Detection of Respiratory Syncytial Virus by RNA-Polymerase Chain Reaction and Differentiation of Subgroups With Oligonucleotide Probes


Department of Virology and WHO Influenza Centre, Erasmus University (A.J.v.M., M.J.W.S., N.M., E.C.J.C.), Department of Virology, Sophia Children’s University Hospital (Ph.H.R., N.M.), and Department of Virology, Dijkzigt University Hospital (Ph.H.R., A.H.B., N.M.), Rotterdam, The Netherlands

The polymerase chain reaction (RNA-PCR) was used for specific detection of respiratory syncytial virus (RSV) genomes in clinical specimens. A set of primers was selected from conserved regions of the 1B and N genes for detection of both subgroups. The primers were found to be RSV specific, all RSV strains generated a 218 bp product, and no RSV specific amplified product was obtained when nucleic acids from a variety of micro-organisms from the respiratory tract were subjected to the RNA-PCR. We took advantage of the sequence heterogeneity of the amplified products to discriminate between the A and B strains by hybridisation with subgroup specific oligonucleotide probes. This additional hybridisation assay increased the sensitivity of the RNA-PCR tenfold. The RNA-PCR was tested on clinical specimens from children with symptoms of an infection of the respiratory tract. The results were compared with isolation of RSV in cell culture and direct immunofluorescence. From 93 specimens tested, 31 were found positive by all three techniques. Six additional positive results were detected using RNA-PCR. From these 37 RSV positive specimens 33 (92%), including all 6 additional positives, were subgroup A and only 4 were subgroup B strains.

Thus, the RNA-PCR is a specific and sensitive technique for the detection and subgroup classification of RSV genomes. © 1994 Wiley-Liss, Inc.

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INTRODUCTION

Respiratory syncytial virus (RSV) is the major cause of severe infections of the lower respiratory tract in infants and young children and is an important pathogen in institutionalised elderly [Falsey et al., 1992]. Early detection of RSV infections is necessary for early intervention, e.g., initiation of antiviral treatment with ribavirin [Hall et al., 1985] and prevention of nosocomial spread. Virus isolation in cell culture is still considered the standard to which all other methods have to be compared [Welliver, 1988]. This technique is time consuming and requires viable virus in the clinical specimens. Rapid immunochemical techniques for direct detection of RSV are much faster, giving results the same day. However, their sensitivity is decreased as compared to the cell culture technique [Johnston and Siegel, 1990; Mendoza et al., 1992; Rothbarth et al., 1991]. Recently, nucleic acid amplification assays for RSV have been described [Cubie et al., 1992; Okamoto et al., 1992; Paton et al., 1992]. Although the sensitivity and specificity of the polymerase chain reaction (PCR) may offer great potential for the diagnostic laboratory, the RSV amplification assays described do not provide a sensitivity superior to cell culture. One of the reasons may be the lack of a confirmatory assay by oligonucleotide hybridisation, which also increases the sensitivity of a PCR assay [Claas et al., 1989].

Two RSV antigenic subgroups, A and B, have been recognised by their reaction with panels of monoclonal antibodies [Anderson et al., 1985; Mufson et al., 1985]. Based on differences in nucleic acid sequences between the two subgroups, nucleic acid hybridisation assays have been developed to differentiate between subgroup A and B RSV [Cubie et al., 1991; Sullender et al., 1990; Sullender and Wertz, 1991]. However, only a few sequences of RSV subgroups have been published.

Based on the nucleotide sequences of RSV subgroup A and B prototypes A2 and CH18537, respectively [Johnson and Collins, 1989], a primer set was selected to enable specific amplification of RSV RNA from both subgroups. After sequence analysis of these amplified products, probes were selected to differentiate the sub-
groups by oligonucleotide hybridisation. The specificity and sensitivity of the RNA-PCR were determined. Subgroup classification by hybridisation was tested on nasopharyngeal aspirates (NPAs) from children with symptoms of an infection of the respiratory tract. The results were compared to the results obtained by direct immunofluorescence (DIF) and isolation of the virus in cell culture.

