Comparison of a Real-Time Reverse Transcriptase PCR Assay and a Culture Technique for Quantitative Assessment of Viral Load in Children Naturally Infected with Respiratory Syncytial Virus

Stephanie M. Perkins, David L. Webb, Shauna A. Torrance, Chadi El Saleeby, Lisa M. Harrison, Jody A. Aitken, Anami Patel, and John P. DeVincenzo

University of Tennessee School of Medicine and Graduate School of Health Sciences, LeBonheur Children’s Medical Center, The Children’s Foundation Research Center, St. Jude Children’s Research Hospital, and Rhodes College, Memphis, Tennessee

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Respiratory syncytial virus (RSV) is the most common cause of lower respiratory infection of children. Understanding RSV pathogenesis and evaluating interventions requires quantitative RSV testing. Previous studies have used the plaque assay technique. Real-time reverse transcriptase PCR (RTrtPCR) offers possible greater sensitivity, stability after freeze/thaw, and lower cost, thus facilitating multicenter studies. We developed RTrtPCR assays based upon the RSV N and F genes. The N-gene assay detected greater RSV quantity and was further evaluated. Standard curves utilized both extractions from RSV culture supernatants of known quantity and cloned purified copies of the target DNA. In vitro, the ratio of RSV subgroup A (RSV-A) genome copies to PFU was 153:1. A total of 462 samples collected quantitatively from 259 children were analyzed in duplicate by RTrtPCR. Results were compared with those of RSV plaque assays performed on fresh aliquots from the same children. Duplicate RTrtPCR results were highly correlated (r² = 0.9964). The mean viral load from nasal washes obtained on the first study day was 5.75 ± standard error of the mean 0.09 log PFU equivalents (PFUe)/ml. Viral load by RTrtPCR correlated with plaque assay results (r² = 0.158; P < 0.0001). Within individuals, upper and lower respiratory tract secretions contained similar viral concentrations. RSV-A-infected children had 1.17 log PFUe higher viral loads than did those with RSV-B (P < 0.0001). RSV quantification by RTrtPCR of the N gene is precise and has significant, though limited, correlation with quantitative culture. The utility of the RTrtPCR quantification technique for clinical studies would be solidified after its correlation with RSV disease severity is established.

MATERIALS AND METHODS

Study subjects, specimens, and statistical analysis. Study subjects were less than 2 years of age and were enrolled over four consecutive winter seasons. Subjects were previously healthy and were enrolled after presentation with respiratory symptoms to an outpatient acute care clinic or an emergency department. A portion of subjects (89.5%) were hospitalized for their acute respiratory illness. Patients with chronic lung disease, hemodynamically significant congenital heart disease, immunodeficiency, or steroid use were excluded. The study was conducted with the approval of the University of Tennessee Institutional Review Board.

This method is time consuming and difficult to perform and has numerous inherent limitations regarding the assay’s ability to accurately and reliably quantify viral concentrations in patient samples. When compared to the specific limitations of quantification by culture, real-time quantitative reverse transcription-PCR (RTrtPCR) offers several theoretic advantages, including the following: (i) a lower threshold of detection, (ii) the potential stability of the assay after specimen freezing and thawing, thus permitting sample batching, (iii) a less subjective assay readout, and (iv) an assay that is unaffected by therapeutic passive neutralizing antibodies or experimental antiviral agents. These potential advantages would facilitate multicenter studies of pathogenesis and therapeutic trials. We therefore sought to develop several quantitative RTrtPCR assays for RSV, to validate these assays in vitro and in vivo, and to compare these assay results with those of fresh plaque assays in studies of infants naturally infected with RSV.
Board and included appropriate informed consent, complying with all relevant federal guidelines and institutional policies. Nasal washes and tracheal aspirates were collected by study team members using a quantified-collection method as previously described (6). Briefly, secretions were collected into 4°C sucrose-containing RSV transport media and placed into the plaque assays within 3 h. Other individual aliquots of each respiratory secretion sample were then snap frozen on dry ice and frozen at −80°C until thawing for the RTrtPCR assay. Respiratory secretions were obtained serially from subjects who remained hospitalized. Statistical analysis was performed using standard techniques, all two-tailed with the level of significance set at 0.05. For normally distributed data, the t test was used. The Mann-Whitney U test was used for data not normally distributed.

