Chapter 24 Detection of Coronaviruses by the Polymerase Chain Reaction*

Janet N. Stewart, Samir Mounir and Pierre J. Talbot

Summary

A simple and reliable method for the amplification and specific detection of human coronavirus nucleotide sequences was developed, based on the synthesis of cDNA, the polymerase chain reaction, and the use of oligonucleotide probes in Southern blots. Regions from several genes of the two prototype strains of human coronavirus (229E and OC43) could be specifically amplified. This powerful technique was applied to clinical specimens, with appropriate controls, to study the tissue tropism of coronaviruses and their possible involvement in diseases other than the common cold. We have obtained preliminary evidence for the detection of the genome of a human coronavirus in central nervous system autopsy tissue from some multiple sclerosis patients.

Introduction

Human coronaviruses are among the causes of respiratory disease in man. They are responsible for 15%–35% of common colds (McIntosh 1990). This acute disease of the respiratory tract is highly prevalent; a large majority of the population is known to seroconvert at an early age. Moreover, the disease can be experimentally reproduced in volunteers (McIntosh 1990). Other disease associations have been suggested but are less well documented. A seroepidemiological study has linked coronavirus infection with some forms of pneumonia, perimyocarditis, meningitis, and radiculitis (Riski and Hovi 1980). A few

Virology Research Center, Institut Armand-Frappier, Université du Québec, Laval, Québec, Canada H7N 4Z3

* This work was supported by grant MT-9203 from the Medical Research Council of Canada to P.J.T. J.N.S. is grateful to the Institut Armand-Frappier for student support. P.J.T. gratefully acknowledges scholarship support from the National Sciences and Engineering Research Council of Canada (NSERC).

Y. Becker et al. (eds.), *Diagnosis of Human Viruses by Polymerase Chain Reaction Technology*

reports of their involvement in severe diarrhea have appeared (Resta et al. 1985; Battaglia et al. 1987). Moreover, coronaviruses belong to a long list of viruses suspected as etiological agents of multiple sclerosis (MS), the most widespread human demyelinating disease. This association stems from the observation of coronavirus-like particles in the brain of a MS patient (Tanaka et al. 1976), the isolation of coronaviruses from MS patients (Burks et al. 1980), the local synthesis of antibodies to human coronaviruses in the central nervous system (CNS) of MS patients (Salmi et al. 1982), and the preferential detection of coronavirus genomes in the brains of MS patients by *in situ* hybridization (Murray et al. 1992). Moreover, murine coronaviruses show diversified tropisms, and some strains have been used in rodents as a model system to study chronic and acute hepatic and neurologic diseases (ter Meulen et al. 1990), which further strengthens the possible involvement of coronaviruses in such diseases in humans. However, contradictory reports have been published on the association of coronaviruses with diseases other than the common cold, which emphasizes the importance of further research on their medical importance. The polymerase chain reaction (PCR) should provide an exquisite tool for that purpose.

Coronaviruses belong to the *Coronaviridae* family of enveloped RNA viruses (Cavanagh et al. 1990). They contain a single-stranded, positive-sense RNA molecule, with a molecular mass of approximately $6-8 \times 10^6$ daltons (Lai and Stohlman 1978; Lomniczi and Kennedy 1977) and an actual size, derived from nucleotide sequences, of 27–31 kb (Boursnell et al. 1987; Lee et al. 1991). The virus-specific mRNAs in infected cells comprise a genomic-sized mRNA plus 5–8 subgenomic mRNA species. These mRNAs are arranged in a 3′-coterminal nested-set structure, in which the sequence of every mRNA is contained within the sequence of the next larger mRNA (Lai 1990). The 5′-unique regions are translated into structural and nonstructural viral proteins. Three or four structural proteins have been identified: the nucleocapsid protein N, the membrane glycoprotein M, the surface peplomer glycoprotein S, and in some strains of coronaviruses such as the OC43 strain of human coronavirus, a surface hemagglutinin-esterase glycoprotein (Cavanagh et al. 1990; Holmes 1990).

The PCR technology was developed for the detection and amplification of individual nucleotide sequences from a single cell or small quantities of sample (Saiki et al. 1988). Convenient *in vitro* amplification of specific DNA sequences with remarkable efficiency is possible by using a thermostable DNA polymerase derived from the bacterium *Thermophilus aquaticus* (Taq) in an automated PCR. Reverse transcription coupled to the PCR (RT/PCR) can be used to amplify specific RNA sequences. The RNA is reverse-transcribed using a primer complementary to the target sequence (antisense primer) to create cDNA copies, which are amplified using sense and antisense primers.

In the present study, we report the use of RT/PCR methodology for the amplification of portions of four genes of two representative strains of human coronaviruses and the application of this technique to clinical specimens of CNS autopsy tissues.

