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Diagnosis of Viral Respiratory Tract Infections in Children by Using a Reverse Transcription-PCR Panel

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Reverse transcription-PCR (RT-PCR) is a sensitive method for detection of RNA virus nucleic acid sequences in clinical respiratory specimens. Previous studies have focused on RT-PCR for a single virus, but this approach is limited by the inability to establish a specific etiology when the RT-PCR result is negative and by the inability to document simultaneous infections involving more than one virus. The purpose of this study was to apply a panel of RT-PCR protocols for respiratory syncytial virus, parainfluenza virus, and picornaviruses to respiratory specimens from 80 children suspected to have acute viral respiratory tract infections and to correlate RT-PCR results with viral culture results and clinical diagnosis. In comparison with viral culture, the RT-PCR panel had a sensitivity of over 94% and showed evidence of simultaneous infections in a significantly greater proportion of specimens (20.0% versus 3.8%; $P < 0.002$). For specimens in which no viruses were detected by culture, the proportion of specimens with positive picornavirus RT-PCR results was significantly greater than the proportion of specimens with positive respiratory syncytial virus or parainfluenza virus RT-PCR results ($P < 0.001$). There were no statistically significant associations between RT-PCR results and clinical diagnosis. In summary, the RT-PCR panel provides an improved approach to obtain new insights into acute viral respiratory tract infections in children.

Reverse transcription-PCR (RT-PCR) is a highly sensitive technique for the detection of nucleic acid sequences from RNA viruses in clinical specimens (5). In comparison with viral culture for the diagnosis of acute respiratory tract infections, RT-PCR is particularly advantageous for detecting fastidious organisms such as rhinoviruses (9) and for characterizing hospital outbreaks of parainfluenza virus (PIV) type 3 infections (11). Previous studies using RT-PCR for the diagnosis of acute viral respiratory tract infections have focused on testing for a single virus of interest; however, the diagnostic utility of RT-PCR for a single virus is limited by the inability to establish a specific etiology when the RT-PCR result is negative and by the inability to document simultaneous infections involving more than one virus. A proposed strategy to overcome these limitations is the use of a panel of RT-PCR protocols that tests for several respiratory pathogens in clinical specimens (10).

For children who have clinical evidence of acute respiratory tract infections, the reported prevalence of simultaneous infections is related to the methods used for viral diagnosis. For example, in children with acute respiratory syncytial virus (RSV) infections (15) or those hospitalized for acute exacerbations of asthma (reviewed in reference 14), there is a 0 to 8% prevalence of simultaneous infections as documented by viral culture. In contrast, McIntosh et al. (12) reported positive serology for more than one virus in 15 of 102 (14.7%) episodes of children hospitalized for acute exacerbations of asthma, but multiple viruses were not detected in cultures of nasopharyngeal and throat swab specimens from these children. Although previous studies (6, 9–11) using RT-PCR in the setting of acute viral respiratory tract infections have suggested that viruses detected by RT-PCR are involved in disease etiology and pathogenesis, none has applied RT-PCR to test for simultaneous infections.

The purpose of this study was to apply a panel of RT-PCR protocols for the detection of nucleic acid sequences from RSV (8), PIV (11), and picornaviruses (9) in respiratory specimens from symptomatic children and to correlate RT-PCR results with viral culture results and clinical diagnosis. For the purposes of this study, a simultaneous infection was defined as an RT-PCR or viral culture positive for more than one virus within a specimen. RSV, PIV, and picornaviruses were selected for the panel because they are common causes of acute respiratory tract infections in children (2). Furthermore, these viruses are commonly associated with acute exacerbations of asthma (14) and are potentially important in triggering the onset of allergy in children of allergic parents (7).