**Quantitative RSV culture.** A HEp-2 cell plaque assay in 12-well plates with 10-fold dilutions in triplicate was used in which plates were overlaid with methylcellulose/medium and fixed and stained at 5 days with hematoxylin and eosin. Plaque assays were performed on fresh specimen within 3 h of specimen collection. HEp-2 cells used in all plaque assays were maintained at low passage number (<18). RSV quantitative standards (RSV-A, Long strain ATCC VR-26) were run in parallel with each plaque assay to assure quantitative precision (5).

**RSV standards.** Separate RSV quantitative standards were prepared for RSV-A (Long strain ATCC VR-26) and RSV-B (ATCC VR-955) in the following manner. HEp-2 cell monolayers at 80% confluency were inoculated with the above laboratory RSV strains (multiplicity of infection, approximately 10) and incubated in 5% CO2 at 37°C for approximately 60 h until optimal cytopathic effect was observed. The flasks were then scraped, and the cells and supernatant were centrifuged. The supernatant was then added to equal volumes of 50% sucrose for stabilization. Individual aliquots were frozen on dry ice and stored at −80°C until use. Individual RSV-A aliquots made in this way were used both as parallel standards for the plaque assay and as the quantitative standards from which the viral RNA was extracted for use in the RTrtPCR assays of RSV-A. RSV-B standards were made in the same manner and were used, after RNA extraction, as the standards for the RSV-B RTrtPCR assays.

**PCR analysis.**

**RNA extraction and reverse transcription.** Viral RNA was extracted from the RSV culture standards described above using silicon-based spin columns (QiAmp viral RNA Minikit) (QIAGEN, Valencia, CA) according to the manufacturer’s instructions and was incubated with an RNase-free DNase. Immediately following extraction, cDNA was constructed through a single reverse-transcription reaction (1 h at 37°C followed by reverse transcriptase inactivation at 95°C for 5 min) using the Omniscript reverse transcription kit (QIAGEN, Valencia, CA) and custom primers (Table 1). The resultant cDNA was placed on ice and then directly utilized as standards in the real-time PCR assay. Viral RNA was isolated from patient samples using this same procedure.

**Design of primers and probes.** All sequences from all RSV genes in the GenBank database in July 2002, except for sequences from mutated research or clinical cultures, were aligned using CLUSTALW (Scientific and Educational Software, Cary, NC). Homologous regions were displayed. The N, M, F, and L genes showed significant regions of complete homology within a single RSV subgroup, but insufficient regions of complete homology between RSV subgroups were found. Therefore, separate primers for RSV-A and RSV-B subgroups were sought. From within the completely homologous regions, Primer Express software (Applied Biosystems International, Foster City, CA) selected minor groove binding primers and probes successfully for the RSV N, M, F, and L genes. Gene targets with the greatest number of comparison sequences available in GenBank were pursued further. Sequences for primers and probes are listed in Table 1 and contained a 5′-6-FAM reporter (6-carboxyfluorescein) and a 3′ TAMRA quencher.

**Real-time PCR.** Real-time PCR was performed in a 96-well format using the ABI Prism 7900HT sequence detection system (Applied Biosystems International, Foster City, CA). The PCR mixture contained the following: 2.25 μl of forward and reverse primers (10 μM), 2.5 μl probe (2.5 μM), 12.5 μl 2× TaqMan universal PCR master mix, 5.0 μl template cDNA, and 0.5 μl of RNase-free water. Reaction mixtures of 25 μl were analyzed in duplicate. Each PCR had a negative control in which water was substituted for cDNA. The PCR employed the following thermal cyler settings: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Six 10-fold dilutions of cDNA, in duplicate, from RNA extracted from single-use, single freeze/thaw aliquots of the RSV standards were used for quantitative standards for all clinical specimens. Since the concentration of RSV in these aliquots was known (7.2 log PFU/ml), the standards contained concentrations of cDNA in the units of PFU equivalents/ml (log PFU/ml). Selected clinical specimens were also run in the PCR with quantitative standards of cloned target DNA.

