Genetic Variability among Group A and B Respiratory Syncytial Virus Isolates from a Large Referral Hospital in New Delhi, India

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Received 26 September 2002/Returned for modification 9 December 2002/Accepted 14 March 2003

Respiratory syncytial virus (RSV) is an important childhood pathogen of acute lower respiratory infections in developed and developing countries. The molecular epidemiology of RSV in India is largely unknown. The present study was undertaken to standardize and evaluate reverse transcription-PCR (RT-PCR) for the rapid and simultaneous detection of RSV groups A and B in clinical samples and to study intragroup genetic variability. RT-PCR was evaluated by comparing the results of seminested RT-PCR with centrifugation-enhanced cultures on 200 nasopharyngeal aspirates from children with acute lower respiratory infections. RSV was isolated in 34 nasopharyngeal aspirates by centrifugation-enhanced cultures and identified in 45 samples by RT-PCR. In 15 samples RSV was identified by seminested RT-PCR alone and in four by centrifugation-enhanced cultures alone. Of the 45 samples positive for RSV by nested PCR, 15 belonged to group A, 29 to group B, and one sample suggested a mixed infection. Group B RSV predominated in both years of the 2-year study. Genetic variability within RSV groups was studied by restriction fragment analysis of 35 PCR products. Among both group A and group B RSV, two different composite patterns were observed. Thus, RSV was found to be a major pathogen of acute lower respiratory tract infections in India, as it was detected in 24.5% of children by RT-PCR. RT-PCR provides a sensitive method for detection and typing of RSV group A and B viruses in clinical samples as well as a means to study intragroup variations. However, a higher sensitivity of detection of RSV in clinical samples can be obtained by its combination with additional techniques, such as virus cultivation.

MATERIALS AND METHODS

Nasopharyngeal aspirates were collected from 200 children attending the Department of Pediatrics at All India Institute of Medical Sciences with signs and symptoms of acute lower respiratory infection (11). Study subjects included children of either sex from 7 months to 60 months of age.

Virus stocks. Standard strains of RSV group A (A2 strain) and group B (8/60 strain) were grown in HEp-2 cells and titrated by 50% tissue culture infectious dose assays. These stocks were used for standardization of RT-PCR, centrifugation-enhanced cultures, and positive controls in both methods. Uninfected HEp-2 cells were used as a negative control.

Isolation of RSV from nasopharyngeal aspirates with centrifugation-enhanced culture. Virus isolation by centrifugation-enhanced culture on HEp-2 cells was carried out on all 200 clinical specimens. The nasopharyngeal aspirates were inoculated in duplicate onto 24-well tissue culture plates with coverslips (12 mm) containing monolayer of HEp-2 cells. Plates were centrifuged at 1,000 rpm for 1 h and incubated at 33°C in a 5% CO2 atmosphere. At 48 h postinoculation, the viral pathogen was identified by immunofluorescent staining with a blend of monoclonal antibodies.
monoclonal antibodies to the fusion (F) and attachment (G) proteins (Chemicon Inc.) of RSV. This method has been described earlier by our laboratory; the samples characterized in the previous publication were analyzed by PCR in this investigation (15).

**Viral RNA extraction.** To standardize the RT-PCR and to determine the sensitivity, tissue culture-grown standard strains (8/60 and A2) of RSV of a known 50% tissue culture infectious dose were taken, and serial log dilutions were made from 10⁻¹ to 10⁻⁶. Viral RNA was extracted from 400 μl of tissue culture lysate or clinical samples (nasopharyngeal aspirates) by the guanidinium isothiocyanate method (3) followed by ethanol precipitation. Four units of RNasin (Promega Corp.) and 10 μg (1 μg/μl) of glycogen (Sigma Chemicals) were added to samples prior to RNA extraction. The RNA pellet was vacuum dried and suspended in 10 μl of 0.1% diethyl pyrocarbonate-treated water and used for cDNA synthesis.

