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Detection of Human Respiratory Syncytial Virus in Respiratory Samples by LightCycler Reverse Transcriptase PCR

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Laboratory diagnosis of human respiratory syncytial virus (hRSV) infections has traditionally been performed by virus isolation in cell culture and the direct fluorescent-antibody assay (DFA). Reverse transcriptase PCR (RT-PCR) is now recognized as a sensitive and specific alternative for detection of hRSV in respiratory samples. Using the LightCycler instrument, we developed a rapid RT-PCR assay for the detection of hRSV (the LC-RT-PCR) with a pair of hybridization probes that target the hRSV L gene. In the present study, 190 nasopharyngeal aspirate samples from patients with clinically recognized respiratory tract infections were examined for hRSV. The results were then compared to the results obtained with a testing algorithm that combined DFA and a culture-augmented DFA (CA-DFA) assay developed in our laboratory. hRSV was detected in 77 (41%) specimens by LC-RT-PCR and in 75 (39%) specimens by the combination of DFA and CA-DFA. All specimens that were positive by the DFA and CA-DFA testing algorithm were positive by the LC-RT-PCR. The presence of hRSV RNA in the two additional LC-RT-PCR-positive specimens was confirmed by a conventional RT-PCR method that targets the hRSV N gene. The sensitivity of LC-RT-PCR was 50 PFU/ml; and this, together with its high specificity and rapid turnaround time, makes the LC-RT-PCR suitable for the detection of hRSV in clinical specimens.

Human respiratory syncytial virus (hRSV) is one of the major causes of viral respiratory tract disease in young children and infants (23). Annually, it causes an estimated 1 million deaths worldwide, primarily in children younger than 5 years of age (9). Furthermore, hRSV remains the most common cause of bronchiolitis in infants, with up to 10% of those infected requiring specialized pediatric care (20, 26). Among infants in the United States it is estimated that hRSV is responsible for 90,000 hospitalizations for bronchiolitis and US$2.25 billion in health care costs each year (24, 25). In adults hRSV infection usually results in a milder form of disease and often remains undiagnosed (12). However, in immunocompromised and elderly patients, hRSV infection can lead to more serious clinical manifestations (4, 6). Virus isolation by cell culture and direct fluorescent-antibody assay (DFA) staining with monoclonal antibodies are the most commonly used laboratory techniques for the detection of hRSV infection, yet both of these methods have significant limitations. Compared to cell culture and reverse transcriptase PCR (RT-PCR), DFA lacks sensitivity, is subjective, and is dependent on the quality and presence of appropriately infected cells (30). Although cell culture is more sensitive than DFA, it requires specimens to be transported and stored under ideal conditions, and the prolonged turnaround time required to obtain results further diminishes its usefulness in patient management (22).

In recent years RT-PCR has proved to be a highly sensitive and specific method for the diagnosis of hRSV infections (1, 7, 18, 19). Unlike DFA and cell culture, the results of RT-PCR are not significantly affected by the loss of viability of the virus during specimen transport or storage and RT-PCR does do not require the presence of intact, infected cells within the specimen. RT-PCR is therefore ideally suited for the detection of hRSV. However, although clinical laboratories have shown an increasing tendency to adopt PCR protocols as part of their testing algorithms, the routine implementation of conventional PCR technology also has limitations. These include the potential for PCR product carryover contamination and technically cumbersome PCR product detection methods (5, 29). Some of these limitations are overcome by real-time PCR instrumentation, which is the latest advance in nucleic acid amplification and detection technology.

In this study we developed a real-time RT-PCR assay for the detection of hRSV using the LightCycler instrument (Roche Diagnostics, Sydney, Australia). This technology applies fluorescence resonance energy transfer (FRET) to two fluorophore-labeled hybridization probes during the continuous monitoring of amplicon development in the amplification process (31). Using the LightCycler RT-PCR assay (the LC-RT-PCR), we examined 190 nasopharyngeal aspirate (NPA) specimens from patients with respiratory infections for the presence of hRSV. The data obtained by this assay were compared with the results obtained by a conventional PCR and a testing algorithm that combined DFA and culture-augmented DFA (CA-DFA).

