Analysis of Respiratory Syncytial Virus in Clinical Samples by Reverse Transcriptase-Polymerase Chain Reaction Restriction Mapping

Angel Valdivia[+], Clara Savón, Danay Chacón, Luis Sarmiento, Luis Morier, Anselmo Otero, Yudira Soto, Suset Oropesa, Angel Goyenechea

Instituto de Medicina Tropical "Pedro Kourí", Apartado 601, Marianao 13, La Habana, Cuba

The aim of this study was to develop a polymerase chain reaction (PCR) for the detection of respiratory syncytial virus (RSV) genomes. The primers were designed from published sequences and selected from conserved regions of the genome encoding for the N protein of subgroups A and B of RSV. PCR was applied to 20 specimens from children admitted to the respiratory ward of "William Soler" Pediatric Hospital in Havana City with a clinical diagnosis of bronchiolitis. The PCR was compared with viral isolation and with an indirect immunofluorescence technique that employs monoclonal antibodies of subgroups A and B. Of 20 nasopharyngeal exudates, 10 were found positive by the three assayed methods. In only two cases, samples that yielded positive RNA-PCR were found negative by indirect immunofluorescence and cell culture. Considering viral isolation as the "gold standard" technique, RNA-PCR had 100% sensitivity and 80% specificity. RNA-PCR is a specific and sensitive technique for the detection of the RSV genome. Technical advantages are discussed.

Key words: respiratory syncytial virus - polymerase chain reaction - restriction mapping - diagnosis

Respiratory syncytial virus (RSV) is well recognized as the single most important pathogen accounting for acute viral infection of the lower respiratory tract in infants and young children. Outbreaks of RSV infection usually occur during the winter and early spring (Spence & Barrat 1968, Kim et al. 1973, Mufson et al. 1973, Sung et al. 1987). Rapid detection of RSV is mandatory for early diagnosis, isolation measures, and, if necessary, antiviral therapy. Several rapid diagnostic methods, including the enzyme immunoassay and immunofluorescence, which rely on detection of the RSV antigen in respiratory secretions, have been increasingly used for that purpose (Smith et al. 1991). There are two subgroups, A and B, of RSV. These were originally defined serologically (Anderson et al. 1985, Mufson et al. 1985). The polymerase chain reaction (PCR) has been used for detection of many pathogens and the technique is very sensitive and specific. Many of its applications for the detection of microbiological pathogens have been described (Wright & Wynford-Thomas 1990, Stoker 1990), and although it has been shown that the technique can be used for clinical samples, there is lack of prospective clinical evaluations. Recently some groups have carried out reverse transcriptase PCR for the detection of RSV genomes (Paton et al. 1992, Cubie et al. 1992). In the present study, we developed a specific RNA-PCR for RSV, which may be used for diagnostic purposes. The specificity and sensitivity of selected primers were evaluated using the "Long" standard strain and other respiratory viruses.

MATERIALS AND METHODS

Viruses and cells - The "Long" strain of RSV and strains of parainfluenza 3, adenovirus 3, measles and influenza types A were obtained from the clinical virology laboratory of the Institute of Tropical Medicine "Pedro Kourí". HEp2 two cell line was grown in minimum essential medium (MEM) containing 10% fetal calf serum, 1% glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate.

Processing of clinical specimens - Twenty nasopharyngeal exudates were obtained from children admitted to the respiratory ward of "William Soler" Pediatric Hospital in Havana City. All samples were suspended in a final volume of 2 ml of MEM containing antibiotics. For PCR analysis, 500 μl were used in a 1.5 ml tube, and the samples stored at -70ºC until being tested.

Cell culture and indirect immunofluorescence were conducted according to previously reported procedures (Parrott et al. 1979). The diluted speci-

mens were centrifuged at 2,000 rpm for 10 min and processed as follows:

Viral isolation - Briefly, two glass tubes covered with monolayers of HEp-2 cells, were inoculated with 0.2 ml of supernatant from each sample. The supernatant from these samples was subcultured three times on HEp-2 cells, until a cytopathic effect (CPE) was observed.