**MATERIALS AND METHODS**

**Virus Strains and Clinical Specimens**

RSV strain A2 (ATCC VR-1302) and strain 9320 (ATCC VR-955), the latter previously identified as a subgroup B strain [Hierholzer and Hirsch, 1979], were used as prototype A and B strain, respectively.

Eighteen strains of RSV were randomly selected during three epidemic seasons: 01/89, 02/89, 03/89, and 01/90 from the 1989/1990 season; 02/90, 01/91, and 02/91 from 1990/1991; 03/91 to 12/91 and 01/92 from 1991/1992. These strains were used primarily for amplification and sequence analysis in order to select the subgroup specific probes.

NPAs supplemented with Dulbecco’s minimal essential medium (Flow Laboratories, Irvine, UK) were obtained from the Sophia Children’s Hospital, Rotterdam. These samples, originating from November and December 1991, were previously tested for the presence of RSV by centrifuged culture with subsequent immunofluorescence (IF) and DIF as described [Rothbarth et al., 1988] and subsequently stored at -70°C.

Bacterial isolates were obtained from the Department of Clinical Microbiology and the viral isolates from the Department of Virology of the University Hospital, Rotterdam. The isolates of rhinoviruses were kindly provided by Dr. A. Dingemans, Municipal Health Services, Rotterdam.

**Cell Culture of RSV**

Monolayers of Hep-2 cells were inoculated with 200 μl of the diluted RSV isolates by centrifugation at 840g for 1 hour. Hep-2 cells were cultured on 24-well flat-bottom plates (Tissue Culture Clusters; Costar, Cambridge, MA) in Dulbecco minimal essential medium containing 1% foetal bovine serum and antibiotics (Flow). The RSV strains A2 and 9320 were also propagated in Hep-2 cells.

The 50% tissue culture infective dose (TCID<sub>50</sub>) values of the A2- or 9320 strain infected Hep-2 cell lysates were calculated according to the method of Reed and Muench [1938]. A titration in eightfold was performed on Hep-2 cells and consisted of serial tenfold dilutions from 10<sup>−1</sup> to 10<sup>−6</sup> of the A2 or 9320 cell lysates. After 7 days of incubation the Hep-2 cells were screened for cytopathic effects (CPE).

**Monoclonal Antibodies**

Isolates were divided in subgroups by an IF assay with monoclonal antibodies [Anderson et al., 1985] on fixed slide preparations of cells from nasopharyngeal washings. The slides were prepared for diagnosis of RSV infection and stored at -70°C. The monoclonal antibodies used were 92-11C and 102-10B (Chemicon International Inc., Temecula, CA) and reacted with subgroup A or B strains, respectively.

**Preparation of Samples for RNA-PCR**

Total RNA was isolated from RSV infected Hep-2 cells by a modification of the method described by Chomczynski and Sacchi [1987]. Briefly, the cell culture monolayer was lysed by adding 150 μl/cm<sup>2</sup> of a guanidinium thiocyanate (GTC) solution containing 4 M GTC, 25 mM sodium citrate, pH 7, 0.5% sarscyol 0.1 M β-mercaptoethanol, and 10 μg poly-A. Total lysate was extracted with 0.3 ml phenol (ultra-pure) and 0.3 ml phenol/chloroform (phenol/chloroform/isoamylalcohol 25:24:1), gently shaking for 10 minutes, and centrifugation at 12,000g for 20 minutes at 4°C. Then the aqueous phase was extracted with phenol/chloroform 1:1 and with chloroform/isoamylalcohol 24:1. After precipitation in ethanol and washing the pellet, the RNA was resuspended in 10 mM Tris, pH; 1 mM EDTA (TE).

Extraction of RNA from the clinical specimens was carried out by adding 500 μl GTC solution to 100 μl of the sample. The rest of the procedure is as described above. The precipitated RNA was resuspended in 100 μl TE and 5 μl was used for the RNA-PCR. Every sixth sample was a negative control, which was carried through the whole procedure. In case of positive results in the negative controls, the experiment was repeated completely.

