

# 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

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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 ( $r^2 = 0.9964$ ). The mean viral load from nasal washes obtained on the first study day was  $5.75 \pm$  standard error of the mean 0.09 log PFU equivalents (PFUe)/ml. Viral load by RTrtPCR correlated with plaque assay results ( $r^2 = 0.158$ ;  $P < 0.0001$ ). 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.**

Respiratory syncytial virus (RSV) is the most common cause of acute lower respiratory tract infection in infants. In the United States, 3% of all previously healthy infants are hospitalized within their first year of life due to RSV infection (1), and this rate is increasing (15), making RSV the single most common cause of infant hospitalization (11). Although infants with chronic lung disease, congenital heart disease, or immunodeficiencies are at increased risk for severe RSV disease, most infants with severe RSV disease requiring hospitalization were previously healthy (1, 18, 19). Furthermore, RSV is also an increasingly recognized major cause of morbidity and mortality in debilitated adults and the elderly (17).

Currently, our understanding of the pathogenesis of RSV in humans is incomplete. Relating viral load and viral dynamics to disease severity will expand our understanding of RSV pathogenesis. Studying RSV pathogenesis and evaluating potential vaccine and antiviral treatment strategies for humans requires quantitative tests for RSV. At present, the quantity of RSV obtained from clinical samples in such studies has been measured by quantitative culture using plaque assays. However,

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 transcriptase 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.

## 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

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

RSV target	Function	Sequence
<b>N gene</b>		
Pan RSV	RT <sup>a</sup> custom primer	5'-ATGGCTCTTAGCAAAGTC-3'
RSV-A	Forward primer	5'-CATCCAGCAAATACACCATCCA-3'
RSV-A	Reverse primer	5'-TTCTGCACATCATAATTAGGAGTATCAA-3'
RSV-A	Fluorogenic probe <sup>b</sup>	5'-CGGAGCACAGGAGAT-3'
RSV-B	Forward primer	5'-CTGTCATCCAGCAAATACACTATTCA-3'
RSV-B	Reverse primer	5'-GCACATCATAATTGGGAGTGTCA-3'
RSV-B	Fluorogenic probe <sup>b</sup>	5'-CGTAGTACAGGAGATAAT-3'
<b>F gene</b>		
RSV-A	RT custom primer	5'-AATGCAGGTGTAACACTACA-3'
RSV-A	Forward primer	5'-AATGATATGCCTATAACAAATGATCAGAA-3'
RSV-A	Reverse primer	5'-TGGACATGATAGAGTAACCTTTGCTGTCT-3'
RSV-A	Fluorogenic probe <sup>b</sup>	5'-CCAACAATGTTCAAATAG-3'

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

<sup>b</sup> Probes incorporated a 5' FAM reporter and a 3' TAMRA quencher.

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 RTTrtPCR 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. Fisher's exact test was used for categorical data.

**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/media 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% CO<sub>2</sub> at 37°C for approximately 60 h until optimal cytopathic effect was observed. The flasks were then scraped, and the cells and supernatant were gently vortexed and 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 RTTrtPCR assays of RSV-A. RSV-B standards were made in this same manner and were used, after RNA extraction, as the standards for the RSV-B RTTrtPCR 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 vaccine strains, were aligned using Clone Manager Suite (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 (6-carboxytetramethylrhodamine).

**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 cycler 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, Karlsruhe, Germany). 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 RTTrtPCR assay.

**Determination of RSV subgroup.** The RSV subgroup was determined by RTTrtPCR. After amplification, if at least one of the duplicate cycle threshold (*C<sub>T</sub>*) 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 *C<sub>T</sub>* 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 RTTrtPCR. 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 RTTrtPCR-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

