Single-Tube, Noninterrupted Reverse Transcription-PCR for Detection of Infectious Bursal Disease Virus

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An assay protocol based on single-tube, noninterrupted reverse transcription-PCR (RT-PCR) for the detection of infectious bursal disease virus (IBDV) is described. After the conditions for RT-PCR had been optimized, a primer set framing a region within the gene coding for IBDV VP2 protein was used to amplify a 318-bp fragment of the IBDV genome. Amplified product was detected with three strains of IBDV, whereas none was obtained from uninfected bursal tissue or seven unrelated avian viruses. The sensitivity of this RT-PCR was tested with purified viral RNA from three strains of IBDV. The detection limit was 10 fg in an ethidium bromide-stained gel. In addition, this assay system was used to detect IBDV in bursal-tissue specimens from commercially reared chickens. The identity of the amplified products from the tissue specimen preparation was determined by using a rapid, simple procedure in which internally nested, end-labeled probes were used.

Infectious bursal disease virus (IBDV) infections cause a variety of disease syndromes in young chickens. These range from loss of feeding efficiency (13) to ablation of the antibody immune response (25). At least two serotypes of the virus are currently recognized (5, 14); the known serotype 1 viruses are pathogenic only in chickens, and pathogenic serotype 2 viruses have not been isolated (2). During the past few years, several workers have described new virulent chicken isolates which represent either a different serotype or variants of the classic strains isolated before 1988 and currently circulating in the field (18, 19). IBDV is classified as a birnavirus (8). The virus genome consists of two segmented double-stranded RNAs (dsRNAs) (16). The larger segment (segment A) is approximately 3.4 kb in length, while the smaller segment (segment B) is approximately 2.9 kb long (1).

The methods for diagnosis of IBDV infection include agar gel precipitation, virus neutralization, and enzyme-linked immunosorbent assay (ELISA). They are designed to measure levels of antibodies to IBDV. In addition, direct immunofluorescence for demonstrating the presence of viral antigen has been routinely used with tissue sections or impression smears (15). Antigen capture ELISA has been described for the detection of IBDV antigens directly from the infected bursal tissues (9, 23). Radiolabeled (6) and nonradiolabeled cDNA probes (7, 9a) used for detecting IBDV RNA have recently been considered more sensitive than the other methods. Laterly, another method, reverse transcription-PCR (RT-PCR), has been described for the diagnosis of IBDV infection (12). The sensitivity of this technique could be further enhanced by hybridization of amplification products after Southern transfer. Attempts have been made to simplify the number of manual manipulations required for processing a large number of samples. Recently, a system was designed whereby all the reagents required for both RT and PCR can be added to a single tube and a simple, noninterrupted thermal cycling program can be carried out for detection of rotavirus (26) and Ross River virus (21). Here we describe a combined RT-PCR assay for the detection of IBDV. The identity of PCR amplification products was determined by mixing the products with an internally nested, end-labeled oligonucleotide probe followed by one cycle of PCR.

MATERIALS AND METHODS

Viruses and cells. Three strains of IBDV representing both serotypes 1 and 2 were used. MO is a serotype 2 turkey isolate (11), while P3009 and 29/11 are isolates locally obtained from chickens. They have been described as serotype 1 viruses (10). All viruses were propagated in chicken embryo fibroblasts (CEF) as described by Lee and Lukert (11). Seven unrelated avian pathogens were used to determine the specificity of the oligonucleotide primers. The six viruses tested were the commercially available vaccinia viruses and included infectious bronchitis virus (Intervet International B. V., Boxmeer, Holland), turkey herpesvirus and Newcastle disease virus (Salsbury Laboratories), avian reovirus strain 1133 (Vineland Laboratories, Vineland, N.J.), and infectious laryngotracheitis virus and fowlpox virus (Nippon Institute for Biological Science, Tokyo, Japan). In addition, an avian influenza virus (20) was also used for the specificity test.

