

Detection of Infectious Pancreatic Necrosis Virus RNA with Radiolabeled and Digoxigenin-labeled cDNA Probes

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Horng-Cherng Wu, Chi-Yao Chang and Jen-Leih Wu (1996) Detection of infectious pancreatic necrosis virus RNA with radiolabeled and digoxigenin-labeled cDNA probes. *Zoological Studies* 35(1): 49-54. A cDNA probe representing 418 base pairs at the center of the genome segment A of the E1S virus (which belongs to the infectious pancreatic necrosis virus-Ab serotype) was used in a dot blot hybridization assay to detect E1S viral RNA from cell culture. The best results of dot blot hybridization were found when E1S viral RNA was denatured by heating in a boiling water bath for 10 min, then quick-chilled in -20 °C ethanol or denatured in 0.1 N NaOH for 5 min. It was concluded that radioactive probes and digoxigenin-labeled probes had the same sensitivity in dot blot hybridization, because the minimum quantity of viral RNA detected by each was 0.1 ng. Therefore, digoxigenin-labeled probes could replace radioactive probes thus providing a safe and effective method for the detection of the E1S virus by dot blot hybridization.

Key words: Birnaviridae, Digoxigenin, Infectious pancreatic necrosis virus.

The infectious pancreatic necrosis virus (IPNV) is an important viral pathogen in shellfish, freshwater fish, and marine fish. It belongs to the family Birnaviridae (Dobos et al. 1979). The virus is an unenveloped, single-shelled, icosahedral capsid. The viral genome consists of two segments of double-stranded RNA (dsRNA). The larger segment, A, contains a large open reading frame capable of encoding a polyprotein that undergoes post-translational cleavage to produce the 2 structural proteins, VP2 and VP3, and a nonstructural protein, NS (Mertens and Dobos 1982, Duncan et al. 1987). The sequence of the coding regions for these 3 proteins at segment A is in the 5'-3' direction: VP2-NS-VP3 (Nagy et al. 1987). The smaller segment, B, encodes a protein designated VP1, which is assumed to be a dsRNA-directed RNA polymerase (MacDonald and Dobos 1981). Three major serological groups of IPNV have been identified (MacDonald and Gower 1981, Hedrick and Okamoto 1982, Okamoto et al. 1983). Two of these serotypes, Sp and Ab, are the predominant

strains found in Europe; the 3rd serotype, VR299, is prevalent in North America. Hill and Way (1983) have discovered 6 additional serotypes constituting a major serogroup. A number of strains isolated from Asian fish are closely related to the Ab strain. These can be differentiated by variation in RNA genome size and by viral polypeptide size (Hedrick et al. 1983, Wu et al. 1987, Hsu et al. 1989, Lipipun et al. 1989). The eel virus strain (E1S), which belongs to the Ab serotype of IPNV, contains a bi-segmented double-stranded RNA genome consisting of a 3.2 kb segment, A, and a 2.9 kb segment, B (Chang 1983, Wu et al. 1987). In recent years, the intensive culture of shellfish and fish has developed very rapidly, and fish disease has become a serious problem; IPNV is one of the most severe viral diseases. Diagnosis of IPNV infection is primarily by isolation of virus from cell culture, and identification in neutralization. There are at least 3 major serological groups of IPNV, which makes diagnosis difficult and time consuming. Improved methods of DNA cloning and

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hybridization have led to the development of diagnostic assays that use nucleic acid probes to detect animal pathogens (Jackwood 1990, Rimstad et al. 1990). The hybridizations often use radioactive probes for their high sensitivity, but the use of radioactive isotopes has several intrinsic difficulties, e.g., short half-lives, long exposure times, and the complexity and danger of handling radioactive substances. Therefore several non-radioactive methods have been developed. One good alternative to radioisotopes is the use of digoxigenin-labeled cDNA probes for hybridization (Keam et al. 1991, Hathcock and Giambone 1992). In this paper, we describe optimal denaturation of E1S viral RNA by heat and alkali. We also compare the sensitivity of digoxigenin-labeled probes with that of ^{32}P -labeled probes for the detection of E1S virus.

MATERIALS AND METHODS

Extraction of viral RNA

A CHSE-214 cell line was infected with E1S virus at an M.O.I. of 0.1. The culture was incubated for 3 to 5 d, until a complete cytopathogenic effect (CPE) was seen. The culture was centrifuged at 2 500 g for 10 min at 4 °C, and then 10% sarcosyl was added to the supernatant to a final concentration of 0.1%. The liquid was then centrifuged at 13 000 g for 1 h at 4 °C. The pellet was resuspended in TNE (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA) containing 0.5% SDS and 200 $\mu\text{g}/\text{ml}$ proteinase K (Sigma Chemical Co., St. Louis, MO), after which proteins were separated from dsRNA by extraction with phenol/chloroform and precipitated with ethanol. Viral RNA was resuspended in TE buffer (0.01 M Tris-HCl, 0.001 M EDTA pH 7.6) and the concentration was estimated by comparing the intensity of the DNA size markers. The quality of the viral RNA was evaluated by electrophoresis on 1% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA) and stained with ethidium bromide. A 1 kb molecular weight ladder (Bethesda Research Laboratories) was used as a size marker.

