JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1995, p. 821–823 Vol. 33, No. 4
0095-1137/95/$04.00+0

Detection of Borna Disease Virus RNA in Formalin-Fixed, Paraffin-Embedded Brain Tissues by Nested PCR

INGRID SORG* AND ALFRED METZLER

Study Group of Environmental Hygiene, Institutes of Parasitology, Veterinary Bacteriology, and Virology, Veterinary Medical Faculty, University of Zürich, 8057 Zürich, Switzerland

Received 22 August 1994/Returned for modification 25 October 1994/Accepted 28 December 1994

A method for detecting Borna disease virus (BDV) RNA in formalin-fixed, paraffin-embedded brain tissue sections was established. By digestion with proteinase K and subsequent extraction with guanidinium thiocyanate, phenol, and chloroform, we were able to efficiently release RNA from the fixed tissues. By reverse transcription of the RNA and nested PCR a 212-bp product was generated, as expected.

Borna disease is a sporadically appearing, infectious, immunopathological disease of the central nervous system characterized by a nonpurulent meningoencephalomyelitis. It occurs naturally in sheep, horses, and rabbits (19, 22, 31). Clinical manifestations in naturally infected cattle have been described only once (4). Typical symptoms of the disease are excitability or apathy, spasms, and paralysis. The disease is usually fatal. Experimentally, a wide variety of animals can be infected, including species phylogenetically distant, such as birds and nonhuman primates (1, 4, 7, 15, 20, 26). Serological investigations indicate that the causative virus is more widely distributed than previously thought and that it possibly plays a role as a human pathogen (2, 24, 26, 27).

The Borna disease virus (BDV) has recently been characterized as an RNA virus (8, 18, 21, 25, 30), with strong evidence for negative polarity (3, 8). Isolation and characterization of BDV-specific cDNA clones revealed the genomic organization of the structural proteins of the virus and allowed the application of molecular techniques for the diagnosis of the infection. PCR, an enzymatic in vitro method for amplification of DNA, has become a valuable tool in the study of the pathogenesis of BDV (23, 28, 29). A reverse transcription (RT)-PCR was established in our laboratory. RT-PCR allows the detection of BDV-specific RNA in the organs of infected animals. Retrospective studies, however, are hampered by the lack of fresh material. For investigations of archival material we describe a method for detecting BDV-specific RNA from routinely formalin-fixed, paraffin-embedded tissues by nested PCR after RT of RNA. The extraction of DNA from fixed tissues is well documented (6, 9, 10, 12, 14, 17), whereas little is known about the extraction of RNA. Detection of RNA is more critical because of the fragility of RNA, the presence of RNases, and the need to carry out RT. For the extraction of RNA we modified a previously described technique (11).

MATERIALS AND METHODS

Cell cultures and virus. MDCK cells persistently infected with BDV (13), kindly supplied by R. Rott, Institute of Virology, University of Giessen, Giessen, Germany, served as a positive control. Cells were subcultured twice each week and were maintained at 37°C in Earle's salts-based Eagle's minimal essential medium buffered with 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and supplemented with 7% fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml).

Tissue samples. Brain tissues from a sheep, a horse, and a cow which showed clinical signs of Borna disease and which represent recent cases of the disease were available. The brains were fixed by immersion in 4% buffered formaldehyde for 48 h or more and were then embedded in paraffin. Sections were investigated histologically and immunohistologically with BDV-specific serum, which confirmed the diagnosis of BDV. Formalin-fixed and paraffin-embedded brain sections of a BDV-negative cow served as a negative control. Tissue sections were kindly supplied by P. Caplazi, Institute of Veterinary Pathology, University of Zürich, Zürich, Switzerland.

RNA extraction. All reagents which were used for RNA extraction and RT were prepared in distilled and diethyl pyrocarbonate (DEPC)-treated water. All glassware was baked at 180°C for 12 h.

For extraction of RNA six sections of formalin-fixed, paraffin-embedded brain tissue were deparaffinized by washing for 30 min in xylene and twice for 5 min in ethanol at room temperature. After air drying, each of the sections was incubated with 1 ml of digestion buffer (1 mg of proteinase K per ml, 20 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 1% sodium dodecyl sulfate) at 45°C for 5 min. The extracts were pooled, and after inactivation of the proteinase for 5 min at 100°C they were subjected to another extraction which was described earlier (5). Briefly, samples were mixed with 6 ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), and the mixture was homogenized by vigorous shaking. The homogenate was then sequentially mixed with 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of water-saturated phenol, and 0.4 volume of a chloroform-isoamyl alcohol mixture (49:1), with thorough mixing by inversion after the addition of each reagent. The final suspension was cooled at 4°C for 15 min. After centrifugation at 10,000 × *g* for 20 min at 4°C the upper aqueous phase was transferred to a fresh tube, mixed with 1 volume of isopropanol, and placed at −20°C for at least 30 min. After centrifugation at 10,000 × *g* for 10 min at 4°C the resulting RNA pellet was dissolved in 300 μl of denaturing solution, mixed with 300 μl of isopropanol, and again placed at −20°C for 30 min. The precipitated RNA was centrifuged at 10,000 × *g* for 10 min, washed twice with ethanol, and further incubated for 15 min with 75% ethanol at room temperature. It was centrifuged again at 10,000 × *g* for 5 min, the resulting RNA pellet was dissolved in 20 μl of DEPC-treated water containing 10 U of human placental RNase inhibitor (Life Technologies, Basel, Switzerland), and the solution was stored at −80°C until further use.