Indirect immunofluorescence assay (IFA) - The infected cells displaying CPE were harvested by scraping each monolayer into 2 ml of MEM and the cells were collected by centrifugation at 2,000 rpm for 10 min at 4°C. The cells were washed twice with phosphate buffered saline (PBS), pH 7.4, resuspended in PBS to 200,000 cells/ml and spotted at 80 cells/250 x microscopic field on 12-well fluorescent antibody slides (Flow Laboratories, McLean, VA).

The slides were allowed to air dry, and the cells were fixed with ice-cold acetone for 10 min. Each slide was previously washed with PBS and drained. The cells were firstly incubated at 37°C with the monoclonal antibodies. After 60 min of incubation at 37°C, the slides were washed twice with PBS for 5 min each. The cells were then treated with fluorescein-conjugated goat anti mouse serum (BIOCEN, Cuba) for 60 min, at 37°C, washed as described above, mounted and viewed with a 25X oil immersion objective using a fluorescent microscope with epiillumination (Ernst Leitz, Wetzler, West Germany). The monoclonal antibodies were used at a dilution of 1:1000. These monoclonals have identified three types of epitopes in the G molecule (Garcia-Barreno et al. 1989, Garcia et al. 1994): (a) variable epitopes, (b) subgroup specific epitopes, and (c) conserved epitopes, shared by subgroups A and B. These monoclonals were 021/1G, 021/19G, 021/686G, 021/786G and they were provided by Professor JA Melero, Department of Molecular Biology, National Center of Microbiology, Virology and Sanitary Immunology, Madrid, Spain.

The plaque test assay - It was performed as described by Kisch and Johnson (1963). The nucleic acid extraction, cDNA (reaction) and PCR were performed as described by Cane and Pringle (1991, 1992).

Nucleic acid extraction - All small-scale RNA extractions were carried out in 1.5 ml microfuge tubes. Half a ml from nasopharyngeal exudate samples were centrifuged in a microfuge for 5 min. The cell pellet was resuspended in 0.5 ml of 3.5 M urea, 200 mM NaCl, 10 mM Tris-HCL pH 7.8, 5 mM EDTA, 0.75 mM MgCl2, 0.5% SDS and 0.35% NP40. Then 0.5 ml of chloroform-phenol (1:1) (buffered with 150 mM NaCl, 10 mM Tris-HCL PH 7.8, 1 mM EDTA) was added, the mix-

ture vortexed for about 5 sec and centrifuged for 10 min. The aqueous layer was extracted again with phenol-chloroform and then 1 ml of ethanol was added; the nucleic acids were precipitated at -20°C for 20 hr, pelleted, washed with 0.5 ml of 70% ethanol, vacuum-dried and resuspended in 20 µl of distilled water.

cDNA reaction - Preparation of cDNA was carried out with approximately 50 ng of RNA (spectrophotometrically quantified) in a 20 µl volume containing 100 ng of each primer. The target RNA was mixed with both primers and placed at 65°C. After 15 min, the solution was placed on ice and the following were added: 100 mM Tris-Hydrochloride, pH 8.3; 500 mM KCl; 25 mM MgCl2; 25 mM (each) dATP, dCTP, dGTP, and dTTP; 20 U RNA sin (Boehringer Mannhein GmbH, Germany) and 5 U of AMV reverse transcriptase (Boehringer Mannhein GmbH, Germany). Incubation was carried out at 42°C for 30 min. Finally, the reaction mixture was placed at 95°C for 5 min and kept on ice until the PCR was carried out. The cDNA reaction and the PCR were performed using the recommended strict protocol with all precautions to prevent contamination (Kwok & Higuchi 1989, Kitchin & Bootman 1993).