Materials and Methods

Viruses and Cells. The 229E strain of HCV (HCV-229E) was propagated at 33 °C in L132 human fetal lung cells (American Type Culture Collection, ATCC, Rockville, MD), which were grown as monolayers at 37 °C in a humidified chamber with 5% (v/v) CO_2 in Earle's minimum essential medium: Hank's M199 (1:1 v/v) supplemented with 0.13% (w/v) sodium bicarbonate, 50 μg/ml gentamicin, and 5% (v/v) fetal bovine serum (reduced to 2% (v/v) for viral infections). The OC43 strain of HCV (HCV-OC43) was propagated at 37 °C in HRT-18 human rectal adenocarcinoma cells, a gracious gift from Drs. Jean-François Vautherot and Jacques Laporte (I.N.R.A., Jouy-en-Josos, France), grown in the same medium. Initial inocula of both human coronavirus strains were obtained from the ATCC.

Purification of RNA. Total cellular RNA from uninfected cells and human coronavirus-infected cells was prepared by disrupting the cells in 4 *M* guanidinium thiocyanate with a Polytron homogenizer and pelleting the RNA through a pad of 5.7 *M* cesium chloride (Chirgwin et al. 1979). The sample was then extracted with phenol/chloroform/isoamyl alcohol and the RNA precipitated by the addition of 0.3 *M* sodium acetate and 2.5 volumes of 100% (v/v) ethanol. After centrifugation, the pellet was washed with 70% (v/v) ethanol and dissolved in water.

For clinical specimens, total RNA was extracted by a more convenient method modified from Chomczynski and Sacchi (1987). Briefly, 50–300 mg of tissue were thawed and homogenized in 0.5 ml of 4 *M* guanidinium thiocyanate, extracted with phenol and chloroform and precipitated with ethanol twice. After washing with 70% (v/v) ethanol, the pellets were air-dried and resuspended in water.

Design of Primers and Probes. The oligonucleotides used to prime the extension of viral RNA were designed to amplify unique portions of the RNA coding for the following viral proteins: the integral membrane protein M, nonstructural (NS) proteins, or the spike glycoprotein S of HCV-OC43, NS proteins encoded by mRNA 4 of HCV-229E, or the nucleocapsid protein N of both HCV-229E and -OC43. These sequences were either based on published sequences of the corresponding viral genomes (HCV-229E N gene: Schreiber et al. 1989; HCV-229E mRNA 4 gene: Raabe and Siddell 1989; Jouvenne et al. 1992; HCV-OC43 N gene: Kamahora et al. 1989), or on unpublished sequences obtained in our laboratory (HCV-OC43 M, NS, and S genes). Oligonucleotides were synthesized by the phosphoramidite method on a Pharmacia-LKB "Gene Assembler Plus" DNA synthesizer (Pharmacia, Baie d'Urfé, Québec). Primers for the S gene of the HCV-OC43 strain were: 5′-GCGATTACCACTGGTTATCGG-3′ (S2H, sense; unpublished data) and 5′-GGGCGTGGCCTTAAGAAC-3′ (S2l, antisense; unpublished data). Primers for the M and NS genes of HCV-OC43 were: 5′-CTGGACACCAGGAGTTAG-3′

(NS-M, sense; unpublished data) and 5′-TCGGCCCACTTGAGGATG3′ (N, antisense, nucleotides 147–165; Kamahora et al. 1989). Primers for mRNA 4 of the HCV-229 strain were: 5′-CCACATACAGTAATGGCTCTAGGT-3′ (229E-4, sense, nucleotides 37–60; Raabe and Siddell 1989; or 42–65; Jouvenne et al. 1992) and 5′-CACTATAAGCACCACACACCAGAG-3′ (#5, antisense, nucleotides 756–780; Raabe and Siddell 1989; or 501–524; Jouvenne et al. 1992). Primers for the N gene of HCV-229E were: 5′-AGGCGCAAGAATTCAGAACCAGAG-3′ (E1, sense, nucleotides 498–521; Schreiber et al. 1989) and 5′-AGCAGGACTCTGATTACGAGAAG-3′ (E3, antisense, nucleotides 783–806; Schreiber et al. 1989). Primers for the N gene of the HCV-OC43 strain were: 5′-CCCAAGCAAACTGCTACCTCTCAG-3′ (O1, sense, nucleotides 215–238; Kamahora et al. 1989) and 5′-GTAGACTCCGTCAATATCGGTGCC-3′ (O3, antisense, nucleotides 497–520; Kamahora et al. 1989). Restriction enzyme cleavage sites were added to the 5′-end of some oligonucleotides for cloning purposes irrelevant to the present study.

