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Discrimination of Respiratory Syncytial Virus Subgroups A and B by Reverse Transcription-PCR

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Reverse transcription (RT)-PCR with shared primers differentiating respiratory syncytial virus (RSV) subgroups A and B was developed for subtyping of RSV isolates. Results of RT-PCR were compared with those of an indirect immunofluorescence test using monoclonal antibodies. Viral RNA isolated from cell cultures infected with RSV served as a template for cDNA synthesis with random primers. For PCR, we used three synthetic oligonucleotides corresponding to the G protein mRNA sequence of subgroup A (bases 248 to 267; 3′ATGCAACAAGCCAGATCAAG), subgroup B (bases 314 to 333; 3′ACTCATCCAAACAACCCACA), or both (bases 511 to 530; 3′GGWACAAARTTGAACACTTC). PCR products of RSV subgroups A and B had molecular sizes of 283 and 217 bp, respectively. Specific cutting sites for RSV A and B in amplified cDNA were demonstrated by restriction fragment analysis with four restriction endonucleases. Our RT-PCR assay divided 68 RSV isolates into 47 strains of subgroup A and 21 strains of subgroup B in full agreement with subtyping by monoclonal antibodies. RT-PCR seems to be a good alternative to subtyping of RSV with monoclonal antibodies.

Respiratory syncytial virus (RSV) is an important respiratory pathogen of young children and infants (11). There may be a relationship between RSV infection and bronchial asthma (14). Originally, RSV was considered to be a monotypic virus, but cross-neutralization studies with hyperimmune sera of animals revealed antigenic differences (4). Two subgroups of RSV were established with monoclonal antibodies (1, 12). Subsequently, characterization of RSV isolates became an important tool in epidemiological investigations of RSV (8, 20). Epidemiologic investigations based on direct analysis of nucleotide sequences of RSV strains (2) or on restriction analysis of amplified cDNAs were published in recent years (2, 17). Currently, however, there exist few comparative data on results from the different methods mentioned above (7). A relationship between subgroups of RSV and different clinical pictures has been discussed in recent years (10, 19). More clinical investigations are needed to resolve this question.

RSV is a single-stranded RNA virus with a genomic molecular mass of about 5×10^6 Da. At least 10 separate mRNAs are transcribed during infection from the RNA of negative polarity (5, 6). cDNA synthesis with reverse transcription (RT) is a necessary step in a PCR assay for RSV. In this report, we describe the development of an RT-PCR assay for the discrimination of RSV subgroups A and B. Results of RT-PCR of 68 RSV strains were compared with previous results of subtyping with two monoclonal antibodies specific for RSV subgroup A or B.

MATERIALS AND METHODS

Cells and viruses. Sixty-eight isolates of RSV were grown in Vero cells in Dulbecco's modified Eagle medium with glucose (GIBCO/BRL, Life Technologies Inc., Gaithersburg, Md.) supplemented with penicillin (10^5 U/ml), streptomycin (100 μg/ml), gentamicin (20 μg/ml), and amphotericin B (0.25 μg/ml). The isolation procedure used and subtyping of strains with monoclonal antibodies have been described previously (20). Briefly, sonified respiratory specimens (October 1990 to June 1993) from infants and children of the pediatric wards of the Kantonsspital Aarau, Switzerland, and of the Kantonsspital Baden, Switzerland, were inoculated into Vero cell tubes. The tubes were observed for the typical cytopathic effect of RSV for at least 10 days. The RSV strains were frozen at −70°C. For characterization of subgroups A and B, two shell vials were inoculated with one RSV strain. After centrifugation at 1,000 × *g* for 30 min at room temperature and incubation at 37°C for 1 or 2 days, cells were washed with phosphate-buffered saline (PBS) and then fixed with acetone-methanol (1:1) for 10 min at room temperature. Indirect immunofluorescence of fixed cells was performed with anti-NP (nucleoprotein) group A-specific monoclonal antibody B90 (12) or anti-F (fusion protein) group B-specific monoclonal antibody 7858 (13) as the first antibody and an anti-mouse fluorescein isothiocyanate conjugate (Clonatec, Paris, France) as the second antibody (20). RSV-specific monoclonal antibodies were produced by the Department of Virology, Statens Bakteriologiska Laboratorium, Stockholm, Sweden.