Polymerase chain reaction - PCR mixture was made up to a volume of 100 µl, containing 100 mM Tris-hydrochloride; pH: 8.3; 500 mM KCl; 25 mM MgCl2; distilled water and 2.5 U of Taq DNA polymerase (Boehringer Mannhein GmbH, Germany). Two drops of mineral oil were added to prevent evaporation. The amplification was carried out in 30 cycles in a Perkin-Elmer Cetus Thermal cycler. Each cycle consisted of denaturation at 93°C for 1.5 min, annealing of the primer at 55°C for 1.5 min, and chain elongation at 72°C for 1.5 min.

Controls - Distilled water, mixed buffer solutions, full-time open vial with final buffer mixture, a nasopharyngeal exudate from an asymptomatic individual and HEp-2 RNA were included as negative controls. RNA from a RSV reference strain ("Long") was prepared as positive control.

Analysis of the amplified products - After the last cycle of amplification, 10 µl of the amplified products were analyzed by electrophoresis on 2% agarose gels with Tris-borate buffer. The remaining of products were diluted with 100 µl H2O, extracted with 150 µl phenol-chloroform, and ethanol precipitated and then digested with PstI, HaeIII, HindIII, and Bgl II. These restriction enzymes were chosen with reference to the sequences of the N protein genes of subgroup A and B RS viruses (Johnson & Collins 1989). The correct interpretation and classification of the restriction patterns obtained was performed according

to previous reports (Cane & Pringle 1991, 1992). All negative samples were subjected to a second amplification reaction with 1 µl of the initial amplification reaction containing all the components described above.

Selection of the primers - The primers originally designed to amplify between nucleotides 858 and 1135 of the human RSV N gene (Collins et al. 1985) have been previously described (Cane & Pringle 1991), namely:

N1: 5'GGAACAAGTTGTTGAGGTTTATGAATATGC 3'
N2: 5'CTTCTGCTGTCAAGTCTAGTACACTGTAGT 3'

The oligonucleotides were prepared with an applied Biosystems 380B DNA synthesizer and purified by HPLC on reverse phase column.

Comparison of RNA-PCR sensitivity with the plaque test assay and IFI - The "Long" strain was cultured on HEp2 cells in flasks with a surface area of 25 cm^2, containing 5 ml of MEM, 5% fetal calf serum, 1% glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate. When extensive CPE was present, the cells were detached into the tissue culture medium by shaking with sterile glass beads, then 10-fold serial dilution of this strain were made. Half a ml of each was put in three different tubes. All tubes were centrifuged at 2,000 rpm. The cell pellet was tested in parallel by plaque test assay, immunofluorescence test and RNA-PCR. PCR products from this experiment were transferred to a nylon membrane (HybondN+, Amershan, Bucks, UK) in a Southern blot experiment (Maniatis et al. 1989) using the same primers as for amplification detection. This experiment was carried out as follows: the reaction was carried out at a final volume of 50 µl, containing: 100 ng of each primer, 1 µl of polynucleotide kinase enzyme (11 U/µl) (Boehringer Mannhein, GmbH, Germany), 5 µl of enzyme buffer (10X), and 50 UCi of Gamma p^{-32} ATP. The volume was completed to 50 µl with distilled water. The tube was placed at 37º C for 1 hr, and afterwards at 80ºC during 10 min in order to inactive the enzyme. The marked primers were hybridated with the PCR products transferred by nylon membrane. This was performed to evaluate the ability of the RNA-PCR method over Southern blot.

RESULTS

Identification of the isolated strains with monoclonal antibodies by IFA - All clinical specimens positive by cell culture were identified to subgroup A.

