NOTE

Single-tube, non-interrupted reverse transcription PCR for detection of infectious pancreatic necrosis virus

Way-Shyan Wang*, Yea-Ling Wi, Jainn-Shyan Lee

Department of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan 402, ROC

ABSTRACT: An assay using a single-tube, non-interrupted reverse transcription-polymerase chain reaction (RT-PCR) was established for the detection of infectious pancreatic necrosis virus (IPNV). Two primer sets, Pr D and Pr F, which framed a region within the gene coding for IPNV VP2 protein were used to amplify specific fragments of the IPNV genome. Amplified products were detected in cultured chinook salmon embryo cells (CHSE-214) infected with different IPNV strains including WB, Ja, Sp, Ab, VR299, 3372, MFK, and CV-HB-1, but not in uninfected CHSE-214 nor in cells infected with IHNV or infectious bursal disease virus (IBDV). The detection limit of this method was estimated with purified viral RNA from the WB strain of IPNV to be between 15 fg and 15 pg in ethidium bromide-stained gels.

KEY WORDS: RT-PCR - IPNV - Fish - Virus


At present, since viral diseases in fish cannot be easily cured by therapeutic reagents, rapid and effective diagnosis of aquatic birnavirus is essential for the control of viral diseases. Despite the fact that serological diagnostic methods such as ELISA (Shankar & Yamamoto 1994), immunodot blot (Hsu et al. 1989), fluorescent antibody test, coagglutination test (Kimura et al. 1984), and cDNA probes (Dopazo et al. 1994) have been developed for IPNV detection, these methods have still been deemed not sufficiently sensitive, rapid, or economically feasible for use under field conditions (Lee et al. 1994, Lopez-Lastra et al. 1994, Wang et al. 1995). Recently, a rapid and sensitive 2-step reverse transcription-polymerase chain reaction (RT-PCR) assay for identifying IPNV in infected cell cultures and fish tissue samples has been described (Rodak et al. 1988, Lopez-Lastra et al. 1994, Blake et al. 1995, Lee et al. 1995, Wang et al. 1995). This method was further modified so that all reagents required for both RT and PCR were added into a single tube and run through a non-interrupted thermal cycling program as described for the detection of rotavirus (Xu et al. 1990) and infectious bursal disease virus (IBDV) (Lee et al. 1994). For routine diagnostic laboratories, the single-tube RT-PCR method is easier and more rapid than the 2-step RT-PCR method. We describe in this paper a non-interrupted RT-PCR in a single tube for the detection of IPNV.

Materials and methods. Viruses and cells. Virus isolation was essentially a modification of the method described by Wang et al. (1995). Eight IPNV strain isolates including WB, Ja, Sp, Ab, VR299, 3372 (isolated from eel in Taiwan), MFK (isolated from milk fish in Taiwan), and CV-HB-1 (isolated from hard clam in Taiwan) were used. These IPNV isolates were propagated in CHSE-214 (chinook salmon embryo cell) with Eagle's minimum essential medium (MEM, Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Gibco BRL Life Technologies Inc., NY, USA) at 18°C for 5 to 6 d. The titer,
Viral RNA purification. Viral RNA was purified according to the method of Lee et al. (1994). The WB strain of IPNV was propagated in CHSE-214 and concentrated with polyethylene glycol 10000 (PEG, Sigma Chemical Co., St. Louis, MO, USA). After centrifuging (10000 × g, 1 h), the pellet was resuspended in TNE buffer (0.01 M Tris-HCl, pH 7.6; 0.1 M NaCl, and 0.001 M EDTA), followed by extraction with Freon TF (DuPont, Sydney, Australia), and the aqueous phase was centrifuged isopycnically in a stepwise gradient of 40%, 30%, and 20% CsCl (Sigma Chemical Co., St. Louis, MO, USA). The viruses banding at a buoyant density of 1.33 g ml⁻¹ in CsCl gradient were collected and pelleted by centrifugation at 132000 × g at 4°C for 2 h. The pellets were resuspended in TNE buffer containing 0.5% sodium dodecyl sulfate, and proteinase K was added to a final concentration of 1 mg ml⁻¹. After incubation at 37°C for 2 h, the viral RNA was extracted with phenol and chloroform (Wang et al. 1995). Viral dsRNA was purified with LiCl (Sigma Chemical Co., St. Louis, MO, USA) precipitation (Diaz-Ruiz et al. 1978) and then washed twice with 70% ethanol to remove the LiCl. Finally, the purified viral dsRNA was resuspended in TE buffer (0.01 M Tris-HCl, pH 8.0; 0.001 M EDTA).

Detection limit assay. Two primer sets, Pr D and Pr F, were used for this assay. The purified IPNV-WB strain RNA template was serially diluted in 10-fold increments with 0.5% DEPC-water (Sigma Chemical Co., St. Louis, MO, USA) from 15 ng to 0.015 fg.

