Rapid Detection of Respiratory Syncytial Virus in Nasopharyngeal Aspirates by Reverse Transcription and Polymerase Chain Reaction Amplification

ADRIENNE W. PATON,1 JAMES C. PATON,1* ANDREW J. LAWRENCE,1 PAUL N. GOLDFATER,1 AND RAY J. HARRIS2

Microbiology Department, Adelaide Children’s Hospital, North Adelaide, South Australia 5006,1 and School of Pharmacy and Medical Laboratory Science, University of South Australia, Adelaide, South Australia 5000,2 Australia

Received 16 August 1991/Accepted 23 January 1992

Respiratory syncytial virus (RSV) is an important cause of acute lower respiratory tract infections in humans, with infants and young children being particularly susceptible. Traditionally, diagnosis of RSV infection has centered on the “gold standard” technique of culture of the virus from nasopharyngeal secretions. However, cell culture methods are generally slow, often taking up to a week before a result is available, and sensitivity may be affected by the lability of RSV (9). The availability of antiviral therapeutic agents effective against RSV and the need to minimize the risk of cross-infection have prompted the development of chemical techniques for direct detection of virus, such as direct immunofluorescence and more recently, antigen-capture enzyme immunoassays (EIA) (9). These methods are much faster than culture, giving results on the same day, but the sensitivity and to a lesser extent the specificity appear to vary considerably (4, 5, 7–9). An alternative diagnostic approach has involved improvement of the speed of RSV culture techniques by the use of immunochromating methods to detect early virus infection of cell cultures, rather than waiting for a cytopathic effect to become apparent (6). However, such tests still take up to 3 days.

A study carried out at the Adelaide Children’s Hospital identified RSV as the single most common cause of nosocomial pediatric respiratory infections during the winter months (2). The results of this study also suggested that the cross-infection rate might be reduced further by improving the sensitivity and specificity of rapid RSV detection assays.

We have developed a rapid method based on reverse transcription (RT) and amplification of viral nucleic acid sequences by the polymerase chain reaction (PCR). This procedure was tested on nasopharyngeal aspirates from children with suspected respiratory infections.

MATERIALS AND METHODS

Patient samples. Nasopharyngeal aspirates (NPAs) were collected from 202 children aged 1 month to 6 years (median age, 14 months) with suspected respiratory infections. The suction catheter was flushed by aspiration of 2 ml of viral transport medium into a specimen trap. A 0.5-ml portion of each specimen was withdrawn and stored at −70°C for subsequent analysis by RT-PCR. The remainder was immediately refrigerated and transported to a nearby central pathology laboratory which provides routine diagnostic virology services to the Adelaide Children’s Hospital. The median delay between collection of a specimen and receipt by the central laboratory was approximately 1.5 h (maximum of 4 h). Immediately after receipt, specimens were inoculated onto HEp-2 and A549 cell monolayers for virus culture and directly tested for the presence of RSV by using an in-house antigen-capture EIA.

Virus stocks. RSV (Long strain) was grown in HEp-2 cells, titrated by 50% tissue culture infective dose (TCID50) assay, and stored in aliquots at −70°C. These samples were used as positive controls in the RT-PCR assay (uninfected HEp-2 cells were used as a negative control). Clinical isolates of RSV and adenovirus (also grown in HEp-2 cells) and parainfluenza virus types 1 and 3 and influenza virus A and B (grown in LLC-MkII cells) were kindly provided by P. Hallsworth.

Preparation of samples for RT-PCR. NPAs (0.5 ml) or cell culture extracts (0.1 ml) were thawed and immediately supplemented with 20 U of RNase inhibitor (Boehringer, Mannheim, Germany). The NPAs were digested by adding 1 M Tris-HCl (pH 7.6) to 12 mM and proteinase K to 0.2 mg/ml...
and incubating at 65°C for 1 h. The digest was then extracted once with phenol and then once with chloroform, and nucleic acids were recovered by precipitation with an equal volume of isopropanol and centrifugation. The pellets were washed in 70% ethanol, centrifuged, dried, and finally resuspended in 10 μl of water containing 20 U of RNase inhibitor.

**RT-PCR.** RT-PCR was carried out in two steps using an RNA PCR kit obtained from Perkin-Elmer Cetus, Norwalk, CT. The RT step involved a reaction mixture (final volume, 20 μl) consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM (each) dATP, dCTP, dGTP, and dTTP, approximately 1 μM of each primer, 1 U of RNase inhibitor per μl, 3 μl of RNA sample, and 2.5 U of avian myeloblastosis virus reverse transcriptase. The reaction mixtures were overlaid with 50 μl of mineral oil, incubated at 42°C for 15 min, and then heated at 99°C for 5 min. For the PCR step, the reaction volume was made up to 100 μl with 10 mM Tris-HCl (pH 8.3)–50 mM KCl and the final concentration of MgCl₂ was adjusted to 2 mM. Taq polymerase (2.5 U) was also added to each tube, and the tubes were subjected to 35 amplification cycles, each cycle consisting of 1.5-min denaturation, annealing, and elongation steps at temperatures of 94, 47, and 72°C, respectively. The sequences of the two primers used were 5'-TTAACCAGCACAAGTGTATGA-3' and 5'-TTGTTATAGCATATCATTT-3'. These primers define a 243-bp segment of the RSV genome encoding the F1 subunit of the fusion (F) protein, and the sequences to which the primers anneal are identical for both the A and B subgroups of RSV (3). The 243-bp amplified product was detected by electrophoresing 20-μl aliquots through 2% agarose gels in the presence of ethidium bromide for approximately 30 min at 10 V/cm and photographing the gels under UV illumination.