Specificity of the primer - The specificity of the primer was further tested on isolates of a variety of respiratory viruses. The extracted nucleic acid was subjected to RNA-PCR. Specific amplification of parainfluenza 3, adenovirus 3, measles and influenza types A were not yielded. We also included one nasopharyngeal exudate from asymptomatic patient (Fig. 1). Twenty clinical samples were tested by the RNA-PCR method, using the selected primers. The products were analyzed by electrophoresis on a 2% agarose gel. All positive clinical samples by RNA-PCR generated a specific amplified product of 277 bp. All positive clinical samples showed a restriction patterns NP4 that belonged to subgroup A thus, these results were similar to those obtained by the antigenic characterization using IFA with monoclonal antibodies.
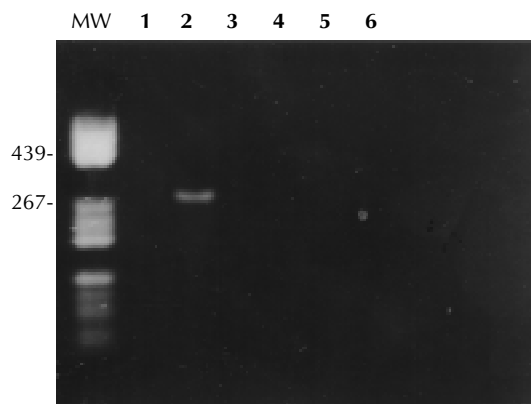

Fig. 1: specificity of the polymerase chain reaction for other respiratory viruses. Position of samples on the gel - 1: nasopharyngeal exudate from an asymptomatic patient. 2: "Long" strain. 3: adenovirus type 3. 4: measles. 5: influenza type A. 6: parainfluenza virus type 3. The molecular weight of some bands are indicated in base pairs.

Sensitivity - (a) RNA-PCR detected the RSV up to 10^{-4} dilution (Fig. 2a); (b) the plaque test assay detected RSV up to 10^{-3} ; (c) the immunofluorescence test detected RSV up to 10^{-3} dilution and (d) by the Southern blot analysis the same dilution (10^{-4}) was detected as by RNA-PCR (Fig. 2b).

Clinical specimens - The results obtained in the analysis of samples are shown in Table I . Viral isolation was positive in 10 samples; 10 samples were also positive by IFA, and 12 were positive by RNA-PCR. The three tests were in complete agreement in 18 samples, of which 10 were positive (50%) and 8 were negative (40%). Only 2 samples were discordant for the three tests employed. These samples were positive by RNA-PCR and negative by cell culture and the IFA test. The PCR products of these two samples were digested with HindIII and Bgl II and were shown to be specific for RSV (Fig. 3). RNA-PCR sensitivity was 100% and the specificity was 80% when compared to virus isolation (Table II).

