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Detection of Respiratory Syncytial Virus by Reverse Transcription-PCR and Hybridization with a DNA Enzyme Immunoassay

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Nasal aspirates from 238 infants hospitalized with acute respiratory infections during the winter of 1994 and 1995 were tested for respiratory syncytial virus (RSV) by immunofluorescence assay (IFA) and the viral isolation technique (VIT) and by two PCR and hybridization methods: reverse transcription PCR 1 (RT–PCR1), which amplifies the RNAs of all RSV strains, and RT–PCR-2, which allows subgroup classification of RSV. RT–PCR-1 and RT–PCR-2 detected viral sequences in 56.7% (135 of 238) and 48.3% (115 of 238) of the samples, respectively, while only 80 (33.6%) samples were found to be positive by IFA and VIT. Of the PCR-positive specimens, 57 were missed by these routine techniques in RT–PCR-1 and 45 were missed in RT–PCR-2. Although the RSV–PCR-1 and RSV–PCR-2 techniques amplified two different sequences of the RSV genome, they gave similar results for 218 (91.6%) nasal aspirates. Compared with conventional methods, the sensitivity, specificity, and agreement were 97.5, 63.9, and 75.2%, respectively, for RT–PCR-1 and 89.7, 71.9, and 77.7%, respectively, for RT–PCR-2, and for these two RT-PCR assays, the positive predictive value (PPV) and the index of agreement (κ) were comparable and moderate, respectively: PPV was 57.8% and κ was 0.52 in RT–PCR-1, and PPV was 60.9% and κ was 0.54 in RT–PCR-2. However, there was a perfect correlation between the two RT-PCRs, with a PPV of 100% and an excellent index of agreement (κ = 0.88). Therefore, most RT-PCR results were really true positive, and VIT and IFA, which missed some of them, appeared to be less sensitive.

As in other countries, winter outbreaks of respiratory syncytial virus (RSV) infections are the leading cause of serious acute lower respiratory viral disease in infants in France (4). Immunofluorescence assay (IFA) of viral antigens in nasal aspirates is largely used for the diagnosis of RSV infections because it is more rapid and sensitive than the virus isolation technique (VIT) (6, 12). The use of two techniques, IFA and VIT, can provide an increase in the proportion of positive results, but a significant number of specimens remain negative, in spite of clinical and epidemiological presumptions of RSV infection. Reverse transcription-PCR (RT-PCR) should be able to improve the sensitivity of RSV detection, but three recently described RSV amplification assays on nasal secretions provide a sensitivity equal or slightly superior to those of IFA and VIT (2, 11, 14). The purpose of the study described here was to compare the standard virus detection techniques, IFA and VIT, and two RT-PCRs with nonisotopic hybridization for the detection of RSV in nasal aspirates of children with acute respiratory tract infection.

During the period from November 1994 to January 1995 nasal aspirates from the upper or lower respiratory tract of infants hospitalized in the University Hospital of Caen with acute respiratory infection were studied. Most children were less than 1 year old. Viral diagnosis was made by IFA and VIT with nasal secretions. Nasal aspirates were collected by hospital staff nurses, transported to the virus laboratory, usually within 3 h, and resuspended in 5 ml of transport medium. Two milliliters was used for the fluorescent-antibody test. The cells were separated by centrifugation, washed in phosphate-buffered saline (PBS), deposited on microscope slides, and fixed in acetone. The test used a fluorescein isothiocyanate conjugate RSV monoclonal antibody reagent (IMAGEN RSV; Dako Diagnostics, United Kingdom). All incubations were carried out at 37°C for 30 min, and the slides were washed two times in PBS. For VIT, cultures of MRC-5 human embryonic lung fibroblasts in 25-cm^2 flasks and cultures of NCI-H292 human lung mucoepidermoid cells in 48-well tissue culture plates were inoculated with 0.2 and 0.1 ml of the resuspended specimens, respectively. In cultures exhibiting a typical RSV cytopathic effect, infected cells were harvested, pelleted by centrifugation, deposited on microscope slides, and fixed in acetone, and the virus was identified by IFA. MRC-5 cells were kept for 4 weeks before a culture was considered negative. NCI-H292 cells were incubated for 5 days, harvested by trypsinization, and stained by the IFA. Blind passages were not done routinely. For RT-PCR and hybridization, 500 μl of nasal aspirate in transport medium was extracted by the RNAzole B method (Bioprobe, France). The first RT-PCR hybridization assay (RT–PCR-1) used two primers previously defined in the human RSV N gene by Cane and Pringle (1): a cDNA primer at positions 1927 to 1956 (GGAA CAAGTTGTTGAGGTTTATGAATATGC) and a reverse primer at positions 2175 to 2204 (CTTGACTTTGCTAAGA GCCATCT). They amplify a fragment of 278 bp from subgroup A and B RSV strains. We selected a 5′-biotinylated probe (5′-GGCCTAGGCATAATGGGAGAGTACAGAG GTACACC-3′) at positions 2072 to 2106 from the sequence of the N gene (8). Primers and probe were synthesized by the Unit of Organic Chemistry, Institut Pasteur, Paris, France. RT was performed in a reaction mixture containing 5 μl of extracted RNA, 4 μl of 5× reverse transcriptase buffer (0.25 M Tris-HCl [pH 8.3], 0.05 M $MgCl_2$, 0.05 M dithiothrei-