MATERIALS AND METHODS

Patient specimens. NPA specimens (n = 190) from patients with respiratory disease that were submitted to our hospital for the detection of respiratory

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TABLE 1. Primers and probes used in this study

Designation	Sequence (5′ to 3′)	Target gene	Position[a]	Reference
Primers				
RSV-LCs	TCTTCATCACCATACTTTTCTGTTA	L	12647–12623	This study
RSV-LCas	GCCAAAAAATTGTTTCCACAATA	L	12478–12500	This study
RSVN3	GGGAGAGGTGGCTCCAGAATACAGGC	N		19
RSVN5	AGCATCACTTGCCCTGAACCATAGGC	N		19
Probes				
RSV-A	Biotin-TTACAAAGGCTTACTACCCAAGGAC	N		19
RSV-B	Biotin-CTACAAGGGCCTCATACCAAAGGAT	N		19
RSV-LC1	GTTGTTCTATAAGCTGGTATTGATGCA-fluoroscein	L	12584–12558	This study
RSV-LC2	LC-Red640-GGAATTCACATGGTCTACTACTGACTGT-phosphate	L	12556–12529	This study

[a] Position refers to the specific nucleotide locations of the primers and probes in the gene coding for the L protein of hRSV (GenBank accession number AF013254).

viruses by DFA and CA-DFA were used in this study. Of these, 82 (43%) were from hospitalized subjects and 108 (57%) were from outpatients. The majority of specimens (n = 180; 95%) were from children younger than 12 years of age (average age, 3.7 years).

Viral RNA was extracted from 0.2 ml of each specimen by using the High Pure Viral Nucleic Acid kit (Roche Diagnostics), according to the instructions of the manufacturer. Purified specimen RNA was eluted from the column in 50 μl of elution buffer (Roche Diagnostics) and stored at −70°C until analysis. RNA extracts were used in both the LC-RT-PCR and the conventional RT-PCR, which were performed with the investigators blinded to the DFA and CA-DFA results.

LC-RT-PCR. The LightCycler instrument provides a platform for the amplification of target nucleic acid and monitors the development of amplification reaction products by fluorescence after the annealing step in each cycle. This technique is based on the FRET principle and was performed with two target-specific hybridization probes that bind to adjacent positions on the target DNA. The upstream oligonucleotide probe (probe RSV-LC1) was labeled with a donor fluorophore, fluorescein, at the 3′ terminus; and the downstream oligonucleotide probe (probe RSV-LC2) was labeled with an acceptor fluorophore, LC-Red640, at the 5′ terminus (TIB-MOLBIOL, Berlin, Germany). Probe RSV-LC2 was also phosphorylated at the 3′ terminus to prevent extension by *Taq* DNA polymerase during the amplification reaction (Table 1). The primers used in the LC-RT-PCR (primers RSV-LCs and RSV-LCas; Table 1) targeted a sequence of the L gene that was conserved across hRSV types A and B and that produced a 170-bp product during the reaction.

The LightCycler RNA amplification kit for hybridization probes (Roche Diagnostics) was used as the basis for the reaction mixture in the LC-RT-PCR, with a 20-μl volume used in each reaction capillary. Briefly, the capillaries were loaded with 0.4 μl of the LC-RT-PCR enzyme mix (reagent 1; Roche Diagnostics), 4.0 μl of the LC-RT-PCR mix hybridization probes (reagent 2; Roche Diagnostics), 2.4 μl of $MgCl_2$ stock solution (25 mM; reagent 3; Roche Diagnostics), 4.0 pmol of primer RSV-LCs, 8.0 pmol of primer RSV-LCas, 4.0 pmol of probe RSV-LC1, 4.0 pmol of probe RSV-LC2, and 2 μl of target RNA extract. Each reaction volume was made up to 20 μl with sterile PCR-grade water (reagent 4; Roche Diagnostics). Every test run included a positive control and three no-target controls consisting of 18 μl of the reaction mixture with 2 μl of unrelated RNA. Reaction capillaries were capped, centrifuged, and placed into the LightCycler carousel. Amplification and detection of amplified DNA were performed by using the following parameters: an initial 25 min of incubation at 55°C for reverse transcription, followed by a denaturation step at 95°C for 30 s. This was followed by 55 cycles of denaturation at 95°C for 5 s, primer and probe annealing at 55°C for 15 s, and extension at 72°C for 25 s. The fluorescence response data were obtained during the annealing period and displayed with the channel setting F2/F1. The fluorescence gain settings were set to 1 for channel F1 and 15 for channel F2. Channel F3 was not used in the analysis of the sample data.