Preparation of probes

DNA probes were prepared from a *pA28* clone (Her 1989). Cloned E1S virus sequences in *pA28* were excised from *pUC18* plasmids by using *EcoRI* restriction endonuclease and separated on 1%

agarose gels in TAE buffer. The cloned segments were electroeluted from gels into TAE buffer before use in random primer labeling reactions. The cDNA segments (60 ng) were random primer labeled with 50 μCi of [^{32}P]dCTP (specific activity > 600 $\mu\text{Ci}/\text{mM}$, Amersham) using Klenow enzyme (Promega), and then purified through a push column (Stratagene). Incorporation of ^{32}P was checked with a β -counter. For comparative studies, cDNA segments were random primer labeled with digoxigenin-dUTP (Boehringer Mannheim) using Klenow enzyme, and then purified with lithium chloride and precipitated with ethanol.

Dot blot hybridization

Viral RNAs were denatured at 100 °C for 10 min and quick-chilled on ice. The denatured RNA samples in a volume of 2 μl or less were placed directly onto nylon membranes (Amersham). Alternately, viral RNAs (1 μl) were placed directly onto nylon membranes, and the membranes were soaked in NaOH solution for 5 min, then soaked twice in 1 M Tris, 1.5 M NaCl (pH 8.0) for 5 min. Nylon membranes containing denatured viral RNA were dried for 20 min and fixed with 2 exposures to ultraviolet radiation with a UV-stratalinker at an intensity of 120 000 microjoules.

The membranes were prehybridized for 2 to 4 h at 42 °C in a hybridization solution containing 5 \times SSC, 5 \times Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 100 $\mu\text{g}/\text{ml}$ boiled calf thymus DNA, and 50% formamide. Heat-denatured digoxigenin-labeled probes were added to the hybridization solution and incubated at 42 °C for 12 h. After hybridization, the membranes were washed twice at room temperature in 2 \times SSC and 0.1% SDS for 5 min, and washed twice in 0.1 \times SSC and 0.1% SDS for 15 min at 68 °C. The membranes were then incubated for 30 min in anti-digoxigenin-alkaline phosphatase (AP) conjugate (Boehringer Mannheim) diluted to 1:5 000 with 1% blocking solution (Boehringer Mannheim) at room temperature. Membranes were washed in 0.1 M maleic acid, 0.15 M NaCl (pH 7.0) for 15 min then equilibrated 5 min in 0.1 M Tris, 0.1 M NaCl, and 0.05 M MgCl_2 (pH 9.5). Membranes were incubated in 0.1 mg/ml 4-methoxy-4-(3-phosphatophenyl) spiro(1,2-dioxethan-3,2-adamantane) (Lumigen PPD) (Boehringer Mannheim) for 5 min. The results were observed using autoradiography. Alternately, membranes were incubated in the dark in substrate buffer containing 0.34 mg/ml 4-nitrobuty-

tetrazolium chloride NBT and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate X-phosphate (Boehringer Mannheim). The color of precipitation on membranes was observed. For comparative studies, dot blot hybridization was performed with 1.6×10^7 Ci min^{-1} probes. The results were observed using autoradiography.

RESULTS

Purification of E1S viral RNA

The viral RNA was extracted by proteinase K digestion; the extraction was completed within 8 h. The 2 segments of E1S viral dsRNA, approximately 3.2 kb and 2.9 kb, are shown in Fig. 1.

Optimal denaturation of E1S viral RNA

RNAs can be denatured by heating and then chilling quickly to avoid renaturation. The E1S viral RNA (10 ng) was denatured by heating in a boiling water bath for 10 min and then chilling quickly with ice, -20°C ethanol, or liquid nitrogen. The denatured RNAs were hybridized with ^{32}P -

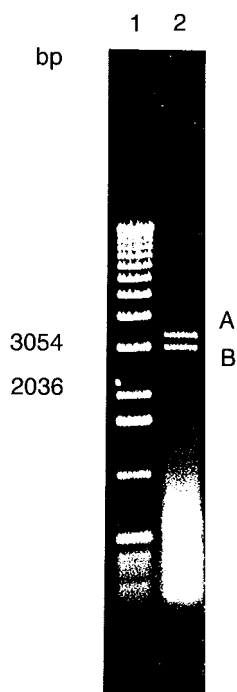


Fig. 1. Agarose gel electrophoresis of E1S genomic RNA. A 1 kb ladder marker (lane 1) was included for size reference. Viral genome segment A (3.2 kb) and B (2.9 kb) purified from IPNV strain E1S (lane 2) are indicated.