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tol, 0.25 M KCl), 2 μl of deoxynucleoside triphosphates (2 mM each), 5.5 μl of DEPC-treated water, 10 U of RNasine (Promega Corp, Madison, Wis.), 2 μl of primer P1 at 10 μM, and 8 U of avian myeloblastosis virus reverse transcriptase (Promega) for 60 min at 42°C. The PCR technique was performed on 5 μl of the cDNA mixture by the addition of 24.5 μl of sterile water, 5 μl of 10× PCR buffer, 5 μl of deoxynucleoside triphosphates (2 mM each), 5 μl of primers P1 and P2 at 10 μM each, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The final mixture was overlaid with mineral oil, and the cDNA was amplified by using an Omnigene thermocycler (Hybaid): 30 cycles of heat denaturation at 93°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 30 s. The PCR product was detected by a DNA enzyme immunoassay based on the hybridization of amplified DNA with a single-stranded DNA probe coated on the wall of a microtiter plate with a streptavidin-biotin bond (GEN-ETI-K DEIA; Sorin). The optimal concentration of the probe required for the test was 0.1 ng/μl, and 20 μl of the denatured amplicon was dispensed into wells. This assay was performed as recommended by the manufacturer. The second RT-PCR hybridization assay (RT–PCR-2) was performed under the same conditions as the first one and used two primers described previously by van Milaan et al. (14): a cDNA primer in the 1B gene at positions 887 to 910 (ATTGGCATTAAGCCT ACAAAGCA) and a reverse primer at the start of the N gene at positions 1083 to 1106 (CTTGACTTTGCTAAGAGCCA TCT). We selected two 5′-biotinylated probes, at positions 961 to 1001, a probe for RSV subgroup A (ATTCACACAATCTA AAACAACAACTCTATGCATAACTATA), and a probe for RSV subgroup B (ACTAACCCATCCAAACTAAGCTATTC CTCAAACAACAGTG) from the sequence of the 1B gene (8). Primers and probe were synthesized by the Unit of Organic Chemistry, Institut Pasteur. RT and the PCR technique were performed under the same conditions used for RT–PCR-1, except that primer annealing was at 52°C for 30 s and the optimal concentration of the probes required for the test 0.5 ng/μl. For statistical analysis, the percent sensitivity, specificity, positive and negative predictive values, and agreement were calculated by standard formulas. The κ statistical method was used for an evaluation of the index of agreement between the three methods (9), and a scale of the variances of the κ estimates allowed us to determine the degree of concordance between them (3, 13).