Oligonucleotides. The sequences of the oligonucleotides used for PCR amplification were based on the published sequences of the G gene of subgroup A and B RSVs (9). The subgroup A-specific 5′ oligonucleotide RS-1 used for PCR amplification was previously described by Sullender and Wertz (18) and corresponds to bases 248 to 267 in the A2 RSV G protein mRNA (ATGCAA CAAGCCAGATCAAG). Subgroup B-specific 5′ oligonucleotide RS-2, chosen from the published sequence of the G protein mRNA of RSV strain 18537 (9), corresponds to bases 314 to 333 (ACTCATCCAAACAACCCACA). The 3′ oligonucleotide RS-3 was specific for both subgroups and was complementary to bases 511 to 530 in the G protein mRNA of the A2 strain with two wobbles, the first at position 522 with W = A and G and the second at position 528 with R = T and A, according to the two mismatches between strains A2 and 18537 in this genomic region (GGWACAAARTTGAACACTTC). Oligonucleotides were commercially synthesized by Microsynth, Windisch, Switzerland. The expected size of the PCR product for subgroup A strains was 283 bp, and that of the PCR product for subgroup B strains was 217 bp.

RNA extraction. Viral RNA was isolated as described by Chomczynski and Sacchi (3). In brief, 25-cm^2 cell culture dishes with RSV-infected Vero cells were washed once with PBS (pH 7.2). Cells were scraped away from the plates and transferred in 1 ml of PBS to a 1.5-ml polypropylene tube on ice. After centrifugation of the tube at 300 × *g* for 12 min at 4°C, the supernatant was removed and discarded. Cells were lysed by adding 500 μl of 4 M guanidinium thiocyanate–25 mM sodium citrate (pH 7)–0.5% sarcosyl and vortexing for 1 min. Phenol extraction was conducted with 500 μl of RNA-grade phenol (GIBCO/BRL) saturated with water and 100 μl of a chloroform-isoamyl alcohol mixture (49:1). The resulting aqueous phase was precipitated with ethanol for 1 h at −20°C and then centrifuged at 10,000 × *g* for 20 min at 4°C. After resuspension of the RNA pellet in 4 M guanidinium buffer as described above, RNA was reprecipitated with ethanol. Immediately before use, the RNA was centrifuged again and the pellet was washed with 1 ml of 70% ethanol, dried for 7 min at 65°C on a heating block, and resuspended at the same temperature in 50 μl of diethyl pyrocarbonate-treated water for 10 min. A 2-μl volume was used for first-strand

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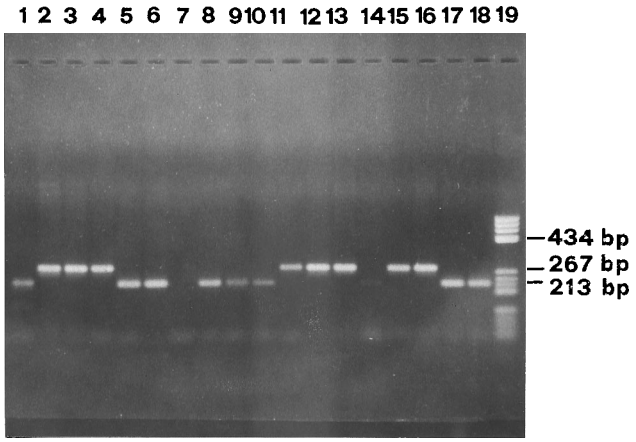

FIG. 1. Ethidium bromide-stained 1.5% agarose gel of RT-PCR-generated DNA products from RSV subtype A and B strains. Lanes: 1, 5, 6, 8 to 10, 14, 17, and 18, RT-PCR-generated products of RSV subgroup B; 2 to 4, 11 to 13, 15, and 16, RT-PCR-generated products of RSV subgroup A. Approximate sizes of standard DNA fragments (pUC19 *Hin*dIII digest) are indicated at the right of lane 19 in base pairs. Molecular sizes of RSV subtype A and B PCR products were 283 and 213 bp, respectively.

cDNA synthesis, and the remaining sample was reprecipitated with ethanol and stored at −70°C.