The oligonucleotides used to prime the extension of control RNAs were located on different exons of the cellular genes, in order to distinguish between amplified RNA and DNA, Primers for myelin basic protein (MBP) RNA were: 5′-AGAACTGCTCACTACGGCTCCCTG-3′ (M1, sense, nucleotides 274–307; Streicher and Stoffel 1989) and 5′-TCCAGAGCGACTATCTCTTCCTCC-3′ (M3, antisense, nucleotides 550–573; Streicher and Stoffel 1989). Primers for γ-actin RNA were 5′-GACCTGACCGACTACCTCATGAAG-3′ (A1, sense, nucleotides 1358–1381; Erba et al. 1988) and 5′-GGAGTTGAAGGTGGTCTCGTGGAT-3′ (A3, antisense, nucleotides 1712–1735; Erba et al. 1988).

The PCR was carried out with a DNA Thermal Cycler (Perkin Elmer-Cetus, Montréal, Québec). The identity of the amplification products of the N genes of HCV-229E and HCV-OC43, as well as of the control RNAs, which were the target for amplification in clinical specimens, was confirmed in hybridization experiments with oligonucleotide probes located on the internal portion of the amplified sequences. The oligonucleotide probe sequences were as follows. For HCV-229E: 5′-ATGAAGGCAGTTGCTGCGGCTCTT-3′ (E2, sense, nucleotides 693–716; Schreiber et al. 1989); for HCV-OC43: 5′-GATGGCAACCAGCGTCAACTGCTG-3′ (O2, sense, nucleotides 419–442; Kamahora et al. 1989); for MBP: 5′-CTGTCCCTGAGCAGATTTAGCTGG-3′ (M2, sense, nucleotides 406-429; Streicher and Stoffel 1989); for γ-actin: 5′-GAAATCGTGCGCGACATCAAGGAG-3′ (A2, sense, nucleotides 1432–1455; Erba et al. 1988).

cDNA Synthesis. The RNA (10–100 ng) was reverse transcribed after initial heating at 65 °C for 7 min in a final volume of 20 µl, containing 1 × Taq DNA polymerase buffer (BIO/CAN Scientific, Mississauga, Ontario), 1 µl of 50 m*M* $MgCl_2$, 2 µl of dNTP mix (10 m*M* of each dNTP), 50 pmol of the antisense primer, 40 U of RNAguard (Pharmacia), and 20 U of Moloney murine leukemia virus reverse transcriptase (Pharmacia). The reaction mixture was incubated at 37 °C for 60 min.

Amplification. The cDNAs produced by RT were amplified using a modification of the original PCR method (Saiki et al. 1988). The 20-µl volume of cDNA was added to 80 µl of the PCR reaction mixture [1 × Taq DNA polymerase buffer, 60 pmol of each primer (antisense and sense), 200 µ*M* of each dNTP, 1.3 m*M* $MgCl_2$, 2.5 U of Taq DNA polymerase (BIO/CAN Scientific)]. Thirty cycles of amplification were performed under the following conditions: denaturation at 94 °C for 1 min, annealing of the primers at 55 °C (cells) or 60 °C (clinical samples) for 2 min, chain extension at 72 °C for 2 min. After the last cycle, the samples were incubated at 72 °C for an additional – 10 min to complete all strands.

For clinical specimens, master mixes of the RT and PCR solutions were prepared containing the same final concentraitons of reagents as described above.

Gel Electrophoresis and Hybridization. Some 5 or 20 µl of the amplified cDNA from cellular or clinical sample RNA, respectively, were electrophoresed on agarose gels in TAE buffer (40 m*M* Tris-acetate, 1 m*M* ethylene diamine tetraacetic acid, pH 7.2), denatured, neutralized, and transferred to nitrocellulose sheets (Hybond-C Extra; Amersham, Oakville, Ontario) according to the method of Southern (1979). Blots were hybridized for 16 h with a purified, [γ-^{32}P]ATP end-labelled oligonucleotide probe (2×10^6 cpm/ml) at 50 °C, in a buffer containing 6 × SSC (1 × SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0), 0.05% (w/v) pyrophosphate, 1 × Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA. The blots were washed three times, for 20 min each time, at room temperature and once for 20 min at 60 °C in 6 × SSC, 0.05% (w/v) pyrophosphate, and exposed to X-ray film (Kodak) at −70 °C.

Results and Discussion

Development of the Method

The RT-PCR was initially developed with continuous cell lines susceptible to HCV infection. The amplified DNA from infected cells was analyzed by agarose gel electrophoresis in parallel with RT-PCR products from uninfected cells. The specificity of the reaction was first based on the amplification of appropriately sized fragments. As shown in Fig. 1, RNA from cells infected with the HCV-229E and -OC43 strains generated cDNA bands of the predicted sizes for the target genes: 1870, 1501, and 729 bp (Fig. 1, lanes 3, 6, and 9, respectively). Uninfected cells (Fig. 1, lanes 2, 5, and 8) or water (Fig. 1, lanes 1, 4, and 7) was used as negative controls. The specificity of the PCR was further confirmed by Southern blot analysis of the 308- and 306-bp amplified products from the N genes of HCV-229E and HCV-OC43 with specific oligonucleotide primers homologous to an internal portion of each target sequence (Fig. 2).