Nasal aspirates collected from 238 infants hospitalized with acute respiratory tract infection from November 1994 to January 1995 were tested simultaneously by the routine techniques IFA and VIT and by two distinct PCR and hybridization methods: RT–PCR-1, which enables amplification of RNAs of all RSV strains, and RT–PCR-2, which allows subgroup classification of RSV genomes. The sensitivities of the two RT-PCRs were examined by analyzing serial 10-fold dilutions of RSV A (Long)-infected MRC5 lysates with a virus titer of $10^{5.5}$ 50% tissue culture infective doses ($TCID_{50}$s) per ml. RSV-amplified products were detected on agarose gels with the $10^{-3.5}$-fold dilution and by hybridization at the $10^{-4.5}$-fold dilution by both RT–PCR-1 and RT–PCR-2 (data not shown). The amount of viral RNA detected by the RT-PCRs was approximately 10 $TCID_{50}$s. The results of the comparison of the two RSV PCR assays with IFA and VIT with nasal aspirates are summarized in Tables 1 and 2. RT–PCR-1 and RT–PCR-2 detected viral sequences in 135 (56.7%) and 115 (48.3%) of the 238 samples tested, respectively, while only 80 (33.6%) samples were found to be positive by IFA and VIT. Of the 135 RT–PCR-1-positive specimens, 78 were IFA and/or VIT positive and 57 were missed by those routine techniques. Of the 115 RT–PCR-2-positive specimens, 70 were IFA and/or VIT positive and 45 were negative by those routine techniques. From the comparisons between IFA-VIT and the two RT-PCR assays, overall similar results were obtained for 179 (75.2%) and 185 (77.7%) samples, respectively. RSV was detected by IFA and VIT only in two and eight samples, respectively. Although the RT–PCR-1 and RT–PCR-2 techniques amplified two different sequences of the RSV genome, they gave similar results for 218 (91.6%) nasal aspirates, which could prove the validity of these new tests for the detection of RSV infections in nasal aspirates of infants. If we consider the association between the two conventional methods, with VIT and IFA as the "gold standards," both RT–PCR-1 and RT–PCR-2 are reliable methods for the diagnosis of RSV infections, because the sensitivity, specificity, and agreement were 97.5, 63.9, and 75.2%, respectively, for RT–PCR-1 and 89.7, 71.9, and 77.7%, respectively, for RT–PCR-2. For the two RT-PCR assays, the positive predictive value (PPV) and the index of agreement (κ) were comparable and moderate, respectively: a PPV of 57.8% and a κ_1 of 0.52 for RT–PCR-1, and a PPV of 60.9% and a κ_2 of 0.54 for RT–PCR-2. However, because the comparison of the two RT-PCR techniques showed a perfect correlation between them, with a total PPV of 100% and an excellent index of agreement ($\kappa_3 = 0.88$), and because the statistical analysis of the index of agreement indicated that κ_1 and κ_2 were identical, we might consider that the results of RT–PCR-1 and RT–PCR-2 were true positives.

TABLE 1. Comparison of IFA-VIT, RT–PCR-1, and RT–PCR-2 for diagnosis of RSV infections in nasal aspirates of 238 infants

Possible result	No. of specimens with the indicated results		
	PCR-1/IFA-VIT	PCR-2/IFA-VIT	PCR-2/PCR-1
+/+	78	70	115
+/−	57	45	0
−/−	101	115	103
−/+	2	8	20

Oligonucleotide hybridization is a confirmation of the specificities of the amplified products, but it can also increase the sensitivity of the PCR assay over that by detection on agarose gels. Using target sequences for amplification and hybridization close to the end of the 1B gene and the start of the N gene, van Milaan et al. (14) found a 10-fold increase in sensitivity over that by detection on agarose gels and that the sensitivity of the RT-PCR was 0.4 to 1.2 $TCID_{50}$s after hybridization. Using the same primers but two different probes with nonisotopic hybridization, we observed a slightly lower sensitivity, but the same 10-fold increase in the sensitivity of hybridization over that of electrophoresis. They also showed that detection of RSV by RT-PCR was superior to that by VIT and IFA, with 39.7% of their clinical specimens testing positive by RT-PCR and 33.3% testing positive by the conventional techniques. In two previously described RT-PCR assays in which confirmation by oligonucleotide hybridization was not carried out, the sensitivity of RSV detection by RT-PCR was not increased over detection by IFA and VIT (2, 11).

One of the previously described RT-PCRs was a nested PCR with detection of the amplicon by agarose gel electrophoresis (2). Using the same outer primers defined by Cane and Pringle (1) (primers P1 and P2) and hybridization with an internal biotinylated probe selected in a highly conserved region of the end of the N gene, we confirmed that in RT–PCR-1, hybridization increases the level of detection of RSV genes 10 times over that by agarose gel electrophoresis for RSV-infected cell

TABLE 2. Sensitivity, specificity, positive and negative predictive values, and agreement of IFA-VIT, RT–PCR-1, and RT–PCR-2 in nasal aspirates of 238 infants

Test	Sensitivity (%)[a]	Specificity (%)[a]	Positive predictive value (%)[a]	Negative predictive value (%)[a]	Agreement (%)[a]	κ index[b]
RT–PCR-1/IFA-VIT	97.5 (78/80)[a]	63.9 (101/158)	57.8 (78/135)	98 (101/103)	75.2 (179/238)	0.52 (0.10)
RT–PCR-2/IFA-VIT	89.7 (70/78)	71.9 (115/160)	60.9 (70/115)	93.5 (115/123)	77.7 (185/238)	0.54 (0.10)
RT–PCR-2/RT–PCR-1	85.2 (115/135)	100 (103/103)	100 (115/115)	83.7 (103/123)	91.6 (218/238)	0.88 (0.04)

[a] Values in parentheses are number of specimens with the indicated result/total number of specimens.
[b] Values in parentheses are ±2 standard deviations.