**RNA-PCR**

RNA-PCR was carried out in two steps. For cDNA synthesis the RNA template and the cDNA primer (Table I) were heated at 80°C for 2 minutes and put on ice. The reaction mixture for the cDNA reaction was added to a final volume of 25 μl, which contained extracted RNA, 50 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM (each) dATP, dCTP, dGTP,
and dTTP, 10 pmol cDNA primer, 1.6 U RNAsin (Promega, Leiden, The Netherlands), and 8 U Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Gibco-BRL, Breda, The Netherlands). This reaction mixture was incubated at 42°C for 45 minutes, heated to 95°C for 3 minutes, and put on ice. For the amplification reaction, addition of 75 µl of PCR reaction mixture made 100 µl of PCR solution containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM (each) dATP, dCTP, dGTP and dTTP, 20 pmol cDNA primer, 20 pmol reverse primer (Table I), and 1 U Taq DNA polymerase (Promega, Woerden, The Netherlands). This solution was overlaid with three drops of mineral oil, heated to 94°C for 4 minutes, and put on ice. Then 36 cycles of amplification were performed with a PCR processor (Biomed, Dittfurth, Germany). Each cycle consisted of denaturing the sample at 94°C for 1 minute, 1.5 minutes annealing of the primers at 52°C followed by an elongation period of 2 minutes at 72°C.

Analysis of the Amplified Products

The amplified products were analysed by electrophoresis of 20 µl aliquots on 2% agarose gels. For Southern blots the gel was denatured for 20 minutes in a solution containing 1.5 M NaCl and 0.5 M NaOH,
neutralised in 3 M NaAc, pH 4.8, for 20 minutes, and soaked in 10× SSC for 5 minutes. The amplified products were transferred to a nylon membrane (Hybond N+, Amersham Buckinghamshire, UK) by diffusion blotting in 10× SSC [Sambrook et al., 1989].

(Pre)hybridisation was performed for 2 hours at 37°C in a solution containing 2.5× SSC, 5× Denhardt (1× Denhardt: 0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyvinyl pyrrolidone), 0.5% sodium dodecyl sulphate (SDS), 75 mM EDTA, and 0.1 mg/ml denatured, sonicated herring sperm DNA.

The Southern blots were hybridised overnight by adding 10⁶ cpm/ml gamma-32P 5′-end labelled oligonucleotide probe. The next day the blots were washed twice for 15 minutes at 37°C in 2× SSC/0.1% SDS and for 15 minutes at 50°C in 2× SSC/0.1% SDS. For dot blot analysis 40 μl of the RNA-PCR product was denatured in 0.5 N NaOH and heated for 10 minutes at 65°C. The samples were put on ice for 5 minutes and before spotting on nylon membranes 30 μl of neutralising ammonium acetate, pH 5.6, was added. The filter was left to dry and hybridised as described for the Southern blots.

Dehybridisation was performed in 0.4 N NaOH at 45°C for 30 minutes followed by 30 minutes at 45°C in 0.2 M Tris/HCl, pH 7.5, 0.1× SSC and 0.1% SDS.

Sequence Analysis of PCR Products

Amplified products were purified by electrophoresis on a 1% GTG agarose gel (FMC BioProducts, Rockland, USA) onto DEAE nitrocellulose membranes (Schleicher and Schuell NA-45) as described [Sambrook et al., 1989].

For double-strand sequencing 0.5 μl NP-40, 0.5 μl Tween-20, and 1 μl DMSO was added [Bachmann et al., 1990] to 15–20 ng DNA and the total reaction volume
adjusted to 10 \mu l with distilled water. After addition of 2 \mu l of annealing buffer and 20 pmol primer the solution was heated at 95°C for 3 minutes and snap cooled in an ethanol/dry-ice bath. Sequence reactions were carried out using a T7 Sequencing kit (Pharmacia LKB Technology) according to the manufacturer's instructions. Finally, 2 \mu l of each reaction was loaded on a 6% polyacrylamide sequencing gel. After electrophoresis for 1.5 hours at 50 mA the gel was vacuum dried and autoradiography carried out on Fuji RX films.

RESULTS

Primer Selection

The cDNA primer and reverse primer (Table I) used in the RNA-PCR assay were selected from nucleic acid sequences conserved between subgroup A and B strains, based on sequence data of strains A2 and CH18537 [Johnson and Collins, 1989]. The primers flank a variable region which offers the possibility for selection of subtype specific probes. The cDNA primer for the A subtype contains one mismatch in comparison to the RNA-PCR assay, generated a DNA band of the predicted size of 218 bp (Fig. 1a).