RT. For RT human placental RNase inhibitor (5 U) was added to 2 μl of the extracted RNA. Annealing was done with 10 μM random primers at 70°C for 10 min, and then the product was chilled on ice. The reaction mixture was completed to a final volume of 20 μl containing 1× RT buffer (50 mM Tris [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$), 10 mM dithiothreitol, 250 μM (each) dATP, dCTP, dGTP, and dTTP, and 200 U of Superscript II reverse transcriptase (Life Technologies). cDNA synthesis was carried out at 45°C for 1 h. The reaction was then stopped by heating for 5 min at 100°C.

Primers. The primers used for PCR and nested PCR were derived from the sequence for cDNA clone pAB5, representing part of the open reading frame for the p38/40 protein of BDV (8, 18, 21). The sequences of the outer set of primers were as follows: P1 (sense), 5′-GTCACGGCGCGATATGTTTC-3′ (bp 150 to 169); P2 (antisense), 5′-GATGACGATCCTATCACAACC-3′ (bp 399 to 419). The sequences of the nested set of primers were as follows: P3 (sense), 5′-GCCCAGCCTTGTGTTTCTAT-3′ (bp 180 to 199); P4 (antisense), 5′-GTAATGAGCAACAATGGCTG-3′ (bp 372 to 391).

PCR. PCR was performed in a 50-μl volume containing 1× PCR buffer (10 mM Tris [pH 9.0], 50 mM KCl, 0.01% [wt/vol] gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 0.2 μM (each) specific primer, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 0.25 U of Supertaq polymerase (Stehelin & Cie AG, Basel,

* Corresponding author. Mailing address: Study Group of Environmental Hygiene, Institutes of Parasitology, Veterinary Bacteriology, and Virology, Veterinary Medical Faculty, University of Zürich, Winterthurerstrasse 204, 8057 Zürich, Switzerland. Phone: 011-41-1-313 00 10. Fax: 011-41-1-312 02 78.

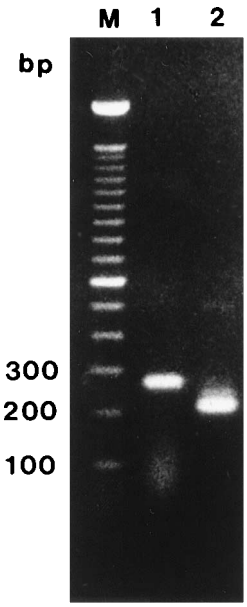

FIG. 1. Gel electrophoresis (1.5% agarose) of BDV-specific RNA extracted from persistently infected MDCK cells amplified by RT-PCR. Lane 1, 270-bp band produced by RT-PCR with the outer set of primers; lane 2, 212-bp band produced by nested RT-PCR with the internal set of primers; lane M, 100-bp DNA ladder.

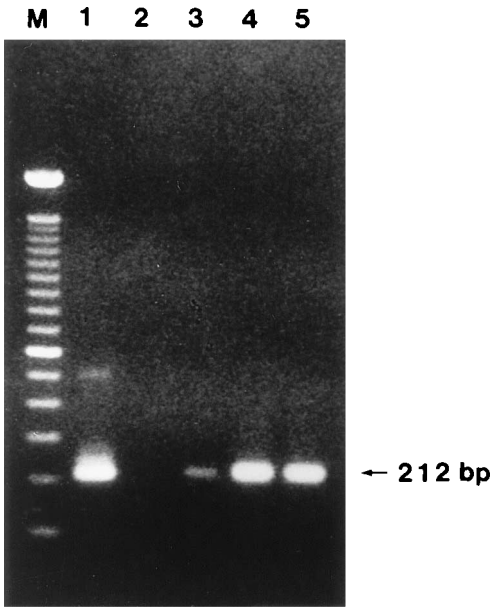

FIG. 2. Electrophoretic patterns of nested RT-PCR products (212 bp) of BDV-specific RNA extracted from formalin-fixed, paraffin-embedded brain tissues. Sections of brains from a BDV-infected horse (lane 3), a BDV-infected sheep (lane 4), a BDV-infected cow (lane 5), and a BDV-negative cow (lane 2) were deparaffinized with xylene, digested with proteinase K, and subsequently extracted with guanidinium thiocyanate, phenol, and chloroform. Lane 1, 212-bp band generated by nested RT-PCR of BDV-specific RNA extracted from persistently infected MDCK cells; lane M, 100-bp DNA ladder.