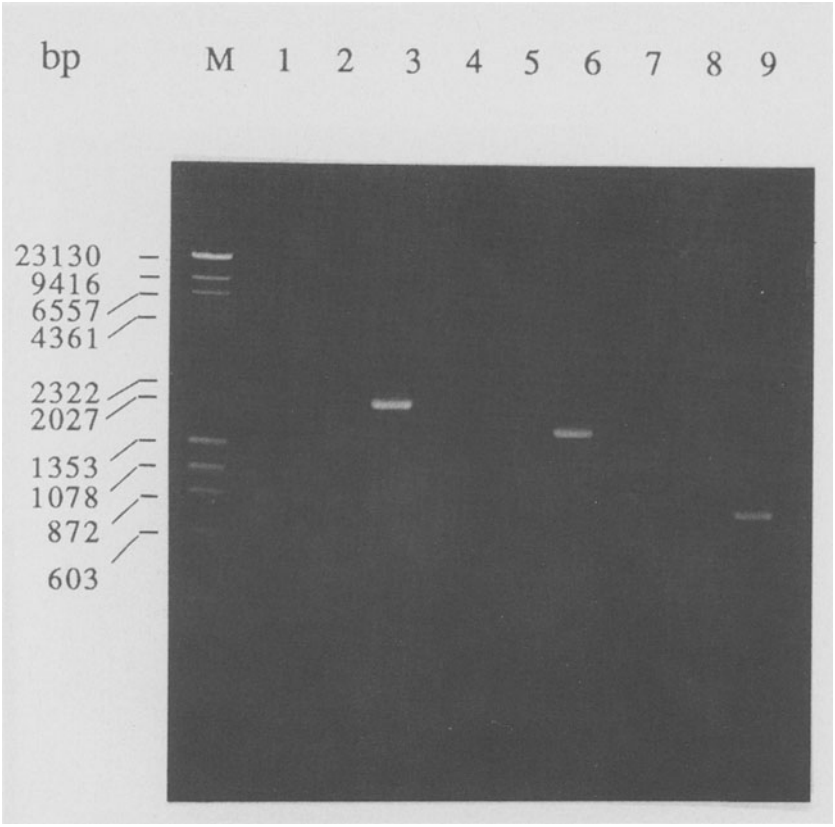

Fig. 1. Detection of various genes of the two prototype strains of human coronavirus (HCV) by *in vitro* amplification and agarose (1% w/v) gel analysis of the amplification products. *Lanes 1, 4 and 7,* H_2O control; *lanes 2 and 5,* HRT-18 cell line used to propagate HCV-OC43; *lane 3*, HCV-OC43 (1870-bp fragment from the S gene); *lane 6,* HCV-OC43 (1501-bp fragment from the M and NS genes); *lane 8*, L132 cell line used to propagate HCV-229E; *lane 9*, HCV-229E (729-bp gene from mRNA 4); *lane M*, *Hin*dIII-digested λ DNA mixed with *Hae*III-digested ϕX174 RF DNA used as molecular size markers. The numbers on the *left* indicate the lengths of fragments in bp

Application to Clinical Specimens

The PCR has made it possible to detect viral nucleic acids in archival tissues, facilitating the study of diseases of possible viral etiology. The distribution of HCVs in different tissues has been little characterized, and RT/PCR provides us with a rapid method for preliminary studies on this subject. Human CNS tissues are of particular interest due to the difficulty of obtaining adequate numbers of fresh specimens for virological studies.

The detection of RNA presents problems that do not occur in the detection of DNA. Formalin-fixed tissues can be studied for DNA content. However, we