**Oligonucleotides.** The oligonucleotides for external PCR and seminested PCR amplification were selected from conserved regions of the G and F protein genes based on primers described by Sullender et al. (24) as well as published sequences. Primer F164 was used as the antisense primer for both external and seminested PCR. The G32 primer was used to amplify both the genogroups in external PCR, and G267 and G399 were used as internal primers for genogroups A and B, respectively (16). The sequence, location, and specificity of primers were as follows: F164(AS)164-186 (F gene), 5'-GTT ATG ACA CTG GTA TAC-3' for primer A, and 5'-GTT ATG ACA CTG GTA TAC-3' for primer B, respectively (16). The sequence, location, and specificity of primers were as follows: G32(AS)378-399 (G gene), 5'-ACA AGC CAG ATC AAG-3' for primer A, and 5'-ACA AGC CAG ATC AAG-3' for primer B, respectively (16). The sequence, location, and specificity of primers were as follows: G399 for group B and antisense primer F164. Amplification was done with the same PCR profile as used for external PCR. The amplified products of the group A and B RSVs were 0.9 kb and 0.78 kb in length, respectively.

**Restriction endonuclease digestion.** The seminested PCR amplicons were purified with a Nanosep microcentrator (Pall Filtron) and subjected to restriction endonuclease digestion with *RsaI, PstI, HincII*, and *AhlI* enzymes (21). Approximately 0.5 to 1.0 μg of DNA was subjected to digestion, and the fragments were analyzed by electrophoresis in 2.5% agarose gels. Within each virus group, the restriction patterns were assigned letter and number designations to facilitate comparisons among the viruses studied. The first letter of the designation represents the restriction enzyme, the second letter represents the group of RSV, and the number represents the pattern number.

**RESULTS**

**Sensitivity and specificity of RT-PCR.** The RT-PCR was first standardized with RNA obtained from tissue culture-grown RSV group A (A2) and group B (8/60) viruses. A 1.1-kb band was obtained with both groups with primers F164 and G32. The specificity of primers was determined by using heterologous RNA from influenza virus A and parainfluenza viruses 1, 2, and 3 as well as nucleic acid from uninfected HEP-2 cells. No amplicons were obtained with the heterologous templates. The detection limit of external PCR for both group A and B RSV was 0.1 TCID₅₀.

**Detection and typing of A and B strains of RSV by RT-PCR and seminested PCR.** RSV was detected in 21 out of 200 nasopharyngeal aspirates by external PCR. However, by seminested RT-PCR, 45 out of 200 nasopharyngeal aspirates were positive for RSV (Fig. 1). Of these 45, 15 were typed as group A and 29 as group B. In one sample, a presumed dual infection was detected, as both the 0.9- and 0.78-kb bands were visualized and the results were confirmed thrice by repeated testing.

**Comparison of seminested PCR with centrifugation-enhanced culture.** RSV was detected in 34 of 200 nasopharyngeal aspirate samples by centrifugation-enhanced culture followed by indirect immunofluorescence (15). On comparison of seminested PCR with centrifugation-enhanced culture in clinical samples, it was found that the results of 30 samples were concordant, whereas discordance was observed in 19 samples. In 15 samples which were positive only by seminested RT-PCR, a repeat seminested PCR confirmed the results. Four samples were negative by seminested PCR, but virus was isolated by centrifugation-enhanced culture (Table 1). With cen-
trifugation-enhanced culture and seminested PCR, RSV could be detected in 49 of 200 nasopharyngeal aspirates from children with acute lower respiratory infections; of these, 34 (70%) were from children less than 1 year of age.