labeled probes (Fig. 2). The dot density of hybridization was scanned with a computing densitometer (Molecular Dynamics). The comparison of dot densities is shown in Table 1. E1S viral RNA chilled with -20°C ethanol had the highest dot density. RNAs can also be denatured with NaOH solution. E1S viral RNA (10 ng) was placed directly onto nylon membranes and then denatured in various concentrations (0.8 N, 0.6 N, 0.4 N, 0.2 N, 0.1 N, and 0.05 N) of NaOH for 5 min. The denatured viral RNAs were hybridized with ^{32}P -labeled probes (Fig. 3). Dot density from treatment with 0.1 N NaOH was 2.98 times greater than that from treatment with 0.8 N NaOH (Table 2).

Sensitivities of radioactive probes and digoxigenin-labeled probes

Radiolabeled probes (1.6×10^7 Ci min^{-1} , 60 ng) and digoxigenin-labeled probes (60 ng) were hybridized with 10, 1, 0.1, and 0.01 ng of E1S viral RNA. The hybridized digoxigenin-labeled probes were immuno-detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody and then developed with chemiluminescent substrates, Lumigen PPD, or with the colorimetric substrates NBT and X-phosphate (Fig. 4). The pA28 probes did not hybridize with calf thymus DNA or fish (*Oreochromis mossambicus*) total RNA. The minimum quantity of E1S viral RNA detected with either labeled probe was 0.1 ng.

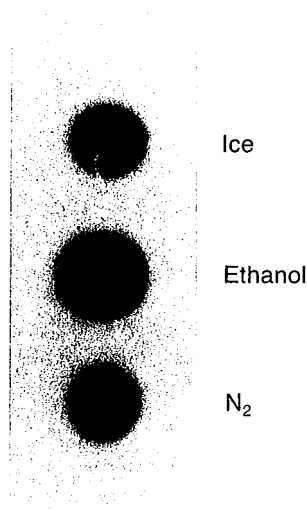


Fig. 2. Effect of chilling temperature on denaturation of E1S viral RNA. Viral RNA was denatured in a boiling water bath for 10 min, and quickly chilled with ice, -20°C ethanol, or liquid nitrogen (N_2). The denatured RNAs were hybridized with a probe.

DISCUSSION

Hybridization techniques are becoming increasingly helpful in the clinical setting, eliminating the need for laborious serological techniques, and in some instances, offering increased sensitivity (Henderson et al. 1991). With the development of non-radioactive labels for nucleic acid probes, these techniques have become practical for the diagnostic laboratory. Up to now, digoxigenin-labeled probes have not been used to detect IPNV. The application described here shows that E1S virus can be sensitively detected with a digoxigenin-labeled *pA28* cDNA probe.

Our previous studies of E1S had shown that E1S viral RNA, denatured on a 95 °C hot plate for 10 min, could not be hybridized with probes (data not shown) because the melting temperature of E1S viral RNA is 83 °C (Her 1989). Viral RNA was denatured in a 100 °C boiling water bath for 10 min, then quick-chilled with ice, -20 °C ethanol, or liquid nitrogen (Jackwood 1990). E1S viral RNA can be hybridized with a *pA28* probe, and chilling with -20 °C ethanol is 2.18 fold more efficient than ice chilling.

Table 1. A comparison of dot density on hybridization. E1S viral genome (10 ng) was denatured in a boiling water bath then quickly chilled with ice, -20 °C ethanol, or liquid nitrogen. Denatured RNAs were hybridized with a probe. Dot densities were measured with a computing densitometer

| Chilling treatment | Pixel value | Relative value |
|--------------------|-------------|----------------|
| Ice | 1 116 | 1.00 |
| -20 °C ethanol | 2 437 | 2.18 |
| Liquid nitrogen | 1 609 | 1.44 |

Table 2. A comparison of dot density on hybridization. E1S viral genome (10 ng) was denatured with different concentrations of NaOH, and hybridized with probes. Dot densities were measured with a computing densitometer

| NaOH concentration (N) | Pixel value | Relative value |
|------------------------|-------------|----------------|
| 0.8 | 866 | 1.00 |
| 0.6 | 908 | 1.06 |
| 0.4 | 1 087 | 1.26 |
| 0.2 | 1 569 | 1.81 |
| 0.1 | 2 584 | 2.98 |
| 0.05 | 2 180 | 2.52 |

We also denatured viral RNA with a NaOH solution at room temperature for 5 min (Rimstad et al. 1990). The optimal concentration was 0.1 N NaOH. Viral RNA could be degraded by higher concentrations of NaOH, but could not be completely denatured in a lower concentration.