Viral RNA purification. Virus particles of IBDV strains P3009, 29/11, and MO were separately purified by methods described previously (9). Briefly, after the viruses had been propagated in CEF and concentrated by polyelectrolyte glycyl 6000 they were pelleted and 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). The aqueous phase was cushioned by 35% sucrose, and the virus-containing pellets obtained were then centrifuged isopycnically in stepwise gradients of 40, 30, and 20% CsCl2. The viruses banding at a buoyant density of 1.33 g/ml in CsCl2 gradient were withdrawn and were pelleted by centrifugation at 132,000 x g at 4°C for 2 h. The pellets were resuspended in TNE buffer containing 0.5% sodium dodecyl sulfate, and protease K (Boehringer Mannheim) was added to a final concentration of 1 mg/ml. After incubation at 37°C for 2 h, the viral RNA was extracted with phenol and chloroform according to the standard procedures. Viral dsRNA was then purified by differential LiCl precipitation (3) and washed twice with 70% ethanol to remove LiCl. Finally, purified viral
FIG. 1. IBDV genome segment A and PCR fragment. The black bar represents genome segment A of IBDV strain Cu-1. The respective regions of the genome encoding viral proteins are indicated by unshaded bars. The segment of VP2 marked 318 bp displays the fragment size amplified by PCR. The arrow indicates the direction and size of the internally nested, end-labeled probe.

dsRNA was resuspended in TE buffer (0.01 M Tris-HCl, pH 8.0; 0.001 M EDTA) until used.

Preparation of nucleic acid from tissue specimens. Bursal-tissue specimens (specimens 1 to 23) were collected from 21 different commercial broiler chicken farms where IBDV infections were suspected. Specimens 2 and 3 have been previously examined with monoclonal antibody probes to IBDV strain P3009 by immunodot assay (9) and RT-PCR (12). Specimen 2 (designated specimen J in previous reports) was identified as IBDV negative, whereas specimen 3 (designated specimen G in previous reports) was IBDV positive. Each sample to be tested contained a pool of two to four bursae, which were homogenized in TNE buffer (3 ml per bursa). Following low-speed centrifugation, a 50-μl volume of the original bursal homogenate was mixed with 1 ml of lysis buffer (8 M guanidinium HCl, 0.1 M EDTA, 0.3 M sodium acetate) for 20 min at 0°C and then centrifuged at 12,000 × g for 20 min at 4°C. The supernatant was collected, and nucleic acids were precipitated with ethanol.

Preparation of nucleic acid from unrelated avian pathogens. The specificity of the primer set for IBDV was further determined by using the IBDV strains (P3009, 29/11, and MO) and the seven unrelated avian viruses mentioned above. The nucleic acids were directly extracted from a minimum of 1,000 infectious virions. The lyophilized viruses were resuspended in TNE buffer. Nucleic acids were released by treatment with lysis buffer and were precipitated with ethanol as described for bursal-tissue specimens.

Oligonucleotide primers for RT-PCR and the internally nested, end-labeled probe. Primers were chosen according to the cDNA sequence of the IBDV strain Cu-1 genome segments reported by Spies et al. (24). Primers were selected in the part of the IBDV genome segment A coding for VP2 capsid protein (Fig. 1). The sequences of primers for RT-PCR are as follows: p1, 5′-GAGATCAGAAACGGGATGCAGCA-3′ (identical to nucleotides 72 to 92, numbered according to reference 24); p2, 5′-GTAGTTGAATACTGGGCCGA-3′ (complementary to nucleotides 372 to 389). The sequence of the oligomer used as the internally nested, end-labeled probe is identical to nucleotides 249 to 263.