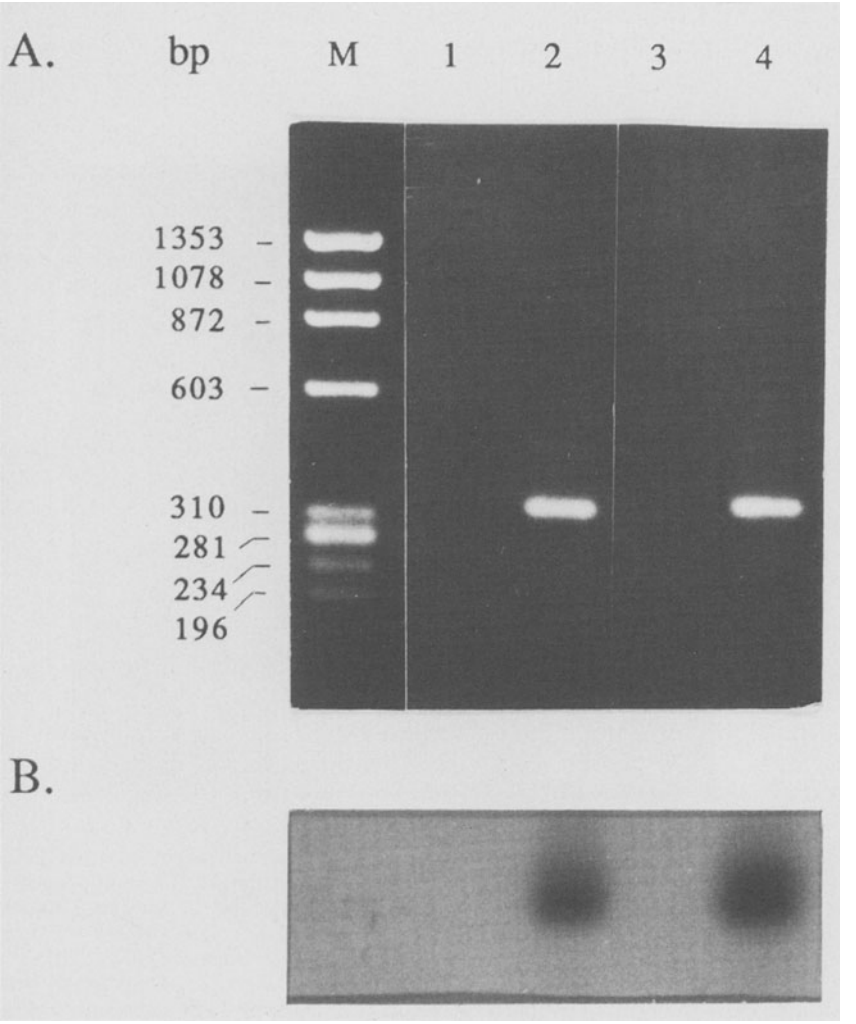

Fig. 2A, B. Detection of the N genes of the two prototype strains of human coronaviruses (HCV) by *in vitro* amplification, followed by agarose (1% w/v) gel analysis of the amplification products (**A**) and identification of amplified sequences by hybridization with oligonucleotide probes (**B**). *Lane 1*, L132 cell line used to propagate HCV-229E; *lane 2*, HCV-229E; *lane 3*, HRT-18 cell line used to propagate HCV-OC43; *lane 4*, HCV-OC43; *lane M*, *Hae*III-digested ϕX174 RF DNA used as molecular size marker. The numbers on the *left* indicate the lengths of fragments in bp

have not been able to extract RNA suitable for amplification from tissues preserved in this manner. RNA is also highly susceptible to degradation by ribonucleases. In our laboratory we analyze RNA extracted from white matter obtained from autopsies which have been preserved at $-70°$ to $-80°$C for several years. Both the length of time between death of the individual and

freezing of the tissue and repeated thawing of the tissues can contribute to RNA degradation. We perform an RT/PCR on each extract of RNA to detect cellular mRNAs to verify that significant degradation of RNA has not occurred. White matter specimens in which mRNA coding for MBP is not detectable are excluded from the study to avoid possible false-negative results due to RNA degradation. The mRNA coding for γ-actin is far more abundant than MBP mRNA in the specimens studied, and therefore we consider MBP mRNA detection a more sensitive indicator of partial RNA degradation in white matter (where MBP is expressed). As shown in Fig. 3, RNA prepared from clinical specimens yielded amplified cDNAs of the expected sizes (300 bp) for both MBP (lane 2) and γ-actin (lane 4). As expected, MBP RNA could not be amplified from lymphocytes (lane 1). Moreover, amplified fragments were shown to be specific by oligonucleotide hybridization. Since γ-actin is present in all cells, we use it for checking RNA extracted from other cells or tissues such as peripheral blood lymphocytes and gray matter. The primers used to amplify cellular control RNAs are located on different exons of their corresponding genes, and as such, RNA and DNA amplification can be distinguished on the basis of the size of the reaction product. An advantage in RNA detection is that viral mRNAs as well as genomes are detected, and since the former are present in much larger quantities, the test becomes more sensitive.

Several rapid methods of sample preparation for DNA amplification are available, but RNA extraction remains a time-consuming activity. Our extraction method is performed using Eppendorf tubes and a microfuge to extract up to 300 mg of tissue per tube. This produces 10–20 μg of RNA per specimen, which is aliquoted for use in multiple tests. No DNA has yet been detected in the RNAs extracted. Several specimens can be prepared simultaneously in 1 day using this method (about 20 in our hands), rendering it applicable to research and clinical investigations but too cumbersome for use in diagnostics, at least until rapid and possibly automated RNA preparation techniques become available.