**Intratypic variations within RSV groups by restriction endonuclease digestion.** A total of 35 RSV strains, (10 group A and 25 group B) were studied. Examples of the restriction fragment patterns from the clinical and prototype samples are provided (Fig. 2 and 3). The group A strains yielded two distinct patterns after digestion with *RsaI* (ra1 and ra2), two patterns with *PstI* (pa1 and pa2), and one pattern with *HincII* (ha1) (Table 2). Group B yielded a single pattern (rb1) after digestion with *RsaI* which was similar to the standard strain and two patterns with *AluI* (ab1 and ab2), i.e., some strains showed digestion, whereas others remained uncut. All group B RSV strains remained uncut with *PstI* and *HincII* (pb1 and hb1, respectively) (Table 3). Overall, restriction fragment analysis revealed two distinct composite patterns with group A and group B RSV strains (Fig. 4). Of 10 group A strains, nine were similar to the standard strain of group A (A2), while one had a distinct pattern. Ten out of 25 group B strains had a pattern similar to that of the group B standard strain (8/60), while the remaining 15 had a different pattern.

**DISCUSSION**

RSV is the major cause of respiratory infections among children in India (12, 15). Information on the genetic heterogeneity of RSV from developing countries is, however, extremely limited. In this study, genetic variability was characterized among RSV strains identified from children with acute lower respiratory infections in a large referral hospital in India over a 2-year period. RSV were identified and grouped into the major antigenic groups A and B by a seminested PCR protocol, and intragroup variations were studied by restriction fragment analysis with RT-PCR-amplified products of the G protein gene.

Direct immunofluorescence immunoassay is a rapid technique for identification of respiratory viruses, but it is less sensitive than culture, and negative specimens may need to be confirmed by culture. Centrifugation-enhanced culture is more rapid and sensitive than standard culture (13, 17). RT-PCR provides a sensitive tool for both detection and typing of RSV into groups (6, 8). In addition, PCR products can be further characterized to study RSV molecular epidemiology. The primers F164 and G 32 (24) were used as external primers to amplify RSV strains. In a previous study, the G32 primer was found to be specific for group B RSV (24). In this application for direct detection of viruses in clinical samples, we sought increased sensitivity. Differences from the earlier assay included lowering of the annealing temperature and the inclusion of a random hexamer primer in reverse transcription. However, under these conditions the G32 primer could amplify both group A and B RSV, yielding a product of 1.1 kb. Therefore we used a seminested approach with the specific primers for group A (G267) (24) and B (G399) (16).

RSV was detected in 30 samples by both centrifugation-enhanced culture and RT-PCR; in 15 samples virus was detected by RT-PCR alone; and in four samples centrifugation-enhanced culture detected virus but RT-PCR was negative. The samples which were RT-PCR positive and culture negative might reflect a false-positive result from RT-PCR testing. However, earlier studies with RT-PCR on clinical samples have suggested that RT-PCR may be more sensitive than culture for detection of virus in clinical samples (5, 28).

![FIG. 2. Agarose gel showing restriction enzyme pattern of group A RSV with *RsaI*, *HincII*, and *PstI* enzymes. Panel A (digestion with *RsaI*): lane M, φX174 *HaeIII* digest (molecular weight marker); lanes 1 and 2, 0.9-kb uncut amplicon and digested amplicon of positive control of group A, respectively, lanes 3 to 6, digested amplicons of group A sample strains. The ra1 pattern was seen in lanes 2 to 5, and the ra2 pattern was seen in lane 6. Panel B (digestion with *HincII*): lane M, φX174 *HaeIII* digest (molecular weight marker); lanes 1 and 2, 0.9-kb uncut amplicon and digested amplicon of positive control of group A, respectively, lanes 3 to 5, digested amplicons of group A sample strains. The ha1 pattern was seen in lanes 2 to 5. Panel C (digestion with *PstI*): lane M, φX174 *HaeIII* digest (molecular weight marker); lanes 1 and 2, 0.9-kb uncut amplicon and digested amplicon of positive control of group A, respectively, lanes 3 to 6, digested amplicons of group A sample strains. The pa1 pattern was seen in lanes 2 to 5, and the pa2 pattern was seen in lane 6.](http://jcm.asm.org/)
nested PCR failed to detect viral RNA in four samples that were positive by centrifugation-enhanced culture. These false-negative results might have occurred for a variety of reasons, including viral genetic variability, degradation of template RNA, or inhibitory substances in the samples (21). A combination of the two techniques was of value in increasing the sensitivity of detection.