MATERIALS AND METHODS

Patient samples. Respiratory specimens (70 nasopharyngeal washes, 6 nasopharyngeal aspirates, and 4 tracheal aspirates) from 80 children (male/female ratio, 40:40; median age [± standard error], 0.80 ± 0.15 years) suspected to have acute viral respiratory tract infections were obtained at British Columbia's Children's Hospital between October 1993 and April 1994. Clinical diagnoses included upper respiratory tract infection ($n = 20$), croup ($n = 20$), acute bronchiolitis ($n = 20$), and pneumonia ($n = 20$). Upon collection, specimens were immediately transported to the Diagnostic Virology Laboratory, such that processing of specimens for viral culture commenced within 1 h of collection.

Viral culture. Five-hundred-microliter aliquots from each specimen were processed for inoculation onto HEp-2, MRC-5, and primary monkey kidney cells (American Type Culture Collection, Rockville, Md.), and the remaining portions were stored at −70°C for subsequent RT-PCR. Cell cultures were maintained for 14 days and observed daily for the appearance of cytopathic effects consistent with those of RSV, picornaviruses (rhinoviruses and/or enteroviruses), adenoviruses, and cytomegalovirus. PIV and influenza virus were detected by hemadsorption. Viruses were identified as follows: RSV by direct fluorescent-antibody assay (Meridian Diagnostics, Cincinnati, Ohio); PIV, adenovirus, and influenza A virus by indirect immunofluorescence (Bartels, Baxter Health Care Corporation, Deerfield, Ill.); picornaviruses by electron microscopy; and cytomegalovirus (CMV) by direct fluorescent-antibody assay (Bartels, Baxter). Specimens were coded such that RT-PCR and viral culture results were not compared until completion of all RT-PCR experiments.

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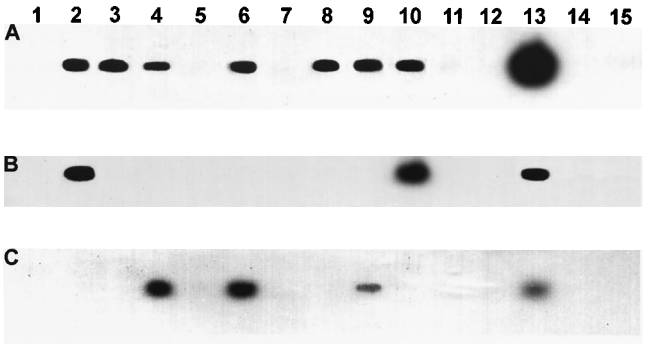

FIG. 1. Autoradiographs (4-day exposures) after filter hybridization of RT-PCR products for RSV (A), PIV (B), and picornaviruses (C). Lanes: 1, RT-PCR of blank reagent controls; 2 to 11, RT-PCR products from 10 respiratory specimens; 12 and 14, empty lanes; 13, positive RNA control samples (RSV-infected HEp-2 cells [A], PIV-infected MK2 cells [B], and rhinovirus-infected MRC-5 cells [C]); 15, negative RNA control samples from uninfected cells (HEp-2 cells [A], MK2 cells [B], and MRC-5 cells [C]). Patient specimens showing positive bands include those in lanes 2 to 4, 6, and 8 to 10 for RSV; those in lanes 2 and 10 for PIV; and those in lanes 4, 6, and 9 for picornaviruses. Specimens in lanes 2 and 10 show coamplification of RSV and PIV; specimens in lanes 4, 6, and 9 show coamplification of RSV and picornaviruses.