The contamination of PCR reaction mixtures with the products of previous amplifications is a well-known source of false-positive results (Persing 1991). An additional risk of contamination is the culture and cloning of the infectious agent in the same laboratory in which nucleic acids are prepared for PCR and the reactions are set up. We follow recommended guidelines (Kwok and Higuchi 1989) to avoid false-positive results arising from contamination. The extraction of RNA, reverse transcription, and the preparation of PCR reactions are performed using positive displacement pipettes in a separate laboratory in which HCVs are not studied. The analysis of reaction products by gel electrophoresis and hybridization is performed in the HCV laboratory.

The results of RT/PCR performed on specimens of white matter to detect the HCV-229E strain can be seen in Fig. 4. Positive controls of both low (lane 1) and high (lane 2) intensity and a negative control of brain RNA (lane 3), extracted at the same time as the tissue being tested, as well as water (not shown) are included in the RT/PCR reactions. A different aliquot of the same RNA extraction giving a positive hybridization signal (lane 4) is retested to

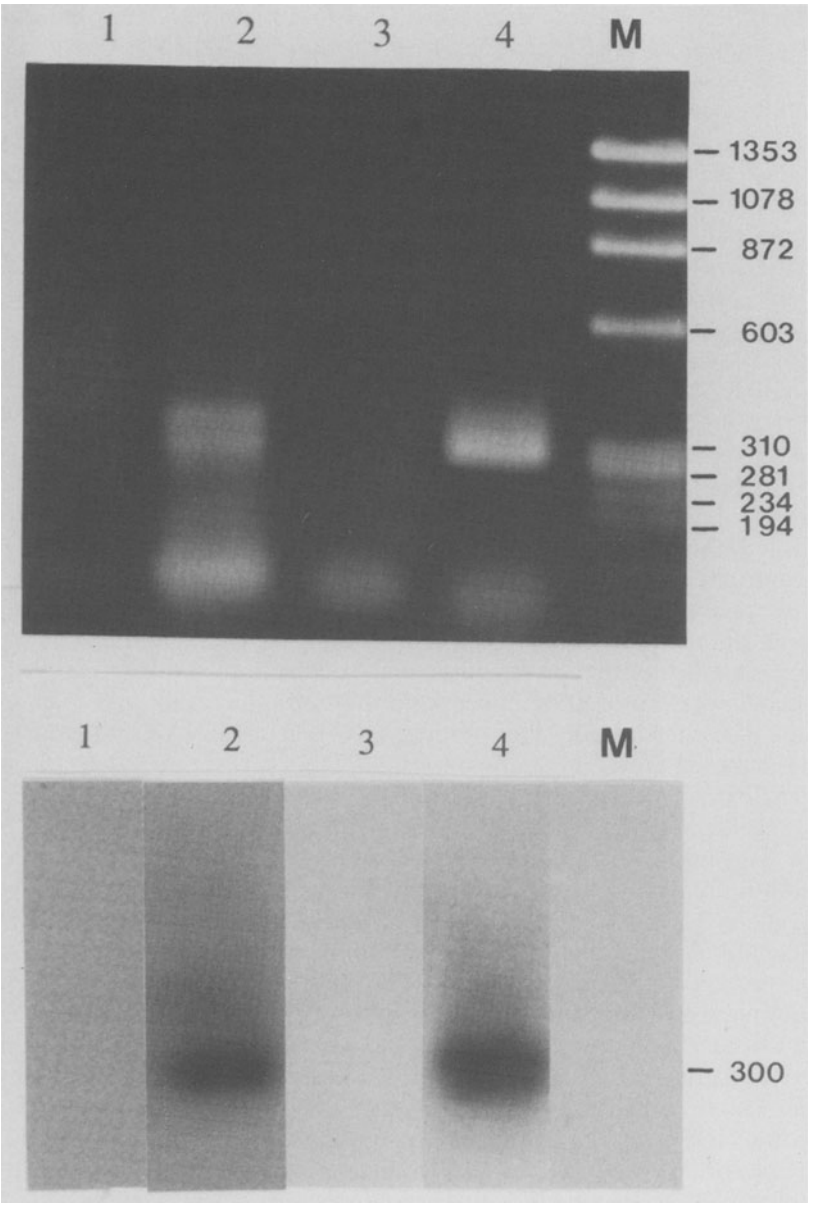

Fig. 3. Agarose (1.5% w/v) gel electrophoresis (*top*) and oligonucleotide hybridization (*bottom*) of amplification products of control mRNAs. *Lanes 1 and 2*, RT/PCR for myelin basic protein mRNA is performed on RNA extracted from human peripheral blood lymphocytes (*1*) and human central nervous system white matter (*2*) *lanes 3 and 4*, RT/PCR for human γ-actin mRNA is performed on H_2O (*3*) and on RNA extracted from human CNS white matter (*4*); *lane M*, *Hae*III-digested ϕX174 RF DNA used as molecular size marker. The numbers on the right indicate the lengths of fragments in bp