RT-PCR. The system was designed so that all components for both RT and PCR could be combined in one 0.5-ml Eppendorf tube and both reactions could be run in a simple noninterrupted thermal cycling program. Standard conditions for RT of the viral RNA and amplification of the 318-bp sequence were as follows. Each reaction tube contained target viral RNA at various dilutions; 0.5 μM each primer; 100 μM each of the four deoxynucleoside triphosphates; 1.5 mM MgCl₂; 1× reaction buffer containing 10 mM Tris-HCl (pH 8.0); 50 mM KCl, 1.5 mM MgCl₂, and 0.1 mg of gelatin per ml; 2 U of Superscriptase (a cloned RNase H free reverse transcriptase) (Bethesda Research Laboratories); and 2.5 U of Taq DNA polymerase (Boehringer Mannheim) in a total volume of 20 μl. The mixture was boiled for 5 min to denature the target RNA and cooled on ice for 2 min before the two enzymes were added. Each reaction mixture was covered with 20 μl of mineral oil (Sigma Chemical Company) and incubated in a Perkin-Elmer Cetus DNA thermal cycler according to the following protocol: 40 cycles of 45°C (to anneal RT) and 5 min at 95°C (for inactivation of Superscriptase and denaturation of DNA); 10 cycles of 94°C for 45 s, 55°C for 35 s (primer annealing), and 72°C for 1 min (primer extension); 45 cycles of 91°C for 45 s, 55°C for 35 s, and 72°C for 40 s; and then a final incubation for 10 min at 72°C. Conditions that were subsequently altered were the concentrations of Superscriptase, Taq polymerase, and MgCl₂ and the number of PCR cycles.

Analysis of the PCR product. A 5-μl volume of the PCR products was separated on a 2% agarose gel (100 V for 30 min in TBE buffer [89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3]). DNA of plasmid pBR322 digested with HaeIII (Boehringer Mannheim) was electrophoresed as a size marker to determine the length of the amplified fragment. An alternative method for analyzing PCR products was essentially as described previously (17). Briefly, the reaction mixture contained 8 μl of the PCR products, 0.2 pmol of the internally nested, 32P-labeled oligomer probe, and 0.5 U of Taq polymerase in a total volume of 10 μl. After addition of 20 μl of mineral oil, the mixture was subjected to one cycle (denaturation for 20 s at 98°C, annealing for 1 min at 50°C, extension for 3 min at 72°C) in a thermal cycler as described above. The PCR products were then separated on a 2% agarose gel and dried for 40 min under a vacuum with a gel dryer and exposed to Kodak X-ray film.

RESULTS

RT-PCR conditions. To determine the optimum ratio of Taq polymerase and Superscriptase for RT-PCR, reactions were performed with various proportions of the two enzymes. Firstly, six RT-PCRs were performed with 2 U of Superscriptase and 0.5, 1.0, 1.5, 2.0, 2.5, and 3 U of Taq polymerase, respectively, with purified RNA of IBDV strain P3009 as the target sequence. All reactions were successful, showing more product formation as Taq polymerase concentration increased, but the amount of nonspecific products slightly increased simultaneously (Fig. 2A). A set of five similar RT-PCRs were performed with 2.5 U of Taq polymerase and 100, 50, 10, 5, and 2 U of Superscriptase, respectively. All these reactions were also successful; however, the band brightness increased as the concentration of Superscriptase decreased (Fig. 2B). Therefore, the concentrations of Taq polymerase and Superscriptase chosen for RT-PCR were 2.5 and 2 U, respectively. In addition, five RT-PCRs were carried out to determine the optimum MgCl₂ concentration. Each reaction mixture contained the optimum ratio of the two enzymes, with the same purified RNA as the target sequence. MgCl₂ at concentrations of 0, 1.0, 1.5, 3.0, and 4.5 mM was added to each of the five reaction mixtures, respectively. The results indicated that all reactions except for the one without addition of MgCl₂ were successful, and more products were formed as the MgCl₂ concentration increased. However, nonspecific products were formed, and the specific product was notably missing in the reaction with the addition of 4.5 mM MgCl₂. Because the RT-PCR buffer originally contained 1.5 mM MgCl₂, the total MgCl₂ concen-
were the 60 cycles. The results showed that all reactions and more amplified used. The detected when nucleic acids from infected with both RNA product. To produced found that was RT-PCR expected 318-bp product was RT-PCR was carried out for detection of IBDV RNA from 23 bursal-tissue specimens. An amplified product of 318 bp was detected from 19 of the specimens following ethidium bromide staining. Amplified product was not obtained when the cell lysis buffer was used as a negative control. Some of the results are indicated in Fig. 3. The identity of the amplification product from the bursal-tissue specimens was further verified by one additional cycle of PCR with the internally nested, end-labeled oligomer probe. Partial results are indicated in Fig. 4. Oligomer extension products were obtained from some of the samples that showed an amplification product at 318 bp.