LightCycler melting curve analysis. The LightCycler hybridization probes were designed to be homologous to a highly conserved region of the L gene of hRSV types A and B. This homology could be confirmed by fluorescent melting curve analysis, which was performed with LightCycler software following the completion of LC-PCR amplification. Briefly, the fluorescence was continuously monitored as the capillaries were heated at a rate of 0.3°C/s, with the heating started at 55°C and going to a final temperature of 95°C. The hybridization probes were designed to dissociate from the amplification product of either hRSV type at a temperature of approximately 62°C, which was calculated by using Primer Premier (version 5.0; Premier Biosoft International, Palo Alto, Calif.). If mutated amplification products were present, they would show a reduction in melting temperature of more than 1°C.

Specificity of LC-RT-PCR. A panel of respiratory viruses and bacteria commonly found in the respiratory tract was used to determine the specificity of the LC-RT-PCR. Genomic nucleic acid was purified from cultures of influenza virus types A and B; adenovirus type 2; parainfluenza virus types 1, 2, and 3; *Bordetella bronchiseptica*; *Bordetella parapertussis*; *Bordetella pertussis*; *Burkholderia cepacia*; *Chlamydia pneumoniae*; *Haemophilus influenzae*; *Klebsiella pneumoniae*; *Legionella pneumophila*; *Mycoplasma pneumoniae*; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; and *Streptococcus pneumoniae* and tested by the LC-RT-PCR under the conditions described above.

Sensitivity of LC-RT-PCR. The limit of sensitivity of the LC-RT-PCR was determined by testing dilutions of an hRSV culture representing a range of viral titers. The number of PFU per milliliter in a culture of an hRSV type A isolate was determined by standard laboratory methods (3, 14, 27). Serial 10-fold dilutions of this isolate ranging from 5×10^6 to 5×10^{-1} PFU/ml were made. Viral RNA was extracted from 0.2 ml of each dilution and tested by the LC-RT-PCR under the conditions described above. The detection limit of the assay was determined as the highest dilution that returned a positive reaction.

DFA. DFA was performed by standard laboratory methods (16, 21, 22). Briefly, substrate slides were prepared from epithelial cells contained within the NPA specimen, dried, and fixed in acetone for 10 min. Each well was incubated with 30 μl of each monoclonal antibody to the seven common respiratory viruses (hRSV; influenza virus types A and B; parainfluenza virus types 1, 2, and 3; and adenovirus) and stained with anti-mouse fluorescein isothiocyanate conjugate (Intracel Corporation, Frederick, Md.). The cells in each well were examined for fluorescence with an epifluorescence microscope (Olympus BX60; Olympus Optical Co. Ltd., Tokyo, Japan) at a magnification of ×400.