First-strand cDNA synthesis. cDNA for PCR was synthesized by RT in a volume of 20 μl with 2 μl of RNA extract, 200 U of RNase H-free reverse transcriptase (Superscript II; GIBCO/BRL), 20 U of RNase inhibitor (Boehringer Mannheim, Schweiz AG), 7×10^{-5} U of optical density at 260 nm of a random primer (GIBCO/BRL), 0.5 mM deoxynucleoside triphosphates (10 mM mixture of dATP, dCTP, dGTP, and dTTP; GIBCO/BRL), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol. The incubation temperature of the samples for RT was 37°C, and the reaction took 45 min. After the reaction was stopped at 95°C for 10 min, the samples were placed on ice until use for further amplification.

PCR. The PCR conditions used were largely those of Saiki (15). Amplifications were conducted in a volume of 50 μl containing 20 mM Tris-Cl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$, 500 μM each dATP, dGTP, dCTP, and dTTP (Boehringer Mannheim); primers RS-1, RS-2, and RS-3 each at 400 nM; 1.75 U of *Taq* polymerase (GIBCO/BRL); and 4 μl of sample. Samples were amplified on a Perkin-Elmer Cetus Thermocycler for the first cycle at 95°C for 5 min, 60°C for 1 min, and 72°C for 1 min and for 39 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. At the end of the last cycle, the temperature was held at 72°C for 7 min.

Analysis (16). Amplification products were analyzed by agarose gel electrophoresis. Ten microliters of product mixed with two microliters of loading buffer (5 × loading buffer is 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% [wt/vol] sucrose) was applied to a 1.5% agarose gel in 0.5× Tris-borate-EDTA buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA [pH 8.4]). The gel was subjected to electrophoresis at 80 V until the bromophenol blue dye marker had migrated 7 cm. Banding of the PCR products of RSV subtypes A and B was detected visually, and subgroup designations were assigned on the basis of DNA fragment size.

Digestion with restriction endonucleases and gel electrophoresis. Specificity of the PCR products was confirmed by restriction endonuclease digestion of eight selected strains. Restriction enzymes were chosen after analysis of both A and B subgroup RSV G gene nucleotide sequences (Recombinant Toolkit, Version 4.0; Biosoft, Cambridge, United Kingdom). For digestion of PCR products (0.2 μg of DNA in a volume of 20 μl), 10 U each of *Hin*fI and *Alu*I and 2 U each of *Mae*II (Boehringer) and *Apo*I (New England BioLabs) were used. Reaction time was 3 h both for *Hin*fI and *Alu*I at 37°C and for *Mae*II and *Apo*I at 50°C. Reaction buffers were delivered by the manufacturers and used in accordance with their recommendations. Gel electrophoresis of restricted DNA products was done as described above for unrestricted PCR products.

RESULTS

RT of extracted RSV RNA yielded cDNA suitable for PCR. PCR products corresponded to expected molecular sizes of 283 and 213 bp for RSV subtypes A and B, respectively. Differences in molecular size between PCR products from RSV subtypes A and B clearly distinguished the two viral subtypes in 1.5% agarose gels (Fig. 1). To prove the specificity of amplified DNA, PCR products of eight selected strains were cut with restriction enzymes *Hin*fI, *Alu*I, *Apo*I, and *Mae*II. On the basis of computer analysis of published RSV subtype A and B G protein nucleotide sequences, there were predicted cutting sites in PCR products of RSV subtype A for restriction enzymes *Hin*fI and *Alu*I but not in PCR products of RSV subtype B. Contrariwise, there were predicted cutting sites in PCR products of RSV subtype B for restriction enzymes *Apo*I and *Mae*II but not in PCR products of RSV subtype A. Banding patterns from PCR products of RSV subtype A showed three detectable restriction fragments after digestion with *Hin*fI and *Alu*I, respectively, and two restriction fragments