RT-PCR. The RT-PCR protocols were based on published methods (8, 9, 11). The target sequence for RSV was a 410-nucleotide sequence of the viral nucleocapsid gene that is common to type A and type B RSV (8). The target sequence for PIV was a 205-nucleotide sequence from the 5′ noncoding region of the F gene of PIV type 3 (11). Results of a GenBank search suggested that PIV type 1 has a similar sequence. The target sequence for picornaviruses was a 380-nucleotide sequence of the 5′ noncoding region of human rhinovirus type 1B that is highly conserved among picornaviruses (9). Each experiment consisted of RT-PCR for 10 clinical specimens and controls using oligonucleotide primers specific to one of the three viruses. Total cellular RNA was extracted from 300-μl aliquots of thawed, archival respiratory specimens by the guanidinium isothiocyanate–phenol-chloroform method of Chomczynski and Sacchi (3). After isopropanol precipitation and washes in 95 and 70% ethanol, RNA samples were dried under vacuum and dissolved in 10 μl of 0.1% diethylpyrocarbonate-containing distilled water. To remove secondary RNA structures, samples were heated to 68°C for 10 min and quick-chilled on ice. RT (37°C; 60 min) occurred in a 20-μl reaction mixture containing the RNA sample, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 0.2 mM pooled deoxynucleoside triphosphates (dNTPs) (Pharmacia, Montreal, Quebec, Canada), 20 U of RNase inhibitor (Pharmacia), 2 μM designated virus-specific oligonucleotide primer (RSV, 5′ GCGATGTCTAGGTTAGGAAGAA 3′; PIV, 5′ AGAGGTCAATACCAAC AACTA 3′; and picornaviruses, OL27 [5′ CGGACACCCAAAGTAG 3′; University of Calgary, Calgary, Alberta, Canada] [9]), and 40 U of Moloney murine leukemia virus reverse transcriptase (Pharmacia). After heat inactivation of reverse transcriptase at 99°C for 5 min, the entire 20-μl RT reaction mixture was used for PCR in a total volume of 50 μl that also included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM dNTPs, 2 μM designated virus-specific flanking primers (RSV, 5′ GCTATGTCCTTGGGTAGTAAGCCT 3′; PIV, 5′ TAGCAGTATTGAAGTTGGCA 3′; and picornaviruses, OL26 [5′ GCACTT CTGTTTCCCC 3′; University of Calgary] [9]) and corresponding virus-specific primers used for RT, and 2 U of *Taq* DNA polymerase (GIBCO BRL, Burlington, Ontario, Canada). Samples were overlaid with mineral oil, and PCR was performed on a Robocycler 40 (Stratagene, La Jolla, Calif.) as follows: for RSV, 35 cycles of denaturation at 94°C for 1 min (5 min during cycle 1) and combined annealing-extension at 70°C for 2 min (10 min during cycle 35); for PIV and picornaviruses, 35 cycles of denaturation at 94°C for 1 min (5 min during cycle 1), annealing at 55°C for 1 min, and extension at 70°C for 1 min (10 min during cycle 35). After PCR, each 50-μl sample underwent electrophoresis on 1.2% agarose gels stained with 0.5 μg of ethidium bromide per ml, followed by Southern blotting and UV light fixation onto Hybond-N nylon membranes (Amersham, Arlington Heights, Ill.). Membranes underwent hybridization with ^{32}P-labeled oligonucleotide probes (RSV, 5′ TAGCTCCAGAATACAGGCATGACTC 3′; PIV, 5′ AAAATTCCAAAAGAGACCGGC 3′; and picornaviruses, JWA-1 [5′ GCATTCAGGGGCCGGAG 3′; University of Calgary] [9]) (specific activity, 4×10^8 to 8×10^8 cpm/μg) that were prepared by the T4 polynucleotide kinase reaction (Pharmacia) (8). Hybridization occurred at 65°C for 18 h in a solution consisting of 6× SSC (0.9 M NaCl, 0.09 M sodium citrate · $2H_2O$ [pH 7]), 5× Denhardt's solution (0.1% Ficoll 400; 0.1 bovine serum albumin, fraction V; 0.1% polyvinylpyrrolidone), 50 mM sodium phosphate (pH 6.8), 0.5% sodium dodecyl sulfate, and 20 μg of yeast tRNA per ml. Posthybridization washes consisted of three 5-min washes in 6× SSC at room temperature and, to increase stringency, a 30-min wash in 6× SSC at 65°C. Membranes were covered with Saran Wrap and placed against Kodak X-ray film in cassettes with regular intensifying screens, and autoradiography was performed at −70°C for 4 h to 5 days.

