Diagnosis of Respiratory Syncytial Virus Infection: Comparison of Reverse Transcription-PCR to Viral Culture and Serology in Adults with Respiratory Illness

Ann R. Falsey,* Maria A. Formica, and Edward E. Walsh

Department of Medicine, Rochester General Hospital, University of Rochester School of Medicine and Dentistry, Rochester, New York

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Respiratory syncytial virus (RSV) is an enveloped, negative-sense RNA virus belonging to the Paramyxoviridae family. It is the major cause of lower respiratory tract illness in young children. In recent years it has been recognized that RSV infection may be severe in certain adult populations, including the elderly, persons with cardiopulmonary disease, and the immunocompromised. Diagnosis of RSV infection by either culture or antigen detection from nasopharyngeal specimens is difficult with adults, presumably due to low viral titers in secretions. In addition, nasal swabs, rather than nasal washes, are frequently obtained from elderly persons because nasal washes are difficult to perform with frail or uncooperative patients. In the elderly, virus can be isolated in fewer than half of illnesses even when samples are processed under optimal conditions. Thus, many investigators have relied primarily on serology for diagnosis of RSV infection in adults. Serologic diagnosis is useful for epidemiological studies but not for patient management because of the delay required for convalescent-phase sera. Reverse transcription-PCR (RT-PCR) is a highly sensitive method for diagnosis of viral infection and has been used successfully in children with RSV. We previously described a novel, single-tube hanging-droplet nested RT-PCR for detection of RSV in respiratory secretions. This method was found to be 100-fold more sensitive than single-round PCR and was capable of detecting 0.05 PFU of tissue culture-passaged virus. The purpose of this report is to define the sensitivity and specificity of this RT-PCR method in clinical samples from adults with respiratory illnesses and to compare the results to those from standard viral culture and serology.

MATERIALS AND METHODS

Study design. Adult volunteers were recruited during the summer and fall of 1999 and 2000 to participate in a surveillance study of respiratory illnesses in Rochester, N.Y. Four cohorts were monitored: young healthy persons aged 18 to 40 years, healthy adults over age 64, high-risk adults with congestive heart failure and chronic obstructive pulmonary disease, and residents of a nursing home. During enrollment subjects underwent a baseline medical evaluation and had a serum sample collected. Between 15 November and 15 April of the two study winters, subjects were evaluated for symptoms of respiratory tract infection that included new or increased nasal congestion, sore throat, cough, sputum production, dyspnea, wheezing, or fever. Illness evaluations consisted of a brief history, physical exam, and collection of nasopharyngeal secretions and acute-phase blood samples. Convalescent-phase serum samples were obtained 4 to 6 weeks later from as many subjects as possible. In addition to the prospective surveillance, adults over age 64 or with underlying heart and lung disease admitted to the hospital during this same period with acute cardiopulmonary conditions were recruited for the study. Subjects were evaluated within 48 h of admission, and nasal swabs and acute-phase blood samples were obtained. Convalescent-phase serum samples were taken 4 to 6 weeks later. The Rochester General Hospital and University of Rochester School of Medicine Institutional Review Board approved the study. All volunteers or their legal guardians gave informed consent.

Laboratory methods. (i) Specimen collection. Nasopharyngeal samples were obtained by gently rubbing the deep nasal turbinates bilaterally with sterile cotton swabs and combining them with a third swab from the posterior pharynx in 3 ml of viral infusion broth. Samples were transported to the laboratory on wet ice and were aliquoted. A fresh aliquot was placed onto cell culture, and the remaining portion was frozen at −70°C for PCR testing at a later time. Only one sample per illness was collected. (ii) Viral culture. Samples (0.4 ml) were inoculated within 4 h of collection onto HEP-2 cells in roller tubes. Cultures were observed daily for 10 days for cytopathic effect. RSV infection was confirmed by immunofluorescence with virus-specific monoclonal antibodies (Bartel’s Diagnostics, Issaquah, Wash.). (iii) Serology. Enzyme immunoassay was used to measure serum immunoglobulin G (IgG) to purified RSV envelope glycoproteins from group A and B viruses. The RSV fusion (F) protein, the attachment protein from the A2 strain of RSV, and the attachment protein of the B1 strain of RSV were purified by...
TABLE 1. Total RSV testing for all subjects

<table>
<thead>
<tr>
<th>Method or diagnosis</th>
<th>No. tested</th>
<th>No. positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>47</td>
<td>1,134</td>
<td>4.1</td>
</tr>
<tr>
<td>PCR</td>
<td>102</td>
<td>1,135</td>
<td>9.0</td>
</tr>
<tr>
<td>Serology</td>
<td>138</td>
<td>1,114</td>
<td>12.4</td>
</tr>
<tr>
<td>RSV by any method</td>
<td>166</td>
<td>1,495</td>
<td>11.1</td>
</tr>
</tbody>
</table>

TABLE 2. Results of RSV diagnostic testing for subjects with all three tests available (n = 1,112)

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of subjects</th>
<th>RSV positive</th>
<th>PCR positive</th>
<th>Serology positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1,112</td>
<td>104</td>
<td>87</td>
<td>43</td>
</tr>
<tr>
<td>Culture</td>
<td>43</td>
<td>37</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>PCR</td>
<td>87</td>
<td>37</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Serology</td>
<td>104</td>
<td>37</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>RSV by any method</td>
<td>43</td>
<td>37</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

RSV was identified by culture, PCR, or serology in 166 samples. Of these, 47 of 132 (37%) were culture positive, 102 of 133 (77%) were PCR positive, and 138 of 151 (93%) were seropositive (Table 1).