**DISCUSSION**

RT-PCR has been developed for detecting IBDV RNA (12), but the protocols always require two separate major stages: RT and a PCR cycle. Following termination of RT-PCR, the identity of the amplified product was usually determined by Southern hybridization. The results of the present studies indicated that RNA of IBDV can be successfully detected by using a simplified RT-PCR protocol in which the two stages are combined and run sequentially in a single tube without removing the tube from the PCR machine. These procedures are easier and faster than the early RT-PCR procedures (12), particularly when a large number of tissue specimens are examined. Furthermore, the identity of the amplification product could be determined by an internally nested, end-labeled oligonucleotide probe with one cycle of PCR. However, oligomer extension products were not obtained from some specimens that showed an amplification product at 318 bp. In general, the signal appeared to be weaker with the oligonucleotide probe (Fig. 4), because the volume of PCR product used for oligomer extension by one cycle of PCR is even more than that used with an ethidium bromide-stained gel (Fig. 3). Therefore, oligomer extension with one cycle of PCR for the identification of PCR product may not be successful under optimum conditions, leading to a decrease in sensitivity.

The sensitivity of the primer set was assayed by using different concentrations of purified viral RNA, and the limit of detection for viral RNA was 10 fg. In addition, this primer set appears to be specific for IBDV RNA, because it did not amplify the DNA sequences of nucleic acid preparations from mock-infected CEF and uninfected bursal tissue. Furthermore, the primer set did not amplify the DNA sequences of nucleic acid preparations from seven unrelated avian viruses tested. In addition to detecting two serotype 1 viruses, this primer set also detects RNA from one strain of IBDV serotype 2 virus.
This suggests that this primer set may also have broad specificity for IBDV strains.

A systematic investigation of the relationship between reverse transcriptase (RTase) and Taq polymerase activities has been described (22). This study demonstrated that RTases from both avian myeloblastosis virus and Moloney murine leukemia virus are able to block Taq polymerase activity if the RTase/Taq polymerase ratio is greater than approximately 3:2. Taq polymerase activity may be completely inhibited when the RTase concentration increases because the amplification product is not obtained. Hence, Sneller et al. (22) suggest that the optimum RT-PCR conditions for the detection of viral RNA are 0.5 U of RTase and 2 U of Taq polymerase. This combination allows combined RT and PCR in one reaction tube without interruption, while minimizing the inhibitory effect of RTase on Taq polymerase. The results obtained in the present study indicated that Superscriptase was able to block Taq polymerase activity, similar to the results reported by Sneller et al. (22) (Fig. 2). However, the maximum Superscriptase/Taq polymerase ratio for inhibiting Taq polymerase activity reported here is different from the ratio reported by Sneller et al. (22). When the Superscriptase/Taq polymerase ratio increases to 10:2.5, Taq polymerase activity seems not to be affected. However, if the ratio increases to 50:2.5 or greater, the amplification activity then decreases significantly but is not completely stopped. Because the Superscriptase used in this study is a cloned RNase H, free RTase originated from Moloney murine leukemia virus, RNase H deletion from the RTase molecule may affect this blocking activity. Alternatively, the number of PCR cycles may also affect observation of the amplified product. When the same RT-PCR mixtures were prepared and 35 cycles were run, the amount of product amplified from each reaction decreased, and no product was obtained with reaction mixtures containing Superscriptase and Taq polymerase at ratios of 50:2.5 and 100:2.5 (data not shown).

Theoretically, the amount of amplification product increases if PCR cycles are extended. However, this increase will be limited by several factors. One of them is the enzymatic stability of Taq polymerase following thermal cycle reactions. The half-lives of Taq polymerase are 130 and 40 min at 92.5 and 95°C, respectively (4). Hence, if an attempt to increase the amount of amplification product (sensitivity) by increasing the number of PCR cycles was made, the temperature for denaturation of the DNA template had to be considered. The results show that the 318-bp amplified product was obtained and the product formation increased in parallel with the number of cycles after 10 cycles at 94°C followed by various numbers of cycles at 91°C for denaturation (Fig. 2B).

Thus, the results show that a sensitive and specific, single-tube RT-PCR seems to be a viable alternative tool for detecting IBDV.

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REFERENCES