Positive RT-PCR controls included total cellular RNA extracted from HEp-2 cells infected with Long strain, type A human RSV; rhesus monkey kidney (MK2) cells infected with PIV type 3; and MRC-5 cells infected with human rhinovirus type 1B (American Type Culture Collection). Negative controls included RNA extracted from uninfected HEp-2, MK2, and MRC-5 cells and blank reagent controls that did not contain nucleic acid.

Statistics. The chi-square test was used to compare the proportion of samples showing a positive RT-PCR result with the proportion of corresponding viral cultures and to compare the proportion of specimens showing a given RT-PCR result with the clinical diagnosis. Statistical analyses were performed by using Systat version 5.1 software (Systat Corporation, Evanston, Ill.), and a P value of <0.05 was considered to be statistically significant.

RESULTS

Figure 1 shows results of autoradiography of 10 patient specimens and corresponding positive and negative controls after filter hybridization with ^{32}P-labeled oligonucleotide probes. Overall, filter hybridization and autoradiography confirmed the specificity of bands visualized on ethidium bromide-stained agarose gels but did not increase sensitivity of detection. Table 1 shows the relationship between RT-PCR and viral culture results. In comparison with viral culture, the sensitivities of RT-PCR were 95.6% for RSV, 94.4% for PIV, and 100% for picornaviruses (one specimen), while the specificities of RT-PCR were 89.5% for RSV, 82.3% for PIV, and 67.1% for picornaviruses. In contrast to viral culture, the RT-PCR panel revealed evidence of simultaneous infections in a significantly higher proportion of specimens (20% versus 3.8%; $P < 0.002$). Picornaviral and PIV nucleic acid sequences were coamplified in 10 specimens, while RT-PCR for all three viruses was positive for 2 specimens.

In 84% of the specimens in which no viruses were detected by viral culture, the RT-PCR panel detected nucleic acid sequences from at least one virus, with RT-PCR for picornaviruses being positive for a significantly higher proportion than RT-PCR for RSV or PIV ($P < 0.001$). There were no statistically significant associations between the clinical diagnosis and the proportion of specimens showing positive RSV, PIV, or picornaviral RT-PCR, nor was there any trend for simultaneous infections or negative RT-PCR results to be associated with a particular diagnosis. In particular, three patients with croup, one patient with acute bronchiolitis, and two patients

TABLE 1. Correlation between RT-PCR and viral culture results

Virus cultured (no. of specimens)	RT-PCR result (no. of specimens)				
	RSV	PIV	Picornavirus	>1 Virus	Negative
RSV (20)	19	2	3	4	1
PIV[a] (17)	2	16	2	4	1
Picornavirus (1)	0	0	1	0	0
Adenovirus (7)	0	0	2	0	5
Influenza A (3)	0	0	1	0	2
CMV (4)	1	1	0	0	2
RSV and PIV type 3 (1)	1	1	1	1	0
RSV and CMV (2)	2	0	0	0	0
Negative (25)	3	8	17[b]	7	4
Total (80)	28	28	27	16[c]	15

[a] Includes 14 PIV type 1 infections and 3 PIV type 3 infections.

[b] $P < 0.001$ in comparison with the proportion of positive RT-PCR results for RSV and PIV obtained with culture-negative specimens.

[c] $P < 0.002$ in comparison with the proportion of simultaneous infections as determined by viral cultures.

with pneumonia had positive picornaviral RT-PCR results as the only evidence (by culture or RT-PCR) of respiratory viral infection in this study.

DISCUSSION

The results of this study show that the RT-PCR panel had over 94% sensitivity for detection of RSV, PIV, and picornavirus nucleic acid sequences in specimens that had positive cultures for these viruses. In addition, the RT-PCR panel frequently coamplified nucleic acid sequences from multiple viruses and could also detect viral nucleic acid sequences in specimens in which no viruses were detected by culture. The results of this study extend previous observations of a high prevalence of positive RT-PCR results for picornavirus in association with negative viral cultures (9), as significantly fewer of these specimens had positive RT-PCR results for RSV or PIV ($P < 0.001$). Furthermore, this study has established that the RT-PCR protocol originally used for detection of PIV type 3 (11) can also be used to detect PIV type 1 in clinical specimens.