**RESULTS**

**Specificity and sensitivity of RT-PCR for RSV.** The oligonucleotide primers used in the RT-PCR assay were chosen on the basis of previously published sequence data, which indicated that the regions to which these primers anneal are identical for both the A and B subgroups of RSV (3). As an initial check on the specificity of these primers for RSV, the oligonucleotides were analyzed for homology with all sequences stored in the GenBank data base (version R64.0) using the DNASIS software package (Pharmacia LKB Biotechnology, Uppsala, Sweden). No homologies likely to result in spurious annealing were detected. Specificity was then examined directly by subjecting cell culture extracts infected with either RSV (Long strain) or clinical isolates of adenovirus, parainfluenza virus type 1, parainfluenza virus type 3, influenza A virus, or influenza B virus to RT-PCR (Fig. 1). A PCR product of the expected size (243 bp) was observed for RSV but not for any of the other virus cultures.

The sensitivity of the RT-PCR assay was examined by analyzing serial 10-fold dilutions (10⁴ to 10⁹-fold) of an RSV-infected HEP-2 lysate. The virus titer of this lysate was approximately 8 × 10⁷ TCID₅₀ per ml. A distinct PCR product was detected when 100-μl aliquots diluted up to 10⁴-fold were tested, and a band of faintly discernible in the reaction from the 10⁴-fold dilution (Fig. 2). The total amount of virus in this reaction was predicted to be approximately 10 TCID₅₀.

**Detection of RSV in NPAs by RT-PCR.** In a preliminary experiment, we analyzed 22 NPAs which had been previously tested for RSV by culture and direct immunofluorescence and had since been stored at −70°C for approximately 3 years. Of these samples, 11 had tested positive for RSV and 10 yielded a PCR product of the expected size in the RT-PCR assay (Fig. 3). No PCR products were detected when the 11 RSV-negative NPAs were similarly analyzed (Fig. 4).

A further 202 freshly obtained NPAs were then examined by RT-PCR, and the results were compared with those for RSV culture and EIA reported by the central pathology laboratory (Table 1). RT-PCR was positive in 118 of 125 (94.4%) NPAs from which RSV was cultured. The RT-PCR test also yielded positive results in a further four of seven samples which were culture negative but EIA positive. RT-PCR was negative in 68 of 70 RSV culture-negative, EIA-negative samples, which included 11 which were positive by culture or EIA for other respiratory viruses (4 for parainfluenza type 2, 2 for parainfluenza type 3, 1 for rhinovirus, 1 for adenovirus, and 3 for influenza B virus).

The EIA was clearly less sensitive than RT-PCR, yielding positive results in only 49 of 125 of the RSV culture-positive NPAs. Microscopic examination of the NPAs had indicated that in a high proportion of samples, there were fewer than 10³ respiratory columnar epithelial cells per ml, which might have contributed to the poor sensitivity of the EIA. It is interesting, however, that the majority of RSV culture-positive, EIA-negative samples, which were detected as positive by RT-PCR, yielded strong positive signals. Figure 5 shows two typical culture-positive EIA-negative NPAs which yielded RT-PCR signals almost as intense as those
obtained from the undiluted RSV-infected cell culture extract used as a positive control.

**DISCUSSION**

In a previous study (1), we have shown that PCR is a very promising method for the rapid detection of a bacterial respiratory pathogen (*Bordetella pertussis*) in NPAs. The PCR test for pertussis is very sensitive, particularly since the target sequence for amplification is reiterated approximately 100 times per genome. In the present study, we have sought to determine whether PCR methodology can also be applied to direct detection of viral respiratory pathogens, particularly pathogens such as RSV which have an RNA genome, thereby necessitating RT before the PCR amplification step. Rapid detection of RSV is of great clinical importance in a pediatric hospital, because it is a major cause of nosocomial infection; also, antiviral therapy is effective against this pathogen (9). The RSV culture procedure used by the central pathology laboratory is extremely sensitive, because it involves testing of cell cultures for virus infection by EIA and examination for cytopathic effect. However, this is a slow procedure; in the present study, it took from 3 to 13 days (median, 7 days) from collection of the NPA to produce a result. This time lag is greater than the mean bed stay of patients with bronchiolitis (the most common RSV infection) at the Adelaide Children's Hospital (4.0 days), and so in the majority of cases the patient would have been discharged from the hospital before the culture result became available. Direct EIA, although yielding a result within 24 h, was of limited clinical value in these cases because of its low sensitivity (approximately 41%).