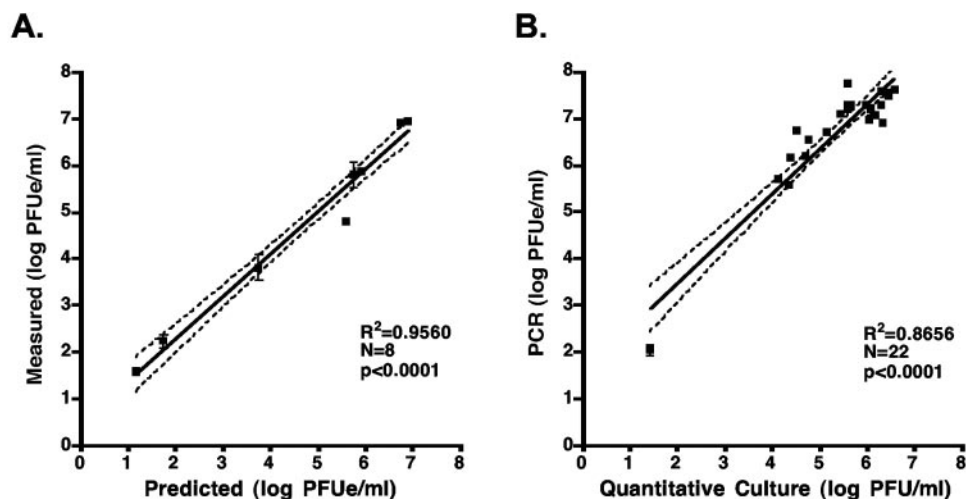


FIG. 1. Preclinical evaluation of RSV RTrtPCR. All *P* values test whether the slope is significantly nonzero. All testing was performed in duplicate. Dotted lines represent the 95% confidence for the slopes. Error bars represent SEM of duplicated PCR results. Panel A: ability of RTrtPCR to predict RSV 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 RTrtPCR. 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.

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 RTrtPCR 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  $C_T$  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 ( $n = 6$ ), 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 RTrtPCR 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 RTrtPCR. Results showed no drop in quantity of RSV measured (data not shown).

To determine whether RTrtPCR 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 RTrtPCR, 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 RTrtPCR. Quantitative culture correlated well with RTrtPCR results ( $r^2 = 0.866$ ;  $n = 22$ ) (Fig. 1B).

To better define the RTrtPCR 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

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

<sup>a</sup> PFUe, PFU equivalents.

<sup>b</sup> 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.

<sup>c</sup> 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.