Sequence Analysis and Probe Selection

To establish a well-considered probe selection, the sequence variation in the amplified products of RSV isolates was determined by double-strand sequence analysis. Eighteen isolates from 1989–1992 were subgrouped on basis of their sequence homology with strains A2 or 18537 (A and B strains, respectively). Fifteen were subgroup A strains and these could be divided in five groups (Fig. 2a) with no sequence variation within these groups: strains 04/91, 06/91, 07/91, and 08/91 belong to group 1; 05/91, 10/91, and 11/91 to group 2; 09/91, 01/89, and 02/90 to group 3; 02/89 and 03/91 to group 4; and 03/91, 11/91, and 01/92 to group 5. Three strains were subgroup B: 01/90, 01/91, and 02/91 (Fig. 2b). The sequences of the subgroup A had a minimum of 92% homology with A2 and the differences are conserved between the strains of three different seasons. The subgroup B strains showed at least 97% homology with the sequence of strain 18537 [Johnson and Collins, 1989].

Based on the variation in nucleotide sequences of the amplified products (Fig. 2), specific probes were selected for the subgroup classification in a hybridisation assay (Table I). Southern blot hybridisation of the amplified products with the subgroup A or subgroup B probes was shown to be specific as no cross-hybridisation was observed (Fig. 1b,c). This subgroup classification was identical to the subgroup classification based on reaction with monoclonal antibodies 92–11C and 102–10B [Anderson et al., 1985] which are directed to the F-protein of subgroup A and B isolates, respectively.

Specificity and Sensitivity

The specificity of the RNA-PCR was examined using nucleic acid isolates of a panel of viruses, bacteria, and fungi which can occur in the respiratory tract (Table II). No specific amplified products were detected by electrophoresis on agarose gel nor by hybridisation of the amplified products (data not shown).

The sensitivity of RNA-PCR was examined by analysing serial tenfold dilutions (10^{-1}–10^{-10}) of A2 or 9320 infected Hep-2 lysates with a virus titre of 10^{8.4} \text{TCID}_{50}/ml for A2 and 10^{4.2} \text{TCID}_{50}/ml for 9320. RSV specific amplified products were detected on agarose gel with the 10^{6}-fold dilution of the A2 lysate (Fig. 3) and the 10^{5}-fold dilution of the 9320 lysate (data not shown). The total amount of virus detected on gel was approximately 12 \text{TCID}_{50} for A2 and 4 \text{TCID}_{50} for 9320. After hybridisation with the specific probes the sensitivity was increased tenfold to 1.2 \text{TCID}_{50} and 0.4 \text{TCID}_{50}, respectively.

Clinical Specimens

RNA extracts from clinical specimens collected in the 1991/1992 winter were subjected to the RSV RNA-PCR, including oligonucleotide hybridisation (Table III).
These specimens were previously tested for presence of RSV by cell culture with subsequent IF as well as DIF. From the 93 specimens tested, 31 were positive by cell culture, DIF, and RNA-PCR. No discrepancies were observed between the results obtained by cell culture and DIF, but six additional positive samples were detected by RNA-PCR. Subgroup classification of the RNA-PCR positive samples showed 33 subgroup A (92%) and 4 subgroup B strains. The specimens which were negative for RSV in all three tests (n = 46) included three parainfluenza I, eight parainfluenza II, and four influenza A positive specimens as determined by cell culture.

**DISCUSSION**

In this study we used RNA-PCR for the detection of RSV genomes. Specific primers were selected in the highly conserved coding regions of the 1B and N genes from RSV [Johnson and Collins, 1989]. Using these primers, no RSV specific products were generated with DNA or RNA from isolates of a variety of other microorganisms which infect the respiratory tract. Therefore, a visible fragment of 218 bp on gel after RNA-PCR reflected the specific presence of RSV-RNA. The sensitivity of the RNA-PCR was found to be 0.4–1.2 TCID₅₀ after hybridisation. Oligonucleotide hybridisation does not only increase the sensitivity of the assay, in this study we found a tenfold increase over detection on agarose gel, and is also a confirmation of the specificity of the amplified products. In none of the RSV amplification assays previously described [Cubie et al., 1992; Okamoto et al., 1992; Paton et al., 1992], confirmation by hybridisation was carried out. However, the increase in specificity and sensitivity makes hybridisation an essential part of any PCR assay.