**Quantitative RSV cloning.** Target cDNA amplified via RT-PCR from RSV-A (ATCC VR-26) and RSV-B (ATCC VR-955) was cloned into the pCR4-TOPO vector and transformed into the TOPO-10 bacterial strain using the TOPO TA cloning kit (Invitrogen). The obtained clones were evaluated by PCR and sequenced. Quantiﬁcation of the plasmid preparations was determined spectrophotometrically in quadruplicate. Aliquots of this quantitative plasmid preparation were used as standards for the RTrtPCR assay.

**Determination of RSV subgroup.** The RSV subgroup was determined by RTrtPCR. After amplification, if at least one of the duplicate cycle threshold (CT) values for the specimen was greater than 30 cycles, a duplicate sample aliquot was then run using both the repeated assay and the alternative subgroup assay. Confirmation of the subgroup was determined by the assay producing the greatest amplification. No specimen producing a CT value less than 30 cycles produced significant amplification when run in the alternative subgroup-specific PCR assay (data not shown). For 134 specimens, alternative subgroup-specific standard PCR (Hexaplex Prodesse Inc., Waukesha, WI) was also used to confirm RSV subgroup. This assay resulted in 93.3% concordance with the final subgroup as determined by RTrtPCR. The subgroup was also confirmed for 61 specimens by direct fluorescent-antibody staining of isolates cultured in HEp-2 cells. The staining used the following monoclonal antibodies: MAB8581 and MAB8582 (F protein 1b and F protein 1c) (Chemicon, Temecula, CA). This direct fluorescent-antibody assay resulted in 93.4% concordance with the final RTrtPCR-determined subgroup.

**Electron microscopic quantification.** Individual aliquots of RSV-A and -B culture standards, as described above, were used for viral particle quantification by electron microscopy (Advanced Biotechnologies Inc., Columbia, MD). RSV standards were mixed with 112-nm latex spheres of known concentration, fixed with buffered glutaraldehyde, washed with ultra-pure water, and stained with

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### TABLE 1. Design of nucleic acid reagents used for real-time reverse transcriptase PCR

<table>
<thead>
<tr>
<th>N gene</th>
<th>Function</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan RSV</td>
<td>RT&lt;sup&gt;a&lt;/sup&gt; custom primer</td>
<td>5′-ATGCGCTTTTAGAATGC-3′</td>
</tr>
<tr>
<td>RSV-A</td>
<td>Forward primer</td>
<td>5′-ATCGGACCATCATAATTCGATGATCA-3′</td>
</tr>
<tr>
<td>RSV-A</td>
<td>Reverse primer</td>
<td>5′-TCTTCGACATCATAATTCGATGATCA-3′</td>
</tr>
<tr>
<td>RSV-A</td>
<td>Fluorogenic probe&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5′-CGGAGCAAGGAGAT-3′</td>
</tr>
<tr>
<td>RSV-B</td>
<td>Forward primer</td>
<td>5′-CTGTACCCAGAAATCACTATTT-3′</td>
</tr>
<tr>
<td>RSV-B</td>
<td>Reverse primer</td>
<td>5′-GCACATCAATTTGGATGTTCA-3′</td>
</tr>
<tr>
<td>RSV-B</td>
<td>Fluorogenic probe&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5′-CTGATGACAGGAGGAA-3′</td>
</tr>
</tbody>
</table>

<sup>a</sup> RT, reverse transcriptase.

<sup>b</sup> Probes incorporated a 5′ FAM reporter and a 3′ TAMRA quencher.
phosphotungstic acid. Virus particle concentrations were determined by counting the ratio of viral particles to latex spheres in random microscopic fields.