Viral RNA detection in fish organs. IPNV free cultured rainbow trout (about 5 to 6 cm) were infected with the WB stain of IPNV (106 TCID₉₀ ml⁻¹) by bath immersion for 30 min. Negative control fish were immersed in MEM-containing water. Four days after post-infection, the infected rainbow trout organs including kidney, gill, intestine, liver, and spleen were collected and homogenized. Then 100 µl of the organ mixture was reacted with 900 µl lysis buffer (8 M guanidinium HCl, 0.1 M EDTA, 0.3 M sodium acetate) and cooled in an ice bath for 20 min. Next, 1 ml of a phenol/chloroform/isoamyl alcohol (25:14:1) mixture was added to the reaction tube, and the combined contents were shaken and centrifuged at 12000 × g for 10 min. The aqueous phase was collected and the same amount of chloroform/isoamyl alcohol (24:1) was added; the solution was again mixed well. After centrifugation, the resulting aqueous phase was collected, and the 2 combined aqueous phases precipitated with 100% ethanol. The pellet RNA was then resuspended in DEPC water for the single tube RT-PCR reaction.

Table 1. Specific primer sets for infectious pancreatic necrosis virus

<table>
<thead>
<tr>
<th>Primer set</th>
<th>VP2 location</th>
<th>Sequence</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr D</td>
<td>419–693</td>
<td>5'-CGGAAATACGACATCCAGAGC-3' 5'-TGCCCTCGGTCTATGGAGCTGG-3'</td>
<td>274</td>
</tr>
<tr>
<td>Pr F</td>
<td>807–1330</td>
<td>5'-GCCGACATCGTCAACTCCAC-3' 5'-GACAGGATCATCTGGCATA-3'</td>
<td>524</td>
</tr>
</tbody>
</table>
Detection limit of the single tube RT-PCR method. Viral RNA, in a series of dilutions in 10 increments ranging from 1.5 ng to 0.15 fg, was tested by RT-PCR for detection by the standard single-tube RT-PCR method described here. The detection limit using the Pr D primer was 15 fg, which correlates to about 200 virus particles (Fig. 3). When the Pr F primer was used, the detection limit was 15 pg, or about 20,000 viral particles (Fig. 4).

Viral RNA detection in fish organs. Viral RNA in fish tissues could be detected by the single tube RT-PCR method without any interference from the RNA of fish cells (Figs. 5 & 6).

Discussion. PCR amplification has become a standard method in molecular biology research, and is used in diagnostic laboratories because of its high sensitivity and specificity. RT-PCR has been developed for detecting IPNV RNA but the protocols always require 2 separate major stages: RT and a PCR cycle. Previous studies have shown that the Pr D and Pr F primers are very specific for IPNV (Lee et al. 1995, Wang et al. 1995). The single tube, non-interrupted RT-PCR method described here is easier and faster than the 2-step RT-PCR procedures described in the earlier reports, particularly with a large number of samples. We have shown that viral IPNV RNA can be successfully detected by using the simplified RT-PCR protocol in which the 2 stages are combined and run sequentially in a single tube. Furthermore, the primer sets identified in the study did not react with nucleic acid from IBDV, IHNV, or uninfected cells.

The detection limits of the assay were 15 fg and 15 pg for primer Pr D and Pr F, respectively. This detection limit is same as that of the 2-step RT-PCR method (Wang et al. 1995). Different primer sets were shown to have different detection limits.
in this single tube RT-PCR. This might be due to the position and length of the gene that was amplified. The Pr F primer amplified a distant region, 807 to 1330 bp, within the VP2 gene of the IPNV, while the Pr D primer amplified the 419 to 693 bp region of the gene. The lower detection limit of Pr D may be due to the length of the amplified product: a shorter gene sequence is much more easily amplified. The relationship of concentrations between reverse transcriptase (RTase) and Taq polymerase activities has been described (Lee et al. 1994). Taq polymerase activity may be completely inhibited when the RTase concentration increases because an amplification product was not obtained. Lee et al. (1994) used different concentrations of RTase and Taq polymerase to reduce the interference between the 2 enzymes in a tube. We have tried unsuccessfully to use mineral oil to separate the RT and PCR reactions, but found that the mineral oil was too light in weight to fully divide the 2 reactions. Finally, we found that wax was firm enough to completely separate the 2 reactions until the reaction temperature reached 45°C. The expected products were consistently amplified by the single-tube method after mineral oil was replaced with wax.

Theoretically, the amount of amplification product increases if the PCR cycles are extended. However, this increase is limited by several factors, one of which is the enzymatic stability of Taq polymerase following thermal cycle reactions. The half-life of Taq polymerase at 92.5 and 95°C is 130 and 40 min, respectively. Hence, the amplification product can be increased by increasing the number of the PCR cycles but the temperature for denaturation of the DNA template may be the limiting factor (Lee et al. 1994).

Our results show that a sensitive and specific, single-tube RT-PCR may be a feasible alternative tool for detecting IPNV.

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