Of the 1,495 samples, 1,112 were evaluated with all three diagnostic tests and were further analyzed to determine the sensitivity, specificity, and positive and negative predictive values of the RT-PCR. One hundred seventeen were positive for RSV by at least one test, and 995 were negative by all three methods. Of the 117, 43 were culture positive, 87 were PCR positive, and 104 were seropositive (Table 2). Subjects were considered to have true infection if they were culture positive or seropositive. All culture-positive specimens were also PCR positive. Therefore, 110 of the 117 were considered to be true positives. Seven samples were PCR positive with negative culture and serology and for the purpose of defining the test characteristics were considered false positives. Given these definitions, the overall sensitivity of RT-PCR was 73% and specificity was 99% (Table 3). The positive predictive value was 91%, and the negative predictive value was 97%.

When each cohort was analyzed separately, specificity remained excellent in all groups at ≥99% whereas sensitivity varied from 60 to 90% depending on the group tested (Table 4). These differences were not statistically significant, but overall the PCR performed better for the outpatient groups than for the hospitalized subjects. Number of days prior to evaluation and rates of pneumonia were examined as possible factors that may have influenced titer or duration of viral shedding to explain differences in PCR results. Although mean number of days of illness prior to evaluation was not significantly longer for the PCR-negative subjects than for the PCR-positive subjects (4.7 ± 5.3 versus 5.0 ± 5.7 days) for the entire group, the number of days with symptoms was higher for the hospitalized group than for the outpatient group (6.0 vs 5.0 days).

TABLE 3. Sensitivity and specificity of RT-PCR for RSV in adults

<table>
<thead>
<tr>
<th>Type of result</th>
<th>No. of samples</th>
<th>PCR negative</th>
<th>PCR positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No RSV</td>
<td>995</td>
<td>7</td>
<td></td>
<td>1,002</td>
</tr>
<tr>
<td>RSV disease</td>
<td>30</td>
<td>80</td>
<td></td>
<td>110</td>
</tr>
<tr>
<td>Total</td>
<td>1,025</td>
<td>87</td>
<td></td>
<td>1,112</td>
</tr>
</tbody>
</table>

* Defined as false-positive PCR results.

RESULTS

A total of 938 subjects were monitored during the 2-year period in the prospective groups, and 626 hospitalized subjects were enrolled. In the prospective studies, 305 healthy elderly adults, 304 subjects with cardiopulmonary disease, 134 nursing home residents, and 195 healthy young adults were enrolled. During the two winter seasons, 1,495 samples were evaluated.

Affinity chromatography (13, 15). Antigens were diluted in bicarbonate buffer and applied as a coating to 96-well enzyme-linked immunosorbent assay plates overnight at 4°C. Serum samples (serially diluted from 1:1,600 to 1:204,800) were incubated overnight in duplicate wells at 37°C. After washing, the bound IgG was detected by alkaline phosphatase-conjugated goat anti-human IgG, followed by washing and addition of substrate. A positive well was defined as an optical density ≥ 0.20 and at least two times the value of the bicarbonate (negative) wells. RSV infection was defined as a ≥4-fold rise in antibody to any of the RSV antigens.

(iv) RT-PCR. Forty micrometers of tRNA (Gibco BRL, Gaithersburg, Md.) was added to each 250-μl sample, which was then extracted with 750 μl of LS Stat (Tel-Test, Inc., Friendswood, Tex.) according to the manufacturer’s instructions. The RNA was precipitated with 100% isopropanol for 15 min at -70°C and centrifuged, and the pellet was washed twice with cold 75% ethanol. After air drying, the RNA pellet was dissolved in 10.5 μl of water for RT-PCR. For the RT step, 10.5 μl of RNA was combined with 2 μl of outer primer 1 (2 μM), 4 μl of 5′ RT buffer (Promega, Madison, Wis.), 2 μl of deoxynucleoside triphosphates (2 mM each), 1 μl (9 U) of avian myeloblastosis virus reverse transcriptase (Promega), and 0.5 μl of RNasin (20 U/μl). The mixture was incubated at 42°C for 1 h, followed by heating to 95°C for 3 min. Five microliters of the resulting cDNA product was used in the PCR.

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Outer primer and inner primer pairs which would result in DNA products of 411 and 263 bp, respectively, were selected from conserved sequences of the published RSV A2 strain F gene (1). The sequences for the primers were as follows: OP1 (outer primer 1), 5′ ATGCAAGGTGTAACAAAGCCCCTTTAAGGACTTTACATGTTAAC 3′; OP2, 5′ GTAAATGTTAACGTTCATAGTGCTCAAAAAATCTGATT 3′; IP1 (inner primer 1), 5′ GATATGCCCTAAACAATGAGTA 3′; and IP2, 5′ GATACTGATCTGCATGTT 3′.