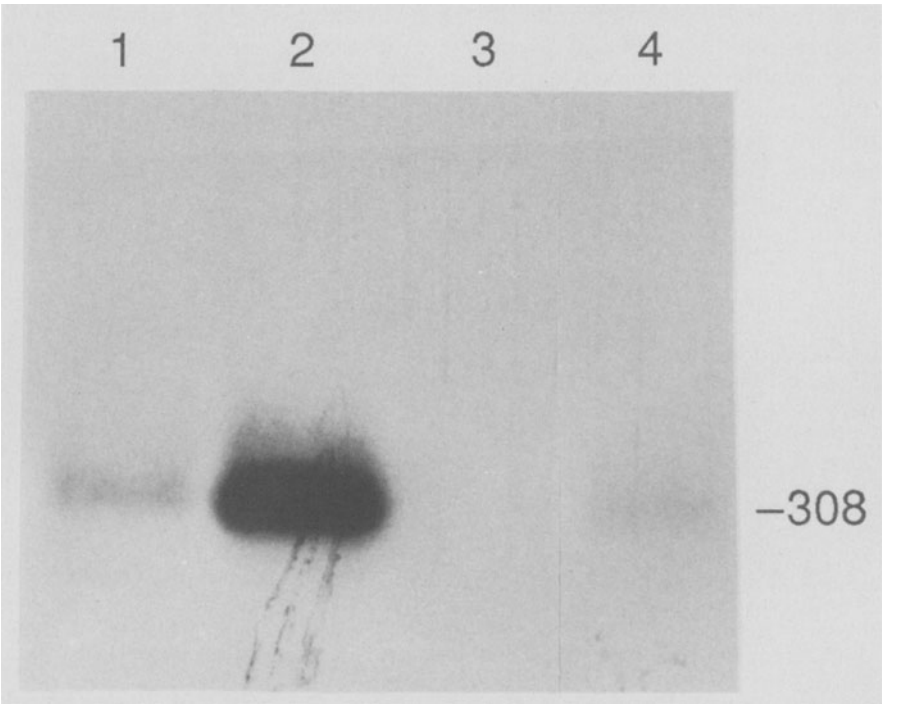

Fig. 4. Detection of the 229E strain of human coronavirus in clinical specimens by oligonucleotide hybridization of reverse transcription/polymerase chain reaction (RT/PCR) 308-bp amplification products from the N gene. *Lanes 1 and 2,* RNA isolated from mouse brain, to which 16 pg (1) or 160 pg (2) total RNA from virus-infected L132 cells was added; *lane 3,* RNA isolated from mouse brain tissue alone; *lane 4,* RNA extracted from human central nervous system white matter obtained from an MS patient

confirm a positive result. It is also possible to use a different set of primer pairs to confirm a positive result (not shown), but the sensitivity of the second set of primers must be similar to the first if reproducible results are to be obtained.

Similar tests using a transcription step prior to cDNA amplification have been developed for several RNA viruses, including rhinovirus (Gama et al. 1988), human immunodeficiency virus (Byrne et al. 1988, Murakawa et al. 1988), rubella virus (Carman et al. 1989), human picornavirus (Hyypia et al. 1989), equine arteritis virus (Chirnside and Spaan 1990), and avian infectious bronchitis virus (Lin et al. 1991). The procedure described in the present study has been repeated for several coronaviruses, and the results are reproducible.

Conclusion

RT/PCR is a relatively rapid method for locating viral nucleic acid in different tissues. However, the technique described in the present study remains restrict-

ed to research applications due to the labor-intensive RNA extraction and Southern blotting steps involved. Nevertheless, it could eventually replace virus isolation from clinical material and nucleic acid hybridization for simple, rapid, specific, and sensitive identification of coronaviruses. Moreover, it is well suited to the study of coronaviruses which exhibit multiple tissue tropisms. Using this technique, *in vivo* infections of different tissue types may be identified, opening up new avenues of research into the specific cell types harboring the virus and eventually into the pathogenic potential of HCVs as primary or secondary agents of diseases other than the common cold.

Acknowledgments. We thank Francine Lambert for excellent technical assistance and Lise Trempe and François Shareck for oligonucleotide synthesis.

References

Battaglia M, Passarani N, DiMatteo A, Gerna G (1987) Human enteric coronaviruses: further characterization and immunoblotting of viral proteins. J Infect Dis 155:140–143

Boursnell MEG, Brown TDK, Foulds IJ, Green PF, Tomley FM, Binns MM (1987) Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J Gen Virol 68:57–77

Burks JS, DeVald BL, Jankovsky LD, Gerdes JC (1980) Two coronaviruses isolated from central nervous system tissue of two multiple sclerosis patients. Science 209:933–934

Byrne BC, Li JJ, Sninsky J, Poiesz BJ (1988) Detection of HIV-1 RNA sequences by *in vitro* DNA amplification. Nucleic Acids Res 16:4165