Switzerland), and 10 μl of cDNA from the RT reaction. Samples were overlaid with 50 μl of mineral oil. After a first cycle (95°C for 5 min, 60°C for 5 min, and 72°C for 1 min) the reaction mixtures were subjected to 40 cycles of amplification by using the following sequence: 95°C for 20 s, 60°C for 20 s, and 72°C for 1 min; this was followed by a final extension step at 72°C for 10 min. For nested PCR, 2 μl of the first PCR product was amplified by using the same reaction conditions used for the single PCR, with the exception that the number of cycles was reduced to 35.

Analysis of amplified DNA. A total of 12 μl of each PCR product was subjected to agarose gel electrophoresis on a 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) for 2 h at 80 V. For staining 250 ng of ethidium bromide per ml was added to the gel, and 125 ng/ml was added to the buffer before electrophoresis. The gel was photographed with UV illumination at 312 nm.

For determination of the size of the PCR products, an ethidium bromide-stainable 100-bp DNA ladder (Life Technologies) was subjected to gel electrophoresis as a marker. It consisted of 15 fragments of between 100 and 1,500 bp in multiples of 100 bp.

RESULTS

Detection of BDV-specific RNA in persistently infected MDCK cells. In order to test the specificity of amplification with the two different sets of primers, PCR and nested PCR were performed with RNA extracted with guanidinium thiocyanate from persistently infected MDCK cells (Fig. 1). PCR with primers P1 and P2 and nested PCR with the primers P3 and P4 generated products with the calculated size of 270 bp (lane 1) and 212 bp (lane 2), respectively, which was estimated according to the sizes of the fragments of the DNA ladder.

Detection of BDV-specific RNA in formalin-fixed, paraffin-embedded tissues. Six sections of formalin-fixed, paraffin-embedded tissues of BDV-infected brains were subjected to deparaffinization with xylene; this was followed by digestion with proteinase K and subsequent extraction with guanidinium thiocyanate. The resulting total RNA was reverse transcribed with random primers and was amplified with a nested set of BDV-specific primers. Figure 2 shows the electrophoretic patterns of the nested RT-PCR products of brain tissues of a BDV-infected horse, a BDV-infected sheep, and a BDV-infected cow (lanes 3, 4, and 5, respectively). As a positive control the nested RT-PCR product of RNA from persistently infected MDCK cells is shown (lane 1). As a negative control formalin-fixed, paraffin-embedded sections of a BDV-negative brain were analyzed (lane 2). Nested RT-PCR with RNA extracted from formalin-fixed, paraffin-embedded tissues as described above resulted in amplification products with the expected size of 212 bp. Nonspecific bands were not observed after nested PCR. No products were generated with the RNA extracted from a BDV-negative brain. Single-step PCR usually did not result in recognizable amplification products. Only in the case of the BDV-infected cow was a very weak signal visible on the gel (data not shown).

DISCUSSION

The aim of the study described here was to establish a PCR method for the detection of BDV-specific RNA in routinely formalin-fixed, paraffin-embedded brain tissue. The results obtained show that the use of deparaffinization with xylene, digestion with proteinase K, extraction with guanidinium thiocyanate, phenol, and chloroform, and then nested RT-PCR is a reliable method. Whereas with guanidinium thiocyanate alone RNA could not be extracted from formalin-fixed tissue, initial digestion with proteinase K released the RNA. The subsequent addition of guanidinium thiocyanate to the digested tissue after the inactivation of the proteinase K allowed further processing of the sample without the need for additional RNase inhibitors. Comparison of single PCR with nested PCR clearly demonstrated the greater sensitivity of the nested PCR method. After tissue digestion, only a few copies of viral RNA were likely to be present in the samples. Because of the high degree of sensitivity of nested PCR, precautions which were suggested previously (16) were observed to exclude the possi-

bility of false-positive results. The possibility of sample-to-sample contamination was controlled by including a negative control. By application of nested RT-PCR we can now confirm the diagnosis of BD, thus adding to the more classical methods of histology and immune histology. Taken together, it is additionally possible to perform retrospective studies with routinely archived materials suspicious for infection with BDV. Such studies, however, would have to consider the possibly detrimental effect of prolonged fixation of brain tissues.

ACKNOWLEDGMENT

This work was supported by the Hermann Klaus-Foundation, CH-8021 Zürich, Switzerland.

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