For the hanging-droplet nested PCR, the droplet mixture was prepared by combining 1 μl of each inner primer (2 μM), 0.5 μl of Taq polymerase, 0.5 μl of a 1:2 dilution of uracil-N-glycosylase (UNG) (0.25 U), and 2 μl of water. This mixture was held at room temperature for 10 min. The UNG was then inactivated at 95°C for 2 min. The following were placed in the reaction tube: 5 μl of RT product, 5 μl of 10× PCR buffer, 8 μl of MgCl2 (25 mM), 5 μl of deoxynucleoside triphosphates (2 mM dTTP replacing dUTP), 2 μl of each outer primer (1 μM), 1 μl of Taq polymerase (5 U/μl), 1 μl of UNG (1 U), and 21 μl of water. The reaction mixture was then covered with 100 μl of oil. Before the tube was closed, the 2-μl droplet was placed in the center of the inside of the reaction tube cap. The tube was closed, held at room temperature for 10 min, and then heated to 95°C for 2 min to inactivate the UNG. The first round of the PCR was carried out for 30 cycles (95°C for 40 s, 42°C for 1 min, and 72°C for 1 min). The reaction mixture was brought to 4°C, and the hanging droplet was incorporated into the reaction mixture by inversion and shaking. The tube was centrifuged briefly to reposition the oil above the reaction mixture, and a second round of 40 cycles was completed using conditions identical to those for the first round with a final 10-min extension at 72°C. PCR products were separated by electrophoresis on 1.5% agarose gels and identified by staining with SYBR green (Molecular Probes, Eugene, Oreg.).

Statistical methods. Proportions were compared using chi-square analysis and means compared by the Student t test and the Welch correction factor as appropriate.
than for outpatients (7.7 ± 7.3 versus 3.0 ± 2.1 days, P = 0.003). The presence of pneumonia was more common in PCR-negative subjects, at 35%, than in PCR-positive subjects, at 12.5% (P = 0.02). The duration of symptoms prior to evaluation was also longer in the pneumonia group than in the nonpneumonia group (8.1 ± 8.7 versus 4.3 ± 4.3 days, P = 0.007).

**DISCUSSION**

The study of RSV infection in adults has been hampered by the inability to make a diagnosis during acute infection. Investigation into the immune response or the study of antiviral treatments has been nearly impossible with the use of viral culture alone for diagnosis. The present data confirm the insensitivity of viral culture for adults and indicate that RT-PCR represents a significant improvement over culture. The ability to diagnose active infection nearly doubled from 39% with culture alone to 73% with RT-PCR. The specificity of the test was excellent at 99%. The seven subjects considered to have false-positive PCR results may have actually been infected with RSV despite negative serology. Although serology has been considered the best test for the diagnosis of RSV in adults, it is not 100% sensitive. In the present study, 6 subjects out of 43 (14%) with culture-confirmed RSV did not show a rise in RSV antibody level. Five of these subjects were healthy young adults, and the other was a healthy elderly person.

The PCR was negative for 29% of seropositive subjects, and this result likely represents a combination of factors that include the presence of inhibitors in secretions and collection of samples after viral RNA had cleared. As expected, the time before evaluation was longer for the hospitalized subjects than for the outpatients and may have been a factor in the lower sensitivity of PCR noted for this group. Interestingly, among RSV-infected persons, pneumonia was more common in PCR-negative than in PCR-positive subjects. One possible explanation is that the RSV infection occurred several weeks prior to admission and the pneumonia represented a bacterial complication. Consistent with this was a mean length of symptoms prior to evaluation of 8 days in this group. The sensitivity of the PCR also appeared somewhat lower for the young healthy group despite no difference in time before evaluation (2.5 days). Although there are no specific data regarding age-related differences in immunity to RSV, it is well documented that cellular immunity diminishes with aging (9). It therefore seems plausible that young healthy individuals may clear infection with RSV more rapidly and thus be PCR positive for a shorter period than older persons.

The present nested RT-PCR is a 2-day procedure and provides a diagnosis more rapidly than does standard viral culture, which typically requires 3 to 5 days for viral isolation (7). Faster results should be achievable using real-time PCR, making same-day diagnosis possible in the future. At present the treatment of RSV in most adults is largely supportive. However, timely diagnosis is important for immunocompromised persons, who may benefit from antivirals and immunoglobulin therapy (16). Rapid diagnosis of RSV may also be useful for the elderly or for hospitalized adults, so that appropriate infection control measures may be taken, inappropriate antibiotic use may be limited, and discrimination from influenza can be achieved.

In conclusion, RT-PCR is a reasonably sensitive and highly specific method for the diagnosis of RSV infection in adults. The use of RT-PCR should provide the means to perform accurate epidemiological studies as well as to investigate the immune response to infection and to study potential antiviral therapeutics for adults.

**ACKNOWLEDGMENTS**

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**REFERENCES**