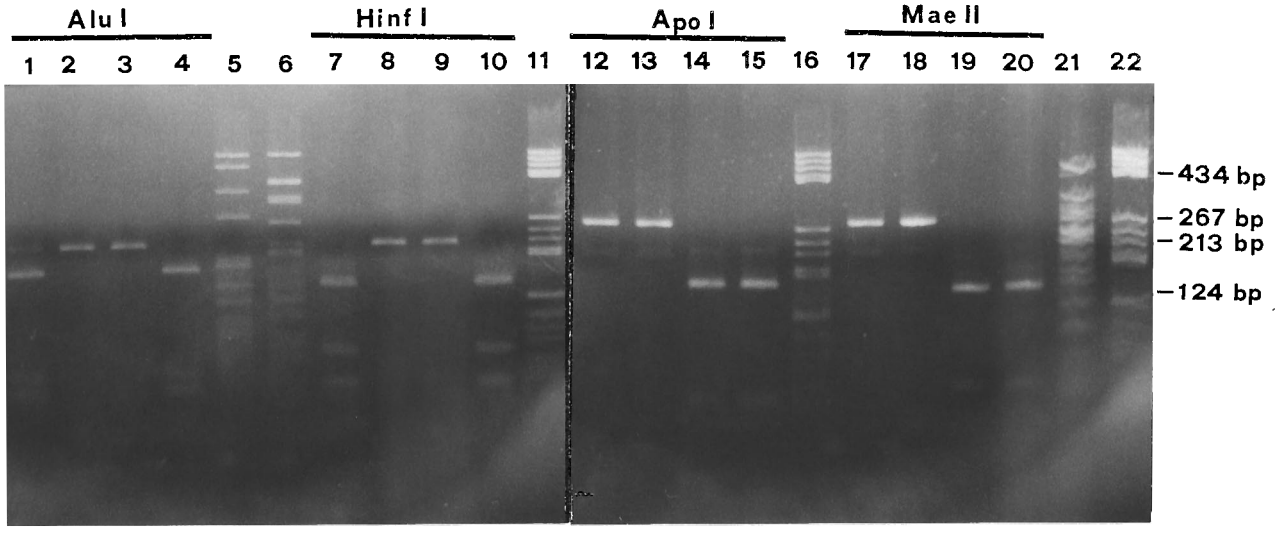

FIG. 2. Electrophoresis of RSV subtype A and B PCR products after digestion with *Alu*I, *Hin*fI, *Apo*I, and *Mae*II, respectively, in 1.5% agarose gels detected by staining with ethidium bromide. DNA in each section was digested with one restriction enzyme, as indicated at the top. Lanes: 1, 4, 7, 10, 12, 13, 17, and 18, digested PCR products of RSV subtype A; 2, 3, 8, 9, 14, 15, 19, and 20, digested PCR products of RSV subtype B; 11 and 22, pUC19 *Hin*dIII digests (Sigma) as a molecular weight marker; 5, 6, 16, and 21, molecular weight marker DNA digested with *Alu*I, *Hin*fI, *Apo*I, and *Mae*II, respectively (digestion control).

from RSV subtype B PCR products after digestion with *Apo*I and *Mae*II, respectively, in line with the predicted DNA restriction fragments (Fig. 2). Sixty-eight RSV isolates were analyzed by RT-PCR. All had been subtyped previously with monoclonal antibodies specific for RSV subtype A or B in an indirect immunofluorescence assay. Forty-seven isolates proved to belong to RSV subtype A, and 21 proved to belong to RSV subtype B in our RT-PCR. Results of RT-PCR and subtyping of RSV with monoclonal antibodies were fully concordant.

DISCUSSION

Molecular epidemiology of RSV is a steadily growing field of investigation. In this context, we decided to develop an RT-PCR for discrimination of RSV subtypes A and B which opens the further possibility of sequencing PCR products. Viral RNA was isolated from cell cultures infected with RSV, and cDNA was made by RT. Amplification of cDNA resulted in clearly distinguishable PCR products for RSV subtypes A and B, respectively. Molecular specificity of PCR products was proven by molecular weight and restriction fragment analyses. There was no difference in specificity between RSV subtyping obtained with monoclonal antibodies and those obtained with RT-PCR. Recent analysis of the RSV G protein nucleotide sequence revealed substantial genetic heterogeneity between strains of RSV subgroup A (7). Apparently this suspected genetic heterogeneity was not sufficient to disturb our RT-PCR, because all viral isolates which could be grown in cells also could be amplified by RT-PCR (results not shown).

Development of monoclonal antibodies is, for various reasons, not always possible in a routine laboratory with limited financial and laboratory resources. Commercial availability of monoclonal antibodies specific for RSV G protein is not certain either. This was an additional reason for our decision to develop an RT-PCR assay for subtyping of RSV. The workload for subtyping of RSV with RT-PCR is higher than that with immunofluorescence. This drawback is limited in epidemiological investigations, but it would be unacceptable in routine diagnosis with large numbers of viral strains. RT-PCR for determination of RSV subtypes A and B is therefore, in any case, a very good method for epidemiological investigations and is the method of choice in some situations.

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