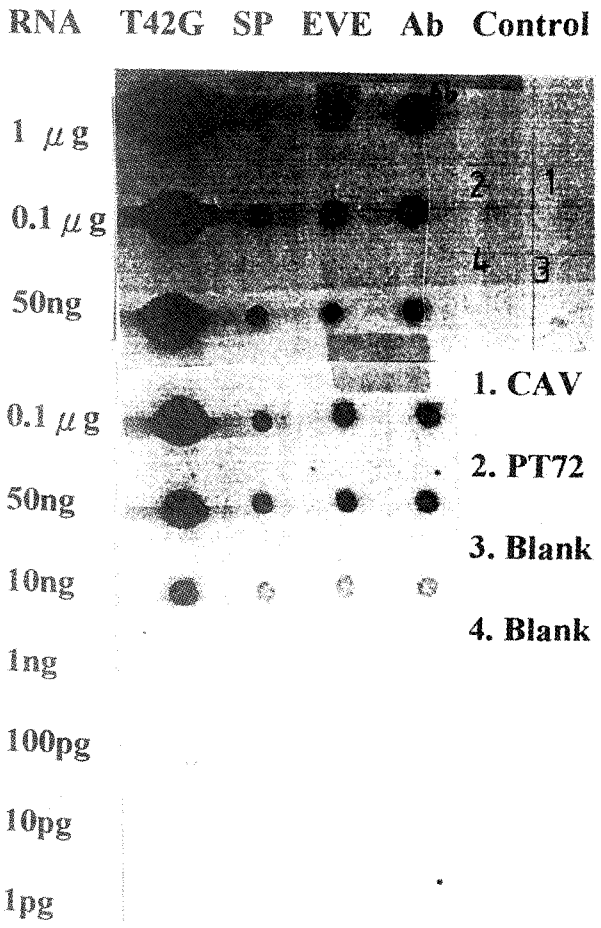


**Figure 6.** DNA-RNA dot blot hybridization of different serotype of IPNV. The denatured RNA of different serotype of IPNV was blotted and fixed on the membrane and hybridized with the $^{32}P$-labeled probe of RT-PCR product of B,D primers and template #2. Controls: CAV (clam aquareovirus) dsRNA, pT72/A dsDNA, Blanks.

From the RT-PCR and the southern blot hybridization, we understood that from the RT-PCR reaction from the B, D primers for these IPNV strains could produce around 300 bp DNA fragment. For the RNA-DNA dot blot hybridization, the $^{32}P$-labeled 300-mer single-stranded DNA probe from T42G RT-PCR from B, D primers, and different strain viral RNA were used, as shown in the Fig. 5, this specific probe could detect the T42G around 10 to 100 pg RNA, SP about 10 ng RNA, EVE about 1 to 10 ng RNA and Ab strain about 10 ng RNA. For the negative control, the CAV (clam aquareovirus) dsRNA (0.5μg) could not be detected. The pT72/A insert DNA (50 ng) could be detected as positive control.

## Discussion

There are many IPNV and IPNV-related isolates. Only three major serotypes exist in IPNVs. The VP2 of IPNV is the major capsid protein with serotypic and neutralizing epitopes (Frost *et al.* 1995; Tarrab *et al.* 1995; Liao and Dobos, 1995). Two conserved regions located at the N-terminal and C-terminal end, the variable region is in the middle part, from 184 amino acid to 331 amino acid (Heppel *et al.* 1993; Havarstain *et al.* 1990; Tarrab *et al.* 1995) which is the serotypic epitopes location (Liao and Dobos, 1995). We designed the PCR primers for two purposes. One is to identify all the IPNV isolates, another one is to classify the serotype. Based on these, the A and C primers located at N-terminal conserved region and the B and D primers in the variable region of VP2 were chosen. Lopez-Lastra *et al.* (1994) designed seven primers located at very similar region as ours for their double-nested RT-PCR. However, Rimstad *et al.* (1990a and b) used 24-mer DNA probe and 310 bp PCR probe based on the sequence of IPNV protease. Both sets of RT-PCR were designed to identify the IPNV, but not for serotyping.

The critical point of RT-PCR is the cDNA synthesis, and the denaturation of dsRNA is the first parameter of IPNV RT-PCR. The dsRNA in the 60% DMSO was heated to 100 ℃ or 60 ℃ for 10 min, which is different from Rimstad group's (95 ℃ for 5 min) (1990a and b), and Lopez-Lastra's group (104 ℃ for 6 min) (1994). This is probably due to the 5' flanking sequence of VP2 contained the secondary structure (Havarstein et al., 1990), which is not easy to be opened. This also could be explained that our presumed 730 bp PCR product from A, C primers obtained so little that could be detected only by hybridization (Fig. 4A and 4B). This also indicated that the locations of primers in the open reading frame would be much easier to be opened for annealing.

For the RT-PCR of IPNV, one specific primer was used for our ds cDNA synthesis. From Fig. 3, the optimal yield of ds cDNA synthesis was the C primer (template #3). However, the data from the RT-PCR, the single presumed size of PCR product was from the template #2 and B, D primers. The yield of template #2 was very little (Fig. 3), but the quantity and quanlity was good enough for synthesizing specific PCR product of IPNV (Fig. 4A and 4B). The time of our ds cDNA synthesis was too long, compared to other groups (Rimstad et al., 1990a and b; Lopez-Lastra et al. 1994). Rimstad's group used one specific primer for ss cDNA synthesis, and the reaction was carried out at 42 ℃ for 30 min. Lopez-Lastra et al. (1994) used two specific primers for ds cDNA synthsis, and this reaction was carried out at 42 ℃ for only 15 min. We should try synthesizing ss cDNA as the template, and combined with new different sets primers for IPNV RT-PCR.

The sensitivity, specificity and speedy are the merits of RT-PCR. These are also the important criteria for diagnosis. Our dot blot hybridization could detect 1pg to 10 ng dsRNA, depending on different isolates. However, the double-nested PCR could detect 1 pg dsRNA by Lopez-Lastra's group (1994), and 0.8 pg dsRNA by Rimstad's group (1990). In general, the identification and serotyping of IPNV could be obtained from our RT-PCR by using template #2 and B, D primers. However, the cDNA synthesis conditions and primers for PCR could be improved for saving time and serotyping.

## Acknowledgement

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# 利用反轉錄－聚合酶連鎖反應及點墨雜交反應作感染性胰臟壞死病毒專一性檢測

林奎甫　洪紋玲　徐亞莉
中央研究院動物研究所

## 摘　要

基於 RT-PCR 原理，檢測IPNV的快速、靈敏且專一的方法發展出來了。病毒 RNA 可自細胞培養中抽取，作為合成 cDNA 的 template。依 IPNV 的 VP2 coding region 設計並合成一專一性的引子合成 cDNA。利用primer B合成之dsDNA為template #2 和 B、D primers，作 PCR，可得單一且專一的約 300bp 的 PCR 產物，此產物大小可作血清型的確定。利用此300bp PCR 產物作探針，DNA-RNA 的點墨雜交反應可檢測到T42G為10到100pgRNA，SP的10ngRNA，EVE的1到10ngRNA，及 AB的10ngRNA。這些方法可以進一步研究而改進。

**關鍵詞**：感染性胰臟壞死病毒、反轉錄－聚合酶連鎖反應、點墨雜交