a

MW 10^{-1} 10^{-2} 10^{-3} 10^{-4}

439-

267-

b
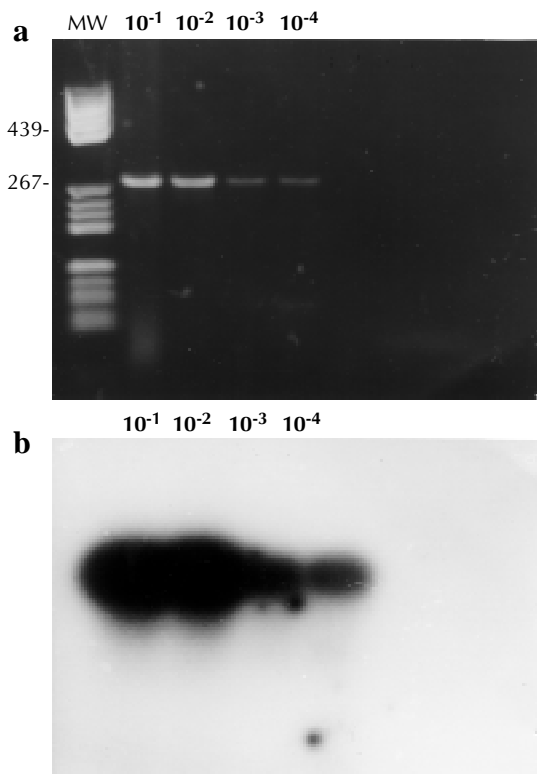
10^{-1} 10^{-2} 10^{-3} 10^{-4}

Fig. 2. a: sensitivity of detection of respiratory syncytial virus ("Long" strain) by polymerase chain reaction (PCR). Lane 1 to 4: PCR products from ten fold serial dilution of infected cells. b: gel was blotted to nylon membrane (Hybond-N-Amersham) and subsequently analyzed by Southern blot hybridization. The sizes of some PBR 322/Hae III's bands of interest are reflected in base pairs.
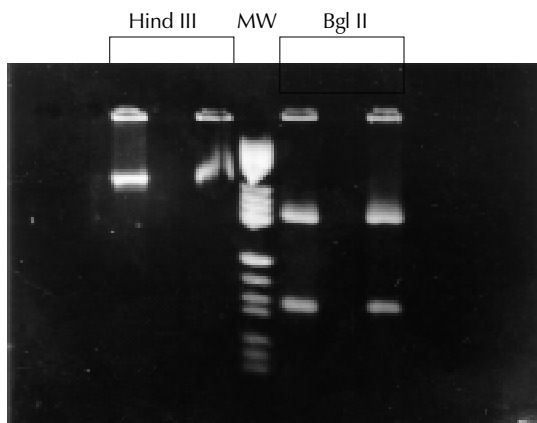
Hind III   MW   Bgl II

Fig. 3: restriction endonuclease digestion with Hind III (left) and Bgl II (right) of amplified products from the two positive samples by RNA-polymerase chain reaction and negative by immunofluorescence and cell culture. PBR 322/Hae III is also shown.

TABLE I

Detection of respiratory syncytial virus in clinical samples

Techniques	Samples	Positives
Viral isolation	20	10
Immunofluorescence	20	10
Polymerase chain reaction	20	12

TABLE II

Comparison of the results obtained by viral isolation, immnufluorescence and polymerase chain reaction

	Viral isolation				
	+	-		+	-
IFA +	10	0	PCR +	10	2
-	0	10	-	0	10

sensitivity: 100%   sensitivity: 100%
specificity: 100%   specificity: 80%
coincidence: 100%   coincidence: 90%

DISCUSSION

A set of primers, previously used for molecular epidemiology studies (Cane & Pringle 1991, 1992, Cane et al. 1992), was used for this method. In this work it was demonstrated that the sensitivity for detection of PCR products employing agarose gel electrophoresis with ethidium bromide was similar to the sensitivity obtained with the Southern blot technique. Therefore, we recommend that the Southern blot technique may be used only in cases in which it is necessary to confirm the diagnosis, this will avoid isotopic risk for a reliable diagnosis.

With respect to the identification of RSV, the restriction analysis of PCR products yielded results which were confirmed by the immunofluorescence detection by monoclonal antibodies.

The determination of the sensitivity was carried out by the plaque test. Those dilutions that did not form plaques, were positive by the RNA-PCR.

A good correlation of RNA-PCR with standard cell culture procedure and false positive results in RNA-PCR have been observed by different authors (Paton et al. 1992, Cubie et al. 1992). We found two samples that were positive by RNA-PCR but negative by the culture method, possibly reflecting the presence of some non infectious viral particles in such specimens. These samples were also negative by IFA and positive by RT-PCR. These could be explained because the RT-PCR have higher sensitivity than IFA, thus we consider that these two cases could be false negative results of the cell culture and IFA techniques.

These findings emphasize the recognized sensitivity of RNA-PCR over conventional methods.

Our study showed that RNA-PCR for the detection of RSV in clinical samples was sufficiently sensitive to allow direct visualization of the resolved PCR product on a 2% agarose gel without the need for nested amplification or Southern hybridization except to further confirm the identity of amplified products. The ease of the interpretation of PCR results and the ability to perform the test on archival samples are additional advantages. We also proved its feasibility for the diagnosis of RSV, considering that RNA-PCR will provide reliable results in a shorter period (1 *versus* 28 to 35 days) than the classical isolation procedures. This is of great relevance for monitoring children under specific therapy. If the technique is applied properly, sensitivity and specificity are the major advantages of the PCR for diagnostic purposes.

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