RESULTS

To determine the relative cumulative sensitivity of the different RTtrtPCR assays, the assay amplifying the N gene was compared to that for the F gene of RSV-A, historically the most frequent subgroup in documented epidemics. RSV-A Long strain culture standards were compared. Over a 4-log range of concentrations, the N-gene assay consistently produced results which were four $CT$ values lower than those for the F-gene assay, indicating greater theoretic sensitivity of the N-gene assay. In a small number of clinical samples tested ($n/1005 = 6$), the correlation of the N-gene assay with fresh plaque assay results was superior to that of the F gene. Therefore, the assay targeting the N gene was used throughout the remainder of the experiments. The lower limit of detection of the N-gene RTtrtPCR assay was 1.15 log PFU/ml (14.13 PFU/ml) as tested using culture supernatants of RSV-A. The assay was evaluated to determine whether it was affected by freezing and thawing. This aspect of the assay is important in its usefulness as a quantitative method for multicenter trials. Identical aliquots of RSV-A standards (see Materials and Methods) were frozen and thawed from one to three times and then tested by RTtrtPCR. Results showed no drop in quantity of RSV measured (data not shown).

To determine whether RTtrtPCR could reliably measure RSV quantity in human respiratory secretions, known concentrations of RSV-A Long was spiked into aliquots of nasal wash fluid obtained from an uninfected volunteer which had been collected in the same manner as the collection of the clinical specimens in the study. Spiked nasal washings correlated well with predicted concentrations ($r^2 = 0.96$) (Fig. 1A). To determine whether different wild-type isolates of RSV could be quantified reliably by RTtrtPCR, viral isolates were obtained from infants infected with RSV. These individual isolates (passage 1) were used to infect individual flasks of HEp-2 cells. Supernatants of infected flasks were tested by fresh plaque assay, and aliquots were frozen. Thawed aliquots were tested in duplicate by RTtrtPCR. Quantitative culture correlated well with RTtrtPCR results ($r^2 = 0.866; n/1005 = 22$) (Fig. 1B).

To better define the RTtrtPCR assay, we utilized various methods to quantify the concentration of virus present in identical aliquots of RSV-A culture standards (Table 2). For every

**TABLE 2. Comparison of quantitative RSV detection methods**

<table>
<thead>
<tr>
<th>Method (units)</th>
<th>Result for $^{a}$ RSV-A standard (ATCC VR-26)</th>
<th>Result for $^{a}$ RSV-B standard (ATCC VR-955)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative culture (PFU/ml)</td>
<td>$1.74 \times 10^7$</td>
<td>$3.16 \times 10^5$</td>
</tr>
<tr>
<td>RTtrtPCR (PFUe/ml$^{b}$)</td>
<td>$1.74 \times 10^7$</td>
<td>$3.16 \times 10^5$</td>
</tr>
<tr>
<td>RTtrtPCR of cloned target (copies $\pm$ SEM/ml)</td>
<td>$(2.66 \pm 0.89) \times 10^9$</td>
<td>$(1.11 \pm 0.08) \times 10^9$</td>
</tr>
<tr>
<td>Electron microscopy (viral particles/ml)</td>
<td>$4.0 \times 10^8$</td>
<td>$2.0 \times 10^8$</td>
</tr>
</tbody>
</table>

$^{a}$ PFUe, PFU equivalents.

$^{b}$ These values are, by definition, the same as the concentration of virus measured by quantitative culture, since the samples used to construct the standard curves were derived from nucleic acid extracted from the RSV-A and RSV-B standards themselves.

$^{c}$ Preparation of RSV standards is described in Methods. Identical aliquots of these laboratory strains grown in HEp-2 cells were used as standards for these assays.