In addition, infections of RSV subgroup A and B viruses could be distinguished by differential hybridisation after amplification. The oligonucleotide probes were selected after sequence comparison of RSV field isolates from 1989 to 1992. The subgroup classification of the isolates by hybridisation was in accordance to their reactivity with monoclonal antibodies.

The sequences of 15 subgroup A isolates from three different epidemic periods could be divided into five groups, with no heterogeneity within these groups. Many of the differences in sequences compared to the A2 sequence are conserved in all groups of subgroup A, and may have accumulated through the years. Sequences of the RNA-PCR products of subgroup B strains were more conserved, although the number of
strains tested is limited. Diversity within the subgroups has also been observed in sequence comparisons of other fragments of the RSV genome or by reaction with monoclonal antibodies [Cane et al., 1991; Cane and Pringle, 1991; Hendry et al., 1986; Storch et al., 1991; Sullender et al., 1991]. Cane et al. [1991] showed that isolates can be divided into distinct lineages based on the 5'-end of the SH gene. The sequence results presented in our study are consistent with the finding that different strains of the same subgroup cocirculate within one epidemic season [Cane and Pringle, 1991; Hendry et al., 1986; Storch et al., 1991]. The clinical relevance for subgroup classification has not yet been established. Therefore, the RNA-PCR with subsequent subgroup hybridisation may have a broad applicability in epidemiological studies.

Heterogeneity of the target sequence can lead to false negative results in the RNA-PCR. Our primers were selected from well-conserved sequences so no false negative results were found. RNA-PCR assays for the detection of RSV infections have been described, using primers which were selected from the F or N gene region [Cubie et al., 1992; Okamoto et al., 1992; Paton et al., 1992]. These genes may be more heterogeneous than was found by sequence comparison of a single A and B strain. Therefore, to avoid false negative results it is of importance to select primers based on a relevant number of sequences from both subgroups.

The RSV RNA-PCR was tested on 93 clinical specimens from children with respiratory illness. From these specimens 31 were previously found positive for RSV in cell culture and DIF. Using RNA-PCR, all of these could be confirmed and an additional six positive results were detected. One of these additional positive specimens originated from a patient who generated a positive result for RSV in cell culture upon resampling 3 days later. Another specimen was from a patient who was positive for RSV 7 days earlier and one was from a patient who had been in contact with a patient positive for RSV. Therefore, a small amount of RSV target may have been present in these samples, which only could be detected by amplification. The appearance of a positive PCR before and after shedding of replicating virus is also described for infections with cytomegalovirus [Gerna et al., 1991].

From the 37 specimens positive for RSV by RNA-PCR 33 were found to be subgroup A viruses and 4 belonged to subgroup B. A dominance of one subgroup over the other in one epidemic period has been previously described and may vary geographically and between epidemic periods [Hendry et al., 1986].

The sensitivity and specificity of the RNA-PCR for the detection of RSV seem superior to cell culture and IF. However an elaborate clinical evaluation remains to be carried out to determine the applicability of the RNA-PCR for routine diagnostic procedures. A comparison will be made between isolation in cell culture and DIF, and RNA-PCR on all respiratory samples of the 1992–1993 season. A PCR for Mycoplasma pneumoniae has already been described [Bernet et al., 1989] and recently a RNA-PCR for influenza was developed in our lab [Claas et al., 1992]. By combining the pathogen specific primers it may be possible to examine one sample simultaneously for more respiratory tract pathogens.

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REFERENCES


Johnson PR, Collins PL (1989): The 1B (NS2), 1C (NS1) and N proteins of human respiratory syncytial virus (RSV) of antigenic subgroups A and B: Sequence conservation and divergence within RSV genomic RNA. Journal of General Virology 70:1539–1547.

Johnson SLG, Siegel CS (1990): Evaluation of direct immunofluorescence, enzyme immunoassay, centrifugation culture, and conven-
Subgroup Detection by RNA-PCR