False-positive results by the RT-PCR methodology were unlikely because blank reagent controls yielded negative results, while possible contamination of specimens during RNA extraction was unlikely because RT-PCR was negative for RNA extracted from uninfected tissue culture cells. Conversely, false-negative RT-PCR results may have been related to sampling error during preparation of aliquots of respiratory specimens, loss of RNA during extraction, RNA degradation from freezing and thawing, digestion by RNases, or the presence of PCR inhibitors (5); however, in comparison with viral culture, the sensitivity of RT-PCR for each virus within the panel was comparable to those in previous studies that used a single viral RT-PCR protocol (6, 9, 10).

The high proportion of specimens showing evidence of simultaneous infections by RT-PCR is similar to previous observations obtained by viral serology of children with acute exacerbations of asthma (12), while the low proportion of specimens showing evidence of simultaneous infections by viral culture in this study is similar to results of previous reports (14, 15). The RT-PCR findings extend previous viral serologic findings by providing direct evidence for nucleic acid sequences from multiple viruses within the respiratory tract and illustrate the additional information that can be gained by using RT-PCR to test for the presence of more than one virus within a specimen.

The lack of association between simultaneous infections and a specific clinical diagnosis in a study that used culture for viral diagnosis has also been reported (15). Although the RT-PCR findings of the present study are nonquantitative and do not indicate the relative importance of each virus detected in disease etiology or pathogenesis, the high prevalence of simultaneous infections documented by RT-PCR could have important clinical implications. For example, in clinical trials evaluating the efficacy of new, virus-specific agents such as soluble intercellular adhesion molecule-1 for rhinovirus infections (13), the persistence of symptoms from a second virus does not necessarily indicate a treatment failure. Similarly, potentially misleading interpretations of treatment outcomes could result for simultaneous infections involving RSV or influenza A virus, respiratory pathogens for which pharmacologic therapy is available. With the publication of RT-PCR protocols for the influenza viruses (4), the RT-PCR panel could be expanded to include pathogens such as influenza A virus that have potential impact on clinical management.

Concerning practical applications of the RT-PCR panel in the diagnostic virology laboratory, a preliminary diagnosis in which RT-PCR products are visualized on ethidium bromide-stained agarose gels can be obtained on the day the specimen is collected, while confirmation by filter hybridization and autoradiography (5) may take up to several days. Thus, the speed of viral diagnosis using the RT-PCR panel is intermediate between that of rapid, antibody-based detection methods and that of viral culture. However, the RT-PCR panel has the potential advantage of a background lower than that observed with some virus-specific antibodies, especially if there is considerable cell necrosis within the respiratory specimen. Secondly, the results of this study confirm the increased sensitivity of RT-PCR over viral culture for the diagnosis of picornavirus infections (9). Furthermore, the comparatively small number of negative results suggests that the RT-PCR panel may be useful as a screening technique; however, the cost-effectiveness of RT-PCR for viral diagnosis remains to be established (1) and further technical advances will be required to facilitate the increased throughput of specimens at a lower unit cost.

In summary, this study has demonstrated that an RT-PCR panel can be used to test for RSV, PIV, and picornavirus nucleic acid sequences in clinical specimens obtained from symptomatic children. In contrast to viral culture, the RT-PCR panel revealed a prevalence of simultaneous infections higher than that previously reported for studies that used culture to document viral respiratory tract infections. Further assessment of the rapidly evolving role of RT-PCR in viral diagnosis may include follow-up sampling of patients, examination of respiratory specimens from asymptomatic subjects, and expansion of the panel to include additional respiratory pathogens. The application of a multivirus RT-PCR panel to clinical specimens offers an approach to obtain new insights into acute viral respiratory tract infections in children.

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