FIG. 1. Preclinical evaluation of RSV RTtrtPCR. All $P$ values test whether the slope is significantly nonzero. All testing was performed in duplicate. Dotted lines represent the 95% confidence for the slopes. Error bars represent SEM of duplicated PCR results. Panel A: ability of RTtrtPCR to predict RSV concentrations in spiked respiratory secretions. Nasal wash secretions from an uninfected person were spiked with known concentrations of cultured RSV. Concentrations of RSV as determined by PCR were compared with the calculated concentrations. Panel B: correlation of viral concentrations in culture supernatants of 22 separate RSV clinical isolates using plaque assay versus RTtrtPCR. Individual points represent duplicate wells. Error bars for each point represent SEM and, on many points, are too small to be seen on the figure.
infectious unit (defined by plaque production), there were 23 viral particles viewed by electron microscopy and 153 copies of the target N gene. There were 6.7 copies of the N gene for each microscopic viral particle. Based on our comparison of F-gene-targeted RTtrPCR, the number of F-gene copies per infectious unit would be expected to be less than that for the N gene. For every infectious unit of RSV-B, there were 633 viral particles viewed by electron microscopy and 3,513 copies of the target N gene. There were 5.6 copies of N gene for each microscopic viral particle.

To assess the precision and reproducibility of the assay, the values obtained from each of the 462 respiratory secretion samples run in duplicate were compared to each other. A high degree of correlation was seen ($r^2 = 0.996$) (Fig. 2A). This was also the case when the standards used for the assay were plasmid cloned N gene ($r^2 = 0.988$). The viral load from nasal wash specimens obtained on the first study day ($n = 259$) quantified by both plaque assay and RTtrPCR were compared. Viral loads were mean ± standard error of the mean (SEM) of $5.75 ± 0.09$ log PFUe/ml (range, 0 to 8.28 log PFUe/ml). Viral load measured by RTtrPCR correlated with that measured by plaque assay ($r^2 = 0.158; n = 259; P < 0.0001$) (Fig. 2B).
Similar correlations were seen when RSV-A and RSV-B subgroups were analyzed separately. RSV-A RTrtPCR results correlated with plaque assay \((r^2 = 0.0913; n = 177; P < 0.0001)\), while the correlation for RSV-B was an \(r^2\) value of 0.3352, \((n = 82; P < 0.0001)\). Eleven of the 259 specimens (4.2%) showed no growth in the quantitative culture but had positive RTrtPCR results. The mean (range) of these 11 RTrtPCR results was 4.90 (2.55 to 7.39) log PFUe/ml, lower than but not significantly different from the mean of the 259 specimens together. Of these 11 specimens which amplified but showed no growth in culture, 5/11 were RSV-A and 6/11 were RSV-B. Only one of the 259 specimens failed to amplify during RTrtPCR. The quantity of RSV in this single specimen as detected by quantitative culture was 5.70 log PFUe/ml. Due to the limitations of the plaque assay itself, the RTrtPCR assay provided a comparatively higher limit of detection within patient samples. The dilutions chosen for use in the plaque assay limited the maximum detectable RSV load to log 6.78 PFU/ml.

Certain infants studied were mechanically ventilated as a result of their RSV infection. These infants' endotracheal tubes provided access to their lower respiratory tracts. Within these lower respiratory tract specimens, there was a somewhat tighter correlation between plaque assay results compared to those of RTrtPCR \((r^2 = 0.3564; n = 13; P = 0.0312)\). We compared the viral loads using RTrtPCR from the nasal wash to those simultaneously obtained from deep tracheal suctioning. For the samples obtained on the first study day, the viral loads in the upper and the lower respiratory tracts were very similar \((r^2 = 0.852; n = 13; \text{slope} = 0.99; P < 0.0001)\) (Fig. 2C), and this similarity remained when all time points were considered \((r^2 = 0.6538; n = 18; P < 0.0001)\).

In clinical specimens, the quantity of RSV-A was compared to that of RSV-B. The mean viral loads in first-collected nasal washes were compared. RSV-A loads were significantly higher than RSV-B loads \((6.12 \text{ versus } 4.95 \log \text{PFUe/ml}; P < 0.0001)\). The nasal wash RSV load as measured by RTrtPCR in the individual subjects assayed over time produced smooth curves over time (Fig. 4A). Likewise, smooth individual curves were generated from the lower respiratory tract (data not shown). In both the upper (nasal wash) and lower (tracheal aspirate) respiratory tracts, most subjects showed a decline in RSV load over the first 24 h with a minority having increasing RSV loads within the first 24 h, followed by a decline thereafter. The mean RSV load obtained from the nasal wash specimens declined by 0.324 log PFUe/ml during the first 24 h and by 0.456 log PFUe/ml during the second 24-h interval (Fig. 4B).