Both group A and B RSV were found during the 2-year study period, with a predominance of group B strains. Most of the other studies on prevalence of group A and B RSV have shown predominance of group A viruses (8, 9). Recent studies from developing countries have begun to define RSV disease burden and epidemiology. In Mozambique, as in this study, group B viruses were found to have a higher prevalence than group A viruses (23). In a rural district hospital in Mozambique, RSV was identified in 8.6% of children presenting to the outpatient department with cough or nasal secretions and 10.6% of children admitted to the hospital with lower respiratory infection (14). Involvement of the lower respiratory tract (59.7%) and hospital admission (18.1%) occurred more often than described in developed countries.

When RSV from rural South African clinics were compared

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<th>TABLE 2. Restriction enzyme analysis of group A RSV strains</th>
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<td><strong>Strain(s)</strong></td>
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<tr>
<td>---------------</td>
</tr>
<tr>
<td>A2</td>
</tr>
<tr>
<td>90, 107, 121, 127, 146, 170, 171, 186, 210, 220 (9 isolates)</td>
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<td>221 (1 isolate)</td>
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<th>TABLE 3. Restriction enzyme analysis of group B RSV strains</th>
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<tr>
<td><strong>Strain</strong></td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>S/60</td>
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<tr>
<td>51, 57, 63, 77, 102, 106, 139, 187, 199, 217 (10 isolates)</td>
</tr>
<tr>
<td>53, 69, 96, 109, 130, 134, 137, 151, 160, 183, 201, 206, 215, 231 (15 isolates)</td>
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to those from hospitalized patients in Soweto, most viruses were not significantly different between the two locations (28).

In Gambia, the incidence rate per 100 infants for acute lower respiratory infections was 9.6 cases per year and 0.83 for severe RSV-associated illness. RSV accounted for 19% of all hospital admissions for acute lower respiratory infections (30). The identification of RSV as the etiologic agent of 24.5% of acute lower respiratory infections in our study exceeds that reported above for Mozambique and the Gambia. This may reflect the heightened sensitivity provided by a combination of culture and molecular detection techniques compared to antigen detection by enzyme-linked immunosorbent assay or immunofluorescence in the other studies.

With the screening technique of restriction fragment analysis, genetic heterogeneity was observed among both group A and B RSV. Two composite patterns were observed in both groups. Earlier studies reported less genetic variability among group B viruses compared to group A RSV (8, 24). However, we observed less variability among the group A viruses than described earlier (1, 2, 4). The restriction fragment analysis employed here was used as a screening tool for genetic variability and is not expected to reveal the full extent of genetic differences. Nucleotide sequence analysis of these samples should provide precise determination of the molecular differences among these viruses.

A detailed understanding of the epidemiology of RSV in India will require prospective, longitudinal, and community- and hospital-based investigations combined with more detailed genetic analyses of the circulating viruses. These approaches will provide insight into the role of viral genetic variability in reinfections. This is the first study from the Indian subcontinent on the use of RT-PCR on clinical samples for detection and typing of RSV. This is also the first report on genetic heterogeneity among group A and B RSV from the Indian subcontinent. In this study, we demonstrate that RT-PCR appears to be more sensitive than centrifugation-enhanced cultures for detection of RSV in clinical samples. Future studies of RSV epidemiology in health care settings and in the community will lay a foundation for efforts to define and ultimately reduce the burden of disease due to RSV in India.

ACKNOWLEDGMENTS

This work was supported through the Indo-U.S. Vaccine Action Program (grant no. BT/MB/VAP/(3/18/98) by the Department of Biotechnology, Govt. of India, and University Grants Commission, India, and by U.S. Public Health Service National Institutes of Health grant AI 50693.

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