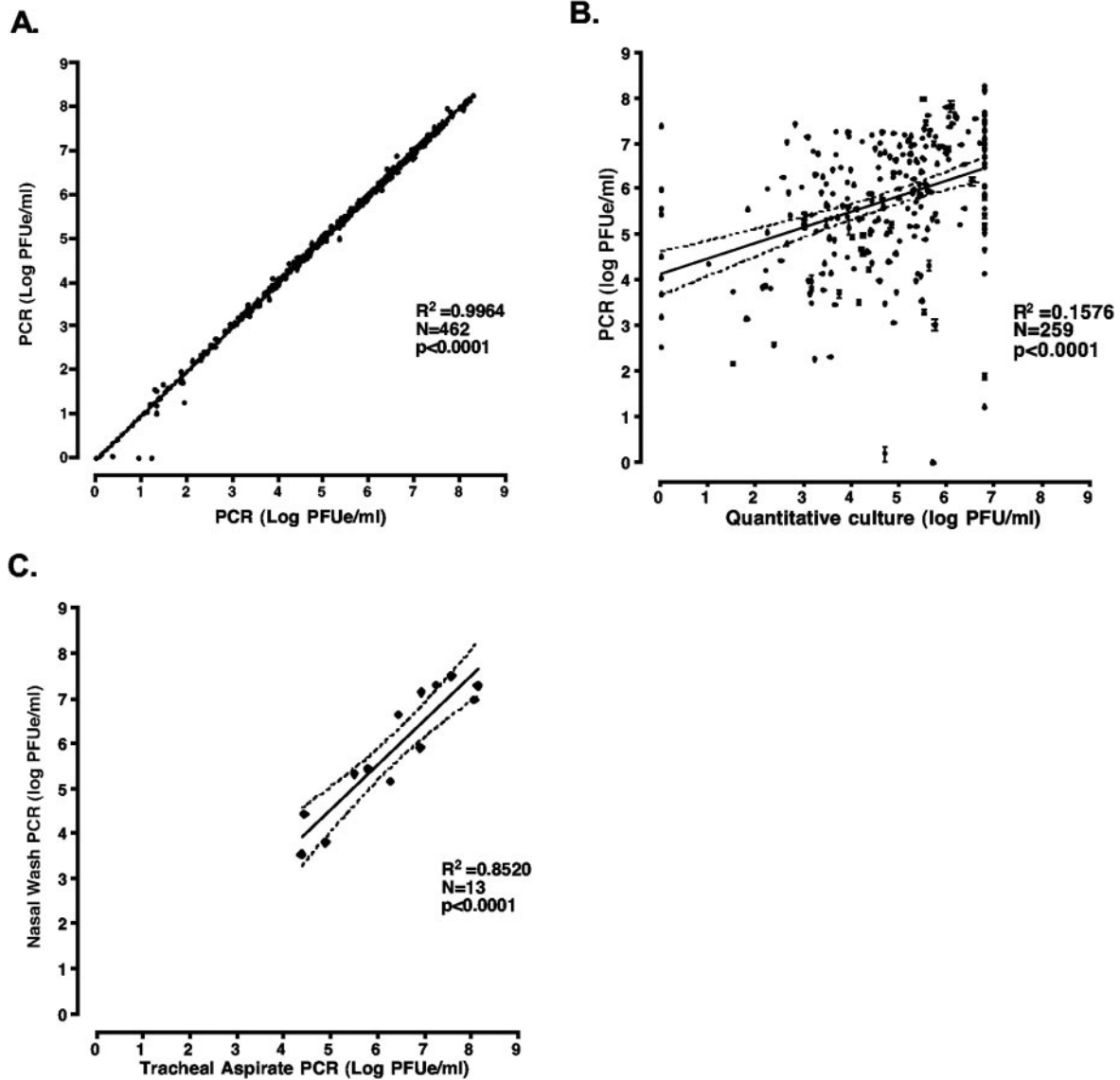


FIG. 2. Evaluations of RSV RTTrtPCR in respiratory secretions of naturally infected infants. All *P* values test whether the slope is significantly nonzero. All testing was performed in duplicate. Error bars represent SEM of duplicated PCR results. Dotted lines represent the 95% confidence for the slopes. Panel A: correlation of duplicate PCR testing showing precision of the assay. Panel B: correlation of RTTrtPCR versus plaque assay. The nasal wash secretions collected upon study enrollment were evaluated by fresh plaque assay and from frozen aliquots by PCR. The slope of the line of correlation is 0.24. When log PFU values of 0 and 6.78 are removed from the analysis ( $n = 204$ ), the  $r^2$  value is 0.1584, the Y intercept is 3.564, and the slope is 0.4628 ( $P < 0.0001$ ). Panel C: correlation between upper and lower respiratory tract RSV concentrations. Upper respiratory secretions were nasal washes, and lower respiratory secretions were deep tracheal aspirates collected upon study enrollment. Points represent upper and lower respiratory RSV loads obtained on the same patient at the same time.

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 RTTrtPCR, 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 RTTrtPCR were compared. Viral loads were mean  $\pm$  standard error of the mean (SEM) of  $5.75 \pm 0.09$  log PFUe/ml (range, 0 to 8.28 log PFUe/ml). Viral load measured by RTTrtPCR correlated with that measured by plaque assay ( $r^2 = 0.158$ ;  $n = 259$ ;  $P < 0.0001$ ) (Fig. 2B).