RT-PCR, on the other hand, was both sensitive and specific for RSV. When extracts of diluted RSV-infected cell cultures were tested, the RT-PCR assay was capable of detecting as few as 10 TCID\textsubscript{50}s, but negative results were obtained for cell cultures infected with other viruses (adenovirus, parainfluenza types 1 and 3, and influenza virus A and B). When NPAs were tested, RT-PCR analysis compared favorably with culture and clearly outperformed EIA. Moreover, the sensitivity of the RT-PCR was such that unlike the EIA, low epithelial cell counts in the NPA did not compromise performance relative to culture. The RT-PCR failed to detect only 7 of 125 culture-positive cases. These false-negative results could be due to RNA sequence variation between strains of RSV, but this is unlikely because the sequences of the regions of the F gene to which the two primers anneal are identical for both the A and B subtypes of RSV (3). Unfortunately, we did not have access to the tissue culture extracts from the RSV culture-positive RT-PCR-negative samples, which would have enabled confirmation of the reactivity of the isolated virus in the RT-PCR reaction. A more likely explanation for the false negatives, however, is that a small proportion of culture-positive NPAs contain so few virus particles that they are below the sensitivity threshold of RT-PCR, which is about 10 TCID\textsubscript{50}.

If true positives are defined as those which are RSV culture positive or culture negative but are positive by both RT-PCR and EIA, then the RT-PCR has a sensitivity of 94.6% (c.f. 96.9% for culture and 41.1% for EIA) and a specificity of 97.1%. The RSV culture-negative NPAs (all but two of which were negative by RT-PCR) included specimens which were positive for adenovirus, parainfluenza types 2 and 3, influenza B virus, and rhinovirus. Interestingly, the only two NPAs which yielded apparent false-positive RT-PCR results were from hospitalized patients from whom no other pathogen was isolated but whose clinical symptoms (a febrile illness with pronounced upper and lower respiratory symptoms and signs) were entirely consistent with RSV bronchiolitis. In both cases, the NPA was collected at least 7 days after onset, and hence viral

**FIG. 3.** RT-PCR of extracts of RSV culture-positive NPAs. Lanes: M, DNA size markers (as in the legend to Fig. 1); +, RSV-infected HEp-2 lysate; 1 to 11, RSV culture-negative NPAs; −, uninfected HEp-2 lysate.

**FIG. 4.** RT-PCR of culture-negative NPAs. Lanes: M, DNA size markers (as in the legend to Fig. 1); −, uninfected HEp-2 lysate; 1 to 11, culture-negative NPAs; +, RSV-infected HEp-2 lysate.

**FIG. 5.** RT-PCR of RSV culture-positive, EIA-negative NPAs. Lanes: M, DNA size markers (as in the legend to Fig. 1); −, uninfected HEp-2 lysate; 1 and 2, culture-positive, EIA-negative NPAs; +, RSV-infected HEp-2 lysate.

<table>
<thead>
<tr>
<th>Culture and EIA result (n)</th>
<th>No. of specimens</th>
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<tbody>
<tr>
<td>Culture +, EIA + (49)</td>
<td>RT-PCR +</td>
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<tr>
<td>Culture +, EIA − (76)</td>
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<tr>
<td>Culture −, EIA + (7)</td>
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* A total of 202 NPAs were analyzed for the presence of RSV by RT-PCR, and the results are compared with those for virus culture and EIA. The number of specimens yielding a positive (+) or negative (−) result for the various assays is indicated.
shedding may have diminished by this stage. Alternatively, RSV viability may have been lost during transport, or infectivity may have been compromised by the presence of secretory antibodies in the NPA capable of blocking virus adsorption. These latter events seem a distinct possibility, since a further four NPAs were culture negative for RSV but were positive by both EIA and RT-PCR. Thus, the above estimate of the specificity of RT-PCR (97.1%) must be considered a minimum.

The major advantage of RT-PCR over virus culture, however, is its speed; an RT-PCR result is available within 8 to 24 h, depending on the time of receipt of the specimen. Thus, RT-PCR is as rapid as the alternative direct detection procedure, EIA, but has vastly superior sensitivity to EIA. The clear outperformance of EIA by RT-PCR was unexpected, because it could be argued that the advantages of amplification of viral nucleic acid might be at least partially offset by the lability of the RNA in the NPA. Also, each virion contains a large number of EIA target molecules but only one RT-PCR target. Notwithstanding these considerations, the results of this study indicate that RT-PCR has great potential for the rapid diagnosis of RSV infection.

ACKNOWLEDGMENTS

We thank Linda Wurfel for technical assistance.

This work was supported by grants from the Channel Seven Children’s Research Foundation and the Adelaide Children’s Hospital Research Foundation.

REFERENCES