Carman WF, Williamson C, Cunliffe BA, Kidd AH (1989) Reverse transcription and subsequent DNA amplification of rubella virus. J Virol Methods 25:21–30

Cavanagh D, Brian DA, Enjuanes L, Holmes KV, Lai MMC, Laude H, Siddell SG, Spaan W, Taguchi T, Talbot PJ (1990) Recommendations of the Coronavirus Study Group for the nomenclature of the structural proteins, mRNAs and genes of coronaviruses. Virology 176:306–307

Chirnside ED, Spaan WJM (1990) Reverse transcription and cDNA amplification by the polymerase reaction of equine arteritis virus (EAV). J Virol Methods 30:133–140

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299

Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Erba HP, Eddy R, Shows T, Kedes L, Gunning P (1988) Structure, chromosome location, and expression of the human γ-actin gene: differential evolution, location and expression of the cytoskeletal β- and γ-actin genes. Mol Cell Biol 8:1775–1789

Gama RE, Hughes PJ, Bruce CB, Stanway G (1988) Polymerase chain reaction amplification of rhinovirus nucleic acids from clinical material. Nucleic Acids Res 16:9346

Holmes KV (1990) Coronaviridae and their replication. In: Fields BN, Knipe DM et al. (eds) Raven, Virology, 2nd edn. New York, pp 841–856

Hyypia T, Auvinen P, Maaronen M (1989) Polymerase chain reaction for human picornaviruses. J Gen Virol 70:3261–3268

Jouvenne P, Mounir S, Stewart JN, Richardson CD, Talbot PJ (1992) Sequence analysis of human coronavirus 229E mRNAs 4 and 5: evidence for polymorphism and homology with myelin basic protein. Virus Res 22:125–141

Kamahora T, Soe LH, Lai MMC (1989) Sequence analysis of nucleocapsid gene and leader RNA of human coronavirus OC43. Virus Res 12:1–10

Kwok S, Higuchi R (1989) Avoiding false positives with PCR. Nature 339:237–238

Lai MMC (1990) Coronavirus: organization, replication, and expression of genome. Annu Rev Microbiol 44:303–333

Lai MMC, Stohlman SA (1978) The RNA of mouse hepatitis virus. J Virol 26:236–242

Lee HJ, Shieh CK, Gorbalenya AE, Koonin EV, La Monica N, Tuler J, Bagdzhadzhyan A, Lai MMC (1991) The complete sequence (22 kilobases) of murine coronavirus gene 1 encoding the putative proteases and RNA polymerases. Virology 180:567–582

Lin Z, Kato A, Kudou Y, Ueda S (1991) A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism. Arch Virol 116:19–31

Lomniczi B, Kennedy I (1977) The genome of infectious bronchitis virus. J Virol 24:99–107

McIntosh K (1990) Coronaviruses. In: Fields BN, Knipe DM et al. (eds) Virology, 2nd edn. Raven, New York, pp 857–864

Murakawa GJ, Zaia JA, Spallone PA, Stephens DA, Kaplan BE, Wallace RB, Rossi JJ (1988) Direct detection of HIV-1 RNA from AIDS and ARC patient samples. DNA 7:287–295

Murray RS, Brown B, Brian D, Cabirac GF (1997) Detection of coronavirus RNA and antigen in multiple sclerosis brain. Ann Neurol 31:525–533

Persing DH (1991) Polymerase chain reaction. Trenches to benches. J Clin Microbiol 29:1281–1285

Raabe T, Siddell S (1989) Nucleotide sequence of the human coronavirus HCV-229E mRNA 4 and mRNA 5 unique regions. Nucleic Acids Res 17:6387

Resta S, Luby JP, Rosenfeld CR, Siegel JD (1985) Isolation and propagation of a human enteric coronavirus. Science 229:978–981

Riski N, Hovi T (1980) Coronavirus infections of man associated with diseases other than the common cold. J Med Virol 6:259–265

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Salmi A, Ziola B, Hovi T, Reunanen M (1982) Antibodies to coronaviruses OC43 and 229E in multiple sclerosis patients. Neurology 32:292–295

Schreiber SS, Kamahora T, Lai MMC (1989) Sequence analysis of the nucleocapsid protein gene of human coronavirus 229E. Virology 169:142–151

Southern E (1979) Gel electrophoresis of restriction fragments. Methods Enzymol 68:152–176

Streicher R, Stoffel W (1989) The organization of the human myelin basic protein gene. Biol Chem Hoppe Seyler 370:503–510

Tanaka R, Iwasaki Y, Koprowski HJ (1976) Intracisternal virus-like particles in the brain of a multiple sclerosis patient. J Neurol Sci 28:121–126

ter Meulen V, Massa PT, Dörries R (1990) Coronaviruses. In: McKendall (ed) Viral disease. Handbook of clinical neurology, vol 12. Elsevier, Amsterdam, pp 439–451