lysates. The RT–PCR-1 assay was also attempted on 238 nasal aspirates from infants hospitalized with respiratory infection, and we noticed that the sensitivity of the RT–PCR-1 was slightly superior to those already found in other studies for RSV RNA detection in nasal aspirates, because 80 (33.6%) specimens were found to be positive by IFA and/or VIT and 135 (56.7%) were found to be positive by the PCR test. RT–PCR-1 failed to detect 2 of 80 IFA- and VIT-positive specimens. These false-negative results could be due to several factors. The presence in nasal aspirates of inhibitors of the enzymatic steps, RT and PCR, cannot be excluded. Unfortunately, we did not have the opportunity to use an internal standard which would have enabled confirmation of the presence of such inhibitors. The sequences of the primers and the probe are unlikely to be variable, because Johnson and Collins (8) have shown that the N gene is the most highly conserved of the five major genes of RSV and that there is a high level of nucleotide sequence identity among subgroups A and B of RSV and intermediate strains. This indicates that N would be the gene of choice for use as a primer or a probe for detecting RSV RNAs and could explain the lowest degrees of sensitivity of those techniques which amplify sequences of other genes: F or 1B, for example (11, 14). Moreover, RT–PCR-1 detected RSV in 57 of 158 IFA- and VIT-negative specimens. They could have been false-positive RT-PCR results because of cross-contamination of the PCR with DNA. The prevention of contamination was rigidly addressed as recommended previously (10) by performing specimen extractions and preparing cDNA and PCR products in separate laboratories. The RT-PCR assay was not performed in duplicate, because it is now used in diagnostic practice, and we do not use isopsoralen or uracil *N*-glycosylase to control for carryover contamination in the PCR test. These two precautions should be necessary if these techniques are to be used for routine diagnostic purposes to improve the specificity of the test. Nevertheless, we applied a second PCR technique, the RT–PCR-2, to the same respiratory samples. RT–PCR-2 amplified a distinctive sequence of the RSV genome. In most cases (79%), RSV sequences were detected by the two PCR techniques, suggesting that the majority of the specimens isolated by RT–PCR-1 could be also linked to false-negative results by VIT and IFA. IFA, a rapid immunological technique, is widely used because it is the most cost-effective and rapid technique available for the diagnosis of RSV infection. Its sensitivity compared with that of culture has been reported to be 45 to 98% (12). However, the use of VIT in conjunction with IFA provides an increase in the proportion of positive results, consequently diminishing the proportion of false-negative results (5). Therefore, we used two cell lines for culturing RSV MRC5 cells and the new NCI-H292 cell line, which is sensitive for RSV detection, in addition to parainfluenza and mumps viruses, as initially reported (7). Because the sensitivity threshold of RT–PCR-1 is a little lower than that of titration in culture, the false-negative results obtained by IFA and VIT could not be due to the fact that some nasal aspirates contained so few viral particles that the number present was below the sensitivity of the RT-PCR.

Because our data demonstrated that the RT–PCR-1 assay detected more samples with positive results for the diagnosis of RSV infection (56.7%) than the conventional methods (33.6%), in the present investigation also we attempted to determine the statistical concordance of the assays with each other and to assess the reliability of the observed agreement between the methods by determination of the κ index. The index of agreement was moderate between IFA and VIT and each of the two RT-PCR assays and was almost perfect between RT–PCR-1 and RT–PCR-2, demonstrating in one other way that the results of RT-PCR were true positive and that the discrepancies between the conventional methods and the PCR assays reflected positive results which were missed by VIT and IFA.

Antigen detection with commercially available fluorescein-labelled monoclonal antibodies is undoubtedly the most cost-effective and rapid technique available for the diagnosis of RSV, but the clear outperformance of IFA and VIT by the two RT-PCR assays used in the present study was unexpected. The children who entered the study were selected on the basis of the presence of respiratory symptoms, and not all of them had an acute lower respiratory tract disease, for example, bronchiolitis. That could be one likely explanation for the low degree of sensitivity of the conventional diagnostic techniques if small amounts of RSV are frequently yielded in patients with respiratory infections but minimal clinical symptoms. On the other hand, however, we observed a lower degree of sensitivity of the RT-PCR assays over titration in infected cell lysates. The clinical relevance of isolated RT-PCR-positive results has not yet been established, and RT-PCR techniques have not been used in large epidemiological studies that include control groups. Therefore, a complete clinical and virological evaluation must be carried out in order to indicate the significance of the presence of RSV RNA sequences in nasal aspirates of infants with and without respiratory symptoms or aspirates from the upper and lower respiratory tract and to indicate if a sensitive RT-PCR assay might one day replace IFA for the routine diagnosis of RSV infections.

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