CA-DFA. CA-DFA was developed in our laboratory as a modification of the standard shell vial technique (11; M. W. Syrmis et al., submitted for publication) with 96-well microtitration plates (Nunc, Roskilde, Denmark). Four continuous cell lines, MRC-5, A549, HEP-2, and LLC-MK2 (Biowhittaker, Walkersville, Md.), which are permissive to infection with respiratory viruses, including hRSV, were cultured in wells of the microtitration plates. Eighty microliters of each NPA specimen was centrifuged onto the cell monolayers at 4,000 rpm for 30 min at 35°C by using a Hettich Rotanta 96R microtitration plate centrifuge (Hettich Zentrifugen, Tuttingen, Germany). The supernatants were aspirated from each well and replaced with 200 μl of maintenance medium (Eagle's minimum essential medium; Biowhittaker) containing 2% (vol/vol) fetal bovine serum (Trace Scientific, Noble Park, Australia), 9.75 U of penicillin G (5,000 U/ml; CSL Biosciences, Parkville, Australia), 10 μg of streptomycin sulfate (5000 μg/ml; CSL Biosciences), and 0.25 μg of amphotericin B (5 mg/ml; Apothecon, Princeton, N.J.). After 40 h of incubation at 37°C in a 5% CO_2 atmosphere, the wells were aspirated and the cell monolayers were fixed with an acetone-methanol mixture (1:1; vol/vol) for 10 min at −20°C. The cell monolayers in the wells were dried and overlaid with 30 μl of appropriate monoclonal antibody (Intracel Corporation) specific for the target respiratory viruses as described above for the DFA method. After incubation with anti-mouse fluorescein isothiocyanate conjugate (Intracel Corporation), respiratory virus was identified with an inverted fluorescent microscope (Eclipse TE200; Nikon, Tokyo, Japan) at a magnification of ×100.

Conventional RT-PCR. The conventional hRSV RT-PCR assay targeted the N gene of the hRSV genome (primers RSVN3 and RSVN5; Table 1) and was described previously (19). We performed this assay in 0.2-ml thin-walled PCR tubes in a model 2400 thermal cycler (Perkin-Elmer Applied Biosystems, Norwalk, Conn.) using the reagents and reaction conditions described by the original investigators (19). All 190 specimens were tested by this assay, and this method was used to determine the type of hRSV isolate detected by LC-RT-PCR, DFA, or CA-DFA.

Conventional PCR amplicon detection. An enzyme-linked amplicon hybridization assay developed in our laboratory was used for amplicon detection (15). This method used a hybridization reaction with two 5′ biotinylated oligonucleotide probes specific for either hRSV type A or hRSV type B (RSV-A and RSV-B, respectively; Invitrogen) (Table 1). Briefly, following amplification, the reaction mixture was added to specific probes diluted in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) and denatured at 94°C. Following denaturation, the contents of the tubes were incubated in streptavidin-coated microwells (Labsystems, Helsinki, Finland), washed, and further incubated with anti-digoxigenin peroxidase conjugate (Roche Diagnostics). The wells were again washed, and tetramethylbenzidine substrate (Moss Inc., Pasadena, Md.) was added to each well. Color development was stopped by the addition of 100 μl of 1 M HCl. The optical density was recorded with a plate spectrophotometer (Murex, Dynex Technology, Channel Islands, Great Britain) at a wavelength of 450 nm, with a wavelength of 690 nm used as a reference. An optical density of 0.2 or greater was indicative of a positive result.

RESULTS

A total of 190 NPA samples from patients with suspected respiratory tract infections were tested by LC-RT-PCR, conventional RT-PCR, and the DFA and CA-DFA testing algorithm. The DFA and CA-DFA testing algorithm consisted of initial screening of the specimens by DFA and then testing of DFA-negative specimens by CA-DFA. In total, 77 (41%) specimens were positive by LC-RT-PCR and conventional RT-PCR, 75 (39%) were positive by the combination of DFA and CA-DFA, and 113 (59%) specimens were negative by all four methods. All 75 specimens that tested positive by use of the DFA and CA-DFA testing algorithm tested positive by the LC-RT-PCR and conventional RT-PCR. Two specimens that were negative by DFA and CA-DFA were positive by the LC-RT-PCR and the conventional RT-PCR method targeting a different gene (N protein). The positive reactions obtained by the conventional PCR for these specimens confirmed the presence of hRSV RNA, and it was shown that both of the specimens contained hRSV type A. Typing of the remaining 75 hRSV isolates identified by the study showed that they consisted of 65 hRSV type A isolates and 10 hRSV type B isolates.