**DISCUSSION**

Our finding that the N-gene PCR detected more copies per unit volume than did the F-gene PCR is not surprising. RSV genes located nearer the promoter are preferentially transcribed (5). Likewise, the high gene copy/PFU ratio and the high particle/PFU ratio are expected and are similar to these ratios estimated for other RNA viruses, such as *Picornaviridae* and *Orthomyxoviridae* (9). The ratio of gene copies or viral particles per culturable virus must depend largely on the conditions of viral replication. It is likely that the lowest ratio would be produced and measured under conditions of a multiplicity of infection of approximately 5 to 10, cultured from the supernatant of a highly competent cell line, and when tested within several hours of inoculation. Similar conditions were used to derive our measured ratios. The ratio would be ex-
These clinical specimens likely contain complete viral particles unable to replicate, partially assembled virions, and whole and fragmented viral genome, as well as fully replication-competent viruses which are inhibited in culture due to some effects of the respiratory secretions themselves. This explains the relatively poor correlation between RTrtPCR and quantitative culture results within respiratory secretions (Fig. 2B), despite the tight correlation when tested in vitro (Fig. 1B). The correlation between RTrtPCR and quantitative culture seen in Fig. 2B is not improved when alternative analyses are performed, such as excluding the quantitative culture results we report here (4.899 versus 4.209 log PFU/ml; P = 0.002; n = 259). The RTrtPCR results are likely a better measurement of quantitative differences between subgroups, since numerous RSV loads were higher than the maximum measurable by our plaque assay and these values were clustered in the RSV-A subgroup. Although this finding is not universally confirmed (2), RSV-A subgroup infections have been shown in several studies to produce more-severe disease than RSV-B (10, 13, 14, 16). RSV load differences may help explain these severity differences, since we have previously shown that increased RSV load correlates with greater disease severity (3, 7).

There are numerous potential advantages of RTrtPCR quantification over quantitative RSV culture. These advantages might be particularly pronounced in clinical trials. We have shown that there is little freeze-thaw effect on the results of RTrtPCR. This is in contrast to a significant and somewhat unpredictable freeze-thaw-induced reduction of quantitative culture results. Thus, specimens can be frozen, batched, and transported from different sites to a central research laboratory for quantification using RTrtPCR. The RTrtPCR assay appears more sensitive than quantification by culture. Significant cost savings can also be achieved over fresh quantitative culture. Another advantage is avoidance of the potential phenomenon of viral neutralization or inactivation by antiviral products being delivered to the respiratory tract. Antiviral products within the respiratory secretions themselves can inhibit viral growth in culture, thus potentially reducing the RSV quantity measured more than the actual reduction in the subject. Quantitative PCR would avoid this problem. When using RTrtPCR for RSV clinical trials, one must note that the natural rate of decline in viral load is less steep than when using quantitative culture. This rate of decline in viral load measured by RTrtPCR may also depend upon the timing of administration of an antiviral agent, the elimination kinetics of viral nucleic
acid in respiratory secretions, and the specific molecular target of the antiviral within the viral replication cycle.

In conclusion, RSV-A and -B quantification by real-time reverse transcriptase PCR of the N gene is highly reproducible, has a broad range of detection, and correlates well with fresh plaque assay with cultured patient isolates. This correlation is less robust for the secretions of naturally infected children. Likewise, measured viral elimination kinetics are different when measured by this technique. There are clear advantages of using this technique of RSV quantification in clinical trials, but the utility of quantitative PCR as a tool in RSV clinical research would be strengthened by an established correlation of PCR viral load with disease severity.

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