Radioisotopic cDNA probes are used to detect homologous viral RNA in the dot blot hybridization technique, which is sensitive and relatively easy. This method, however, needs a radioactive probe to achieve this sensitivity and thus suffers from

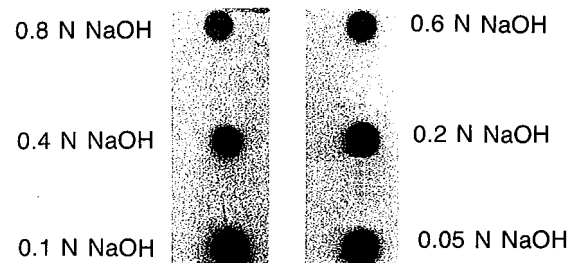


Fig. 3. Hybridization of E1S viral RNA denatured with different concentrations of NaOH solution.

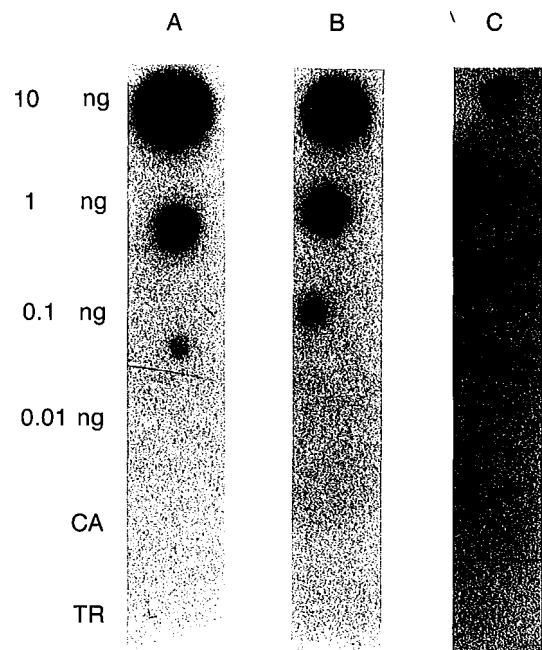


Fig. 4. Hybridization of E1S viral RNA with a ^{32}P -labeled probe and digoxigenin-labeled probe. E1S viral RNA (10, 1, 0.1, and 0.01 ng) was denatured using 0.1 N NaOH for 5 min, then hybridized with a ^{32}P -labeled probe (A; $1.6 \times 10^7 \text{ Ci m}^{-1}$, 60 ng), digoxigenin-labeled probe developed with chemiluminescent substrate (B) or colorimetric substrates (C). Calf thymus DNA (CA) and fish (*Oreochromis mossambicus*) total RNA (TR) were the negative controls.

the instability and hazards associated with radioactive substances. For this reason, several non-radioactive methods have been developed (Davis et al. 1994, Lopez-Lastra et al. 1994, Melby and Christie 1994), and one good alternative to radioisotopes is the use of digoxigenin-labeled nucleotide probes to detect viral RNA (Hathcock and Giambone 1992, Jackwood et al. 1992). This article describes E1S viral RNA detection with digoxigenin-labeled cDNA probes in dot blot hybridization; the minimum quantity of viral RNA detected was 0.1 ng. The sensitivity of a digoxigenin-labeled probe is equal to that of radioactive probes (in this study). The smallest quantity of viral RNA detected with radioactive probes was from 100 ng to 0.1 ng, depending on the specific activity of the radioactive probe (Jackwood et al. 1989, Jackwood 1990, Rimstad et al. 1990, Dopazo et al. 1994). The radioactive probe used in this study was more sensitive than those used by these other researchers, and thus digoxigenin-labeled probes for dot blot hybridization can be substituted for detection methods using radioactive substances.

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利用放射性探針與非放射性探針檢測魚類感染性胰臟潰瘍病毒

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從感染性胰臟潰瘍病毒-E1S病毒中製成的cDNA，含有E1S病毒基因體A片段，中間部位418個鹼基。此cDNA經放射線物質或digoxigenin標記後，可用點雜交法，偵測到由細胞培養而來的E1S病毒。

E1S病毒最好的加熱變性條件是100 °C水浴加熱10分鐘，馬上在零下20 °C酒精中冷卻；而最好的鹼性變性條件是0.1 N氫氧化鈉處理5分鐘。

經放射物質或digoxigenin標記後探針，利用在點雜交法上有相同的靈敏度，最少能檢測到0.1 ng的E1S病毒。Digoxigenin標記後的探針可以取代放射物質標記的探針，以避免處理放射物質之不便。

關鍵詞：兩段核糖病毒，感染性胰臟潰瘍病毒。

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