Of the 75 positive specimens in which hRSV was identified by DFA or CA-DFA, the initial DFA screen identified 70 (93%) positive samples, and subsequent testing by CA-DFA identified 5 more positive samples. Compared to the results of LC-RT-PCR, this gave the initial DFA screen a sensitivity of 91% and the DFA and CA-DFA testing algorithm a sensitivity of 97%. There was complete agreement between the results of the LC-RT-PCR and the conventional RT-PCR assays. The limit of sensitivity of the LC-RT-PCR was determined by testing dilutions of an hRSV type A isolate and was found to be 50 PFU/ml of specimen, which is equivalent to 0.25 PFU per reaction mixture.

In the LC-RT-PCR quantification curve analysis, the threshold cycle (C_T) was the cycle number at which the sample's fluorescence curve became exponential. The C_T values for hRSV-positive specimens ranged from 17 to 36, reflecting a range of concentrations of target RNA. The two specimens that were LC-RT-PCR positive and DFA and CA-DFA negative showed C_T values of 31 and 34, respectively, reflecting low viral loads. Negative samples did not produce exponential quantification curves during the PCR. Fluorescent melting curve analysis by LC-RT-PCR of the 77 LC-RT-PCR-positive specimens and the hRSV type A-positive and hRSV type B-positive controls revealed melting temperatures ranging from 62 to 63°C. The fact that the melting temperatures varied by only 1°C suggests that the hybridization probe targets are highly conserved among the isolates identified by this study.

Testing of a panel of genetically unrelated viruses and bacteria commonly found in the respiratory tract failed to produce any positive reaction, thus confirming the specificities of the primers and probes used in the LC-RT-PCR.

DISCUSSION

Rapid laboratory diagnosis of hRSV infection in children and infants can be critical for effective patient management by focusing appropriate drug treatment, reducing unnecessary use of antibiotics, and preventing nosocomial spread. This, combined with an increasing awareness within the clinical community of rapid PCR-based diagnostics, has led to greater pressure for diagnostic laboratories to adopt molecular biology-based diagnostic protocols. The LightCycler instrument is a recently developed, commercially available nucleic acid amplification and detection system which enables PCR technology to be easily incorporated into diagnostic laboratories. It is designed to decrease the PCR assay time by monitoring the amplification of target sequences in real time by FRET analysis. For our laboratory, the LightCycler instrument offered significant benefits for the diagnosis of hRSV infections. First, the LC-RT-PCR could generate results within 2 h of receipt of the specimen, which was significantly faster than the minimum time of 2 days required to complete the DFA and CA-DFA testing algorithm.

Second, the LC-RT-PCR was more sensitive than the combination of DFA and CA-DFA. Although the initial screen by DFA could be performed within 3 h, it failed to detect hRSV in seven specimens that were LC-RT-PCR positive. CA-DFA detected hRSV in five of these specimens, leaving two specimens negative by both tests. However, the presence of hRSV RNA in these two specimens could be confirmed by a conventional RT-PCR method targeting a gene on the hRSV genome different from the gene targeted by the LC-RT-PCR. The specificities of all methods were 100%, as no false-positive results occurred with the clinical samples tested, and the LC-RT-PCR failed to give a positive reaction in assays with any of the unrelated organisms. In summary, in comparison to the traditional DFA and CA-DFA assays, the LC-RT-PCR had a faster turnaround time in terms of the time required to obtain results, improved sensitivity, and equivalent specificity.

When designing the LC-RT-PCR we used the sequence data in a publicly available sequence database (GenBank accession numbers AF254574, AF013255, AF013254, U63644, AF035006, U50363, U50362, U27298, M74568, M75730, U39661, U39662, and U35343) to identify primer and probe sequences that would provide the optimal sensitivity and specificity for the assay. One of the critical factors for ensuring the optimal sensitivity of any diagnostic PCR assay is the identifi-

cation of primer and probe sequences which are highly conserved among the various isolates and types of the target organism. For hybridization probe assays, this is made more difficult by the fact that the sequences of two primers and two probes must be specific for genomic sequences in close proximity (preferably within 200 bp) to allow maximum amplification efficiency. One of the few conserved areas of the hRSV genome that satisfied this requirement was the gene coding for the L protein, and it was for this reason that we chose the L gene as the target for our assay (13). In choosing this gene we were conscious of the fact that limited sequence data are publicly available for the hRSV L gene and that the transcription rate of this gene is lower than those for genes located at the 3′ end of the viral genome.