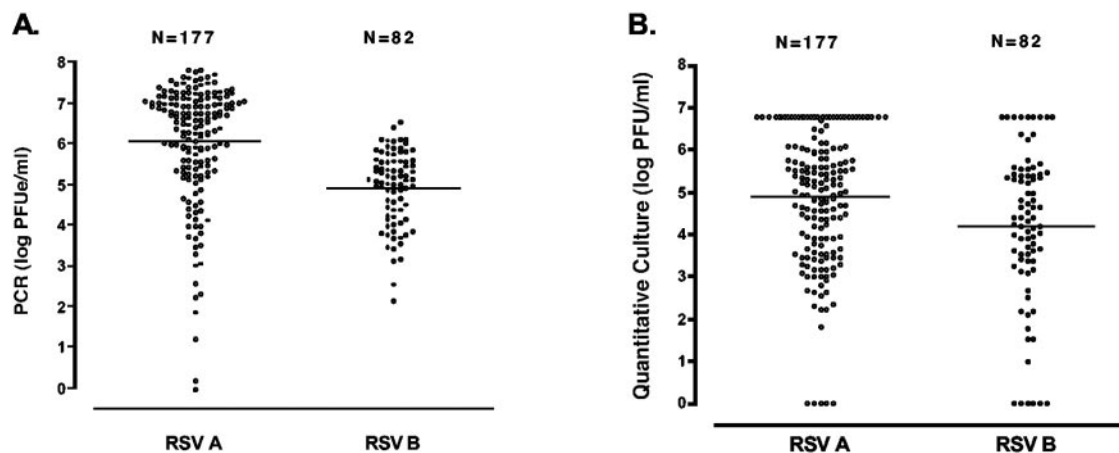


FIG. 3. RSV subgroup differences in nasal wash viral loads. Nasal washes collected from subjects upon enrollment were analyzed according to RSV subgroup. Panel A: quantification by RTTrtPCR showing RSV loads significantly higher in subjects infected with RSV-A than in those infected with RSV-B. The mean viral loads were  $6.12 \pm \text{SEM } 0.112$  log PFUe/ml for RSV-A and  $4.95 \pm 0.101$  log PFUe/ml for RSV-B. The difference between the means was  $1.17$  log PFUe/ml (95% CI: 0.817 to 1.516;  $P < 0.0001$ ). Panel B: quantification by plaque assay showing RSV loads also significantly higher in subjects infected with RSV-A than in those infected with RSV-B. Some of the data in panel B have been reported previously (6). The mean viral loads were  $4.90 \pm \text{SEM } 0.118$  log PFU/ml for RSV-A and  $4.21 \pm 0.201$  log PFU/ml for RSV-B. The difference between the means was  $0.69$  log PFU/ml (95% CI: 0.258 to 1.123;  $P = 0.0020$ ).

Similar correlations were seen when RSV-A and RSV-B subgroups were analyzed separately. RSV-A RTTrtPCR 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 RTTrtPCR results. The mean (range) of these 11 RTTrtPCR 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 RTTrtPCR. The quantity of RSV in this single specimen as detected by quantitative culture was  $5.70$  log PFU/ml. Due to the limitations of the plaque assay itself, the RTTrtPCR assay provided a comparatively higher upper 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 RTTrtPCR ( $r^2 = 0.3564$ ;  $n = 13$ ;  $P = 0.0312$ ). We compared the viral loads using RTTrtPCR 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 ( $r^2 = 0.852$ ;  $n = 13$ ; 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 ( $6.12$  versus  $4.95$  log PFUe/ml;  $P < 0.0001$ )

(Fig. 3A). The RSV-A RTTrtPCR assay uses different primers and probes than the RSV-B assay. To assess whether the observed difference in RSV load between subgroups was due to quantitative efficiency differences in the assays themselves, viral loads were compared by subgroup using the plaque assay results. Plaque assays confirmed that mean RSV-A loads were higher than RSV-B loads ( $4.90$  versus  $4.21$  log PFU/ml;  $P = 0.002$ ) (Fig. 3B).

The nasal wash RSV load as measured by RTTrtPCR 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-

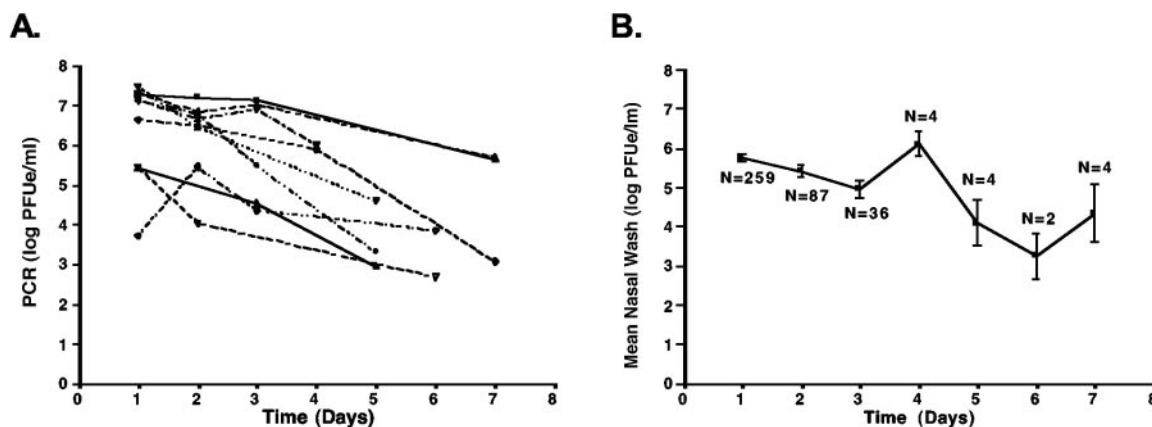


FIG. 4. Individual and mean RSV loads over time as measured by RTTrtPCR. Nasal washes collected from subjects over multiple time points were analyzed. Panel A: individual subjects' RSV loads are plotted as separate single lines. Smooth viral loads are measurable in individual infected children. Panel B: mean viral loads are plotted over time. Error bars represent SEM. As time increases, subjects are dropped from the study as they are discharged and therefore not available for further sample acquisition. The mean viral load declines by 0.39 log PFUe/ml/day over the first 2 days. Small numbers cause large scatter after this third-day time point.

pected to increase when measured under other conditions, especially in clinical specimens several days after illness onset. 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 RTTrtPCR and quantitative culture results within respiratory secretions (Fig. 2B), despite the tight correlation when tested *in vitro* (Fig. 1B). The correlation between RTTrtPCR and quantitative culture seen in Fig. 2B is not improved when alternative analyses are performed, such as excluding the quantitative culture results of zero and those above the detection limit of the plaque assay ( $r^2 = 0.158$ ;  $P < 0.0001$ ; slope, 0.463;  $n = 204$ ).

In this paper, we have again found that the quantity of virus in the upper respiratory tract (nasal secretions) is nearly equivalent to that found in the lower respiratory tract. We have previously reported this finding using quantitative culture (4, 12). This tight correlation between upper and lower respiratory tract viral loads has been observed and tested rigorously only with groups of infants whose age predicts primary RSV infection, whose measurements have occurred while the RSV disease is severe enough to require mechanical ventilation, and while the viral load is already dropping. It is possible that viral loads in the nose rise first, followed later by increasing concentrations within the lower respiratory tract. There may also be a disconnect between upper and lower respiratory tract viral loads in children or adults experiencing mild disease or their second or third RSV infection. Indeed, much higher viral loads are seen in the lungs of immunocompromised adults than in the nose (8). Likewise, different antiviral or immunologic interventions may have a different effect on either one of these replication compartments.

Our results reported here show significant differences between viral loads of RSV-A and RSV-B, differences which we observed using both RTTrtPCR and quantitative culture methodologies. A single other report of RSV subgroup quantification in natural human infection has been published (6). Using

quantitative culture, this smaller, previous study failed to show a significant difference between RSV subgroups. However, a trend towards higher RSV-A viral loads was observed (4.77 versus 4.68 log PFU/ml;  $n = 102$ ), which can be compared to the quantitative culture results we report here (4.899 versus 4.209 log PFU/ml;  $P = 0.002$ ;  $n = 259$ ). The RTTrtPCR 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 RTTrtPCR 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 RTTrtPCR. 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 RTTrtPCR. The RTTrtPCR 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 RTTrtPCR 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 RTTrtPCR 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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#### REFERENCES

1. Boyce, T. G., B. G. Mellen, E. F. Mitchel, Jr., P. F. Wright, and M. R. Griffin. 2000. Rates of hospitalization for respiratory syncytial virus infection among children in Medicaid. *J. Pediatr.* **137**:865–870.
2. Brouard, J., F. Freymuth, S. Constantini, J. Petitjean, G. de Schrevel, and J. F. Duhamel. 1993. Prevalence and clinical aspects of A and B subgroups of respiratory syncytial virus infection. Observation of 8 consecutive epidemics between 1982 and 1990. *Arch. Fr. Pediatr.* **50**:639–643. [In French.]
3. Buckingham, S., A. Bush, and J. DeVincenzo. 2000. Nasal quantity of respiratory syncytial virus correlates with disease severity in hospitalized infants. *Pediatr. Infect. Dis. J.* **19**:113–117.
4. Buckingham, S. C., H. S. Jafri, A. J. Bush, C. M. Carubelli, P. Sheeran, R. D. Hardy, M. G. Ottolini, O. Ramilo, and J. P. DeVincenzo. 2002. A randomized, double-blind, placebo-controlled trial of dexamethasone in severe respiratory syncytial virus (RSV) infection: effects on RSV quantity and clinical outcome. *J. Infect. Dis.* **185**:1222–1228.
5. Collins, P. L., K. McIntosh, and R. M. Chanock. 1996. Respiratory syncytial virus, p. 1313–1350. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fields virology*, 3rd ed, vol. 1. Lippincott-Raven Publishers, Philadelphia, Pa.
6. DeVincenzo, J. P. 2004. Natural infection of infants with respiratory syncytial virus subgroups A and B: a study of frequency, disease severity and viral load. 2004. *Pediatr. Res.* **56**:914–917.
7. DeVincenzo, J. P., A. J. Bush, J. Aitken, and L. Harrison. 2003. Respiratory syncytial virus (RSV) load correlates with disease severity: results of a prospective study of previously healthy naturally infected infants. *Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother.*, abstr. V-788.
8. Englund, J. A., P. A. Piedra, A. Jewell, K. Patel, B. B. Baxter, and E. Whimbey. 1996. Rapid diagnosis of respiratory syncytial virus infections in immunocompromised adults. *J. Clin. Microbiol.* **34**:1649–1653.
9. Flint, S. J., L. W. Enquist, R. M. Krug, V. R. Racaniello, and A. M. Skalka. 2000. *Principles of virology: molecular biology, pathogenesis, and control*. ASM Press, Washington, D.C.
10. Hall, C. B., E. E. Walsh, K. C. Schnabel, C. E. Long, K. M. McConnochie, S. W. Hildreth, and L. J. Anderson. 1990. Occurrence of groups A and B of respiratory syncytial virus over 15 years: associated epidemiologic and clinical characteristics in hospitalized and ambulatory children. *J. Infect. Dis.* **162**:1283–1290.
11. Leader, S., and K. Kohlhasse. 2002. Respiratory syncytial virus-coded pediatric hospitalizations, 1997 to 1999. *Pediatr. Infect. Dis. J.* **21**:629–632.
12. Malley, R., J. DeVincenzo, O. Ramilo, P. Dennehy, H. Meissner, W. Gruber, P. Sanchez, H. Jafri, J. Balsley, D. Carlin, S. Buckingham, L. Vernacchio, and D. Ambrosino. 1998. Reduction of respiratory syncytial virus (RSV) in tracheal aspirates in intubated infants using humanized monoclonal antibody to RSV F protein. *J. Infect. Dis.* **178**:1555–1561.
13. McConnochie, K. M., C. B. Hall, E. E. Walsh, and K. J. Roghmann. 1990. Variation in severity of respiratory syncytial virus infections with subtype. *J. Pediatr.* **117**:52–62.
14. Mufson, M. A., B. Akerlind-Stopner, C. Orvell, R. B. Belshe, and E. Norrby. 1991. A single-season epidemic with respiratory syncytial virus subgroup B2 during 10 epidemic years, 1978 to 1988. *J. Clin. Microbiol.* **29**:162–165.
15. Shay, D. K., R. C. Holman, R. D. Newman, L. L. Liu, J. W. Stout, and L. J. Anderson. 1999. Bronchiolitis-associated hospitalizations among US children, 1980–1996. *JAMA* **282**:1440–1446.
16. Walsh, E. E., K. M. McConnochie, C. E. Long, and C. B. Hall. 1997. Severity of respiratory syncytial virus infection is related to virus strain. *J. Infect. Dis.* **175**:814–820.
17. Walsh, E. E., D. R. Peterson, and A. R. Falsey. 2004. Risk factors for severe respiratory syncytial virus infection in elderly persons. *J. Infect. Dis.* **189**:233–238.
18. Wang, E. E., B. J. Law, F. D. Boucher, D. Stephens, J. L. Robinson, S. Dobson, J. M. Langley, J. McDonald, N. E. MacDonald, and I. Mitchell. 1996. Pediatric Investigators Collaborative Network on Infections in Canada (PICNIC) study of admission and management variation in patients hospitalized with respiratory syncytial viral lower respiratory tract infection. *J. Pediatr.* **129**:390–395.
19. Wang, E. E., B. J. Law, and D. Stephens. 1995. Pediatric Investigators Collaborative Network on Infections in Canada (PICNIC) prospective study of risk factors and outcomes in patients hospitalized with respiratory syncytial viral lower respiratory tract infection. *J. Pediatr.* **126**:212–219.