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Evaluation of quantitative and type-specific real-time RT-PCR assays for detection of respiratory syncytial virus in respiratory specimens from children

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Abstract

Background: Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract morbidity in young children and immunosuppressed patients. **Objectives:** To rapidly and accurately quantify and subtype RSV in respiratory samples, we developed and evaluated two real-time RT-PCR assays. **Study design:** A quantitative assay was designed using primers for a consensus region of the matrix protein gene and a subtype-specific assay for RSV-A and RSV-B detection was designed using primers for the polymerase gene. Quantitative RSV RT-PCR results of pediatric nasal wash samples submitted to the University of Washington Virology Laboratory from December 2002, through May 2003, were compared to those of an indirect fluorescent antibody RSV antigen detection assay (FA). **Results:** Specificity of the RT-PCR assay was high, with no amplification of eleven common respiratory viruses and eight herpes viruses. Among 751 samples, RSV was detected in 267 (35.6%) by FA and in 286 (38.1%) by RT-PCR. Median RSV copy number in nasal wash samples that were positive by both FA and RT-PCR was 2.5×10^7 copies/mL versus a median of 3.0×10^4 copies/mL for samples positive by RT-PCR only ($P < 0.001$). The detection and quantity of RSV in respiratory specimens was associated with younger age, but not with gender or hospitalization. Among positive samples from this Seattle cohort, 52% were subtype A and 48% were subtype B. Both subtypes were detected with similar viral loads among all patient groups (stratified by age, gender, and hospitalization), and throughout the specimen collection period. **Conclusions:** These real-time RT-PCR assays provide a rapid, specific, and highly sensitive alternative for detecting, quantifying, and subtyping RSV in clinical specimens.

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Keywords: Respiratory syncytial virus; Polymerase chain reaction; Quantitative; Type-specific

1. Introduction

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection and disease in young children, especially those less than 6 months old, and in immunocompromised individuals of any age (Englund et al., 1988; Shay et al., 2001). RSV, a member of the viral genus Pneumovirus, contains a negative sense RNA genome that is classified into two genetic subtypes, A and B. RSV in-

fection is commonly diagnosed by the detection of RSV antigens in respiratory secretions using enzyme immunoassays or fluorescent antibodies (FA), or by culturing respiratory specimens in traditional cell cultures or using rapid shell vial methods. Culture tests are labor intensive, technically challenging, and time consuming. Furthermore, both culture and antigen detection assays may lack sensitivity, especially for samples from adult patients (Falsey et al., 1996).

Reverse transcription (RT) and polymerase chain reaction (PCR) amplification assays that detect RSV RNA and distinguish between subtypes have been developed (Abels et al., 2001; Tang et al., 1999). Because they do not require viable virus or intact cells, RT-PCR for RSV has been shown to be more sensitive than conventional methods for RSV detection in clinical samples from both children

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; mL, milliliter; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; μ L, microliter; mM, millimolar; μ g, microgram; min, minutes; bp, base-pair

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and adults (Erdman et al., 2003; van Elden et al., 2002). Real-time PCR technology has been used not only to rapidly detect but also to quantify the amount of RSV in a sample (Gueudin et al., 2003; Hu et al., 2003; van Elden et al., 2003).

This study evaluates the performance of two real-time RT-PCR assays that provide absolute quantification and subtyping of RSV in respiratory specimens. The quantitative assay also includes an amplification control in the same reaction as the diagnostic target, which provides the added advantage of allowing evaluation of sample quality and of ruling out false negative results due to inhibition of amplification by sample components. The quantitative assay was compared to FA for detection of RSV in a large number of pediatric respiratory samples submitted over a 6-month period to a clinical virology laboratory. Detection of RSV using these sensitive, rapid, and accurate quantitative and type-specific RT-PCR assays can facilitate an earlier diagnosis and improve patient management.

2. Methods

2.1. Clinical specimens

From December 2002, through May 2003, 751 pediatric specimens (including 702 nasal wash samples, 26 nasal swabs, 16 tracheal aspirates, and 7 bronchoalveolar lavage (BAL) specimens) that were submitted to the University of Washington Virology Laboratory for respiratory virus FA or FA and culture, and contained sufficient residual material were tested for RSV by real-time RT-PCR. Specimens were submitted from both hospitalized (77%) and non-hospitalized (23%) patients. The age of the 685 patients from whom samples were tested ranged from 1 day to 20 years (mean age = 27 months, S.D. = 44 months). Fifty-five percent of samples were from male and 45% were from female patients. Fifty-two patients provided more than one specimen (mean interval = 27 days, range = 5–97 days).

2.2. RSV antigen detection (FA)

Specimens were tested for RSV using an indirect fluorescent antibody assay. In brief, cells obtained from patients' samples by centrifugation were suspended in buffer and spotted onto slides, air-dried, fixed in acetone, and incubated with mouse anti-RSV monoclonal antibody (Chemicon, Temecula, CA). After washing, goat anti-mouse fluorescein-conjugated monoclonal antibodies (ICN, Biomedicals, Inc., Costa Mesa, CA) were applied to the sample, and the slides were incubated, washed, and read using a fluorescent microscope. The presence of bright green fluorescence within intact cells was considered positive for RSV.

2.3. Sample preparation for RT-PCR assay

Total nucleic acids were isolated from 200 μ L of each respiratory specimen by adding 400 μ L of lysis buffer consisting of 68% guanidine thiocyanate, 50 mM Tris, pH 7.5, 1.3 mM dithiothreitol, and 40 μ g/mL glycogen. To ensure that negative results were not due to poor RNA extraction or inhibition of the PCR assay, 50,000 copies/mL (1000 copies/RT-PCR reaction) of EXO external control, a 130 base RNA transcript derived from jellyfish DNA (Limaye et al., 2001), were added to the lysis buffer. After incubation for 10 min at 60°C, 600 μ L of isopropanol was added and the samples were centrifuged at 13,000 \times g for 15 min. The pellets were washed with 1 mL of 70% ethanol and suspended in 200 μ L of RNase free water (Sigma, St. Louis, MO). One positive control with 2×10^4 – 1×10^5 copies/mL (200–1000 copies/RT-PCR reaction) of RSV harvested from HL cell culture and diluted in minimal essential medium, and one negative control consisting of harvested, uninfected HL cells were extracted with each batch of clinical specimens.

2.4. Design of primers and probes

The RSV RT-PCR primer and probe sequences were designed using aligned RSV sequences obtained from the NCBI database, using Primer Express software (Applied Biosystems, Foster City, CA), and are shown in Table 1. Three TaqMan primers and one probe were designed to amplify 80 and 81 bp fragments of the RSV type A and type B matrix protein genes, respectively. The RSV probe was labeled on the 5' end with 6FAM and on the 3' end with a minor groove binder non-fluorescent quencher (MGBNFQ) (Applied Biosystems). A second primer set and VIC-labeled probe, which amplified and detected the exogenously added EXO RNA molecules, were added to the RSV reaction (Limaye et al., 2001). The probe was modified from the published sequence by shortening it five bases on the 3' end to accommodate an MGBNFQ label. The RSV type specific assay