In practice, however, it was shown that the assay was very sensitive, detecting 0.25 PFU of hRSV per reaction mixture, as well as detecting hRSV in all samples that were positive by the conventional RT-PCR assay. Other studies have also reported increased sensitivities of PCR-based assays for the detection of hRSV, with some showing a significant improvement over DFA or culture-based methods (2, 10, 28). The LC-RT-PCR described here showed a 9% improvement over DFA, which was somewhat less than expected on the basis of the results of the other studies. However, we believe that the sensitivity of the LC-RT-PCR may have been limited by the use of the one-step RT-PCR kit (LightCycler RNA amplification kit; Roche Diagnostics). Previous experiments in our laboratory have shown that RT-PCR assays that use these one-step reagents are less sensitive than assays that perform an initial step of reverse transcription followed by a separate cDNA amplification step (data not shown).

Melting curve analysis showed that the sequences targeted by the hybridization probes were well conserved across the hRSV isolates detected. The melting temperatures of the 65 hRSV type A isolates and the 10 hRSV type B isolates varied by only 1°C, ranging from 62 to 63°C. This suggests that false-negative results are unlikely to occur as a result of sequence variation.

In recent years, many PCR protocols for the detection of infectious agents in the clinical diagnostic laboratory have been described. The advent of real-time PCR has created further potential to expand the use of PCR for disease diagnosis. The LC-RT-PCR described here successfully detected hRSV RNA in NPA specimens from patients with suspected respiratory tract infections and therefore offers a suitable alternative to conventional techniques for the detection of these infections. The results showed that the LC-RT-PCR detected hRSV in all specimens that were positive by DFA or CA-DFA, as well as in seven additional DFA-negative specimens and two specimens that were negative by both DFA and CA-DFA. This confirmed that the LC-RT-PCR is more sensitive than DFA alone, as well as the combined DFA and CA-DFA testing algorithm. In addition to improved sensitivity and a high degree of specificity, we found that the LC-RT-PCR offered other significant advantages, including a faster turnaround time in terms of the time required to obtain results and therefore greater cost-effectiveness. The turnaround time for the LC-RT-PCR result was approximately 2 h, which was marginally faster than that for DFA (3 h) and a significant improvement over that for CA-DFA (48 to 72 h). However, DFA can identify multiple viral pathogens in a single test and is relatively cheap to perform. Therefore, in laboratories in which DFA might remain the first test choice, LC-RT-PCR may provide a suitable alternative to cell culture-based assays.

A significant improvement of the LC-RT-PCR over conventional viral detection methods may be in the detection of hRSV infection in older children and adults, particularly in cases of reinfection. In these subjects the productive viral load during infection is significantly reduced and disease is more difficult to diagnose by DFA and culture-based assays. This was supported by the results of this study, which showed that six of the seven specimens that were DFA negative and LC-RT-PCR positive were from subjects older than 12 years of age. In addition, the two LC-RT-PCR-positive specimens that were DFA and CA-DFA negative were collected from adults (ages, 20 and 31 years). These results suggest that the improved sensitivity of the LC-RT-PCR becomes significant in the diagnosis of hRSV infections in older subjects with low viral loads. In addition, the failure of DFA and CA-DFA to detect virus in a clinical specimen might be due to the presence of nonviable virions in the sample as a result of either an insufficient amount of specimen or the use of inappropriate transport or storage conditions (8). The LC-RT-PCR is not affected by these limitations and relies on the presence of viral nucleic acid rather than viable or intact virions (17, 19).

Although the hRSV LC-RT-PCR described here presents several advantages over conventional viral detection methods, it is limited by its ability to detect a single target only. We are examining the LightCycler technology with a view to developing duplex and multiplex real-time PCR assays for the detection of multiple respiratory viruses in a single reaction. These assays will offer a significant improvement over existing virus detection methods and should receive a wider application in the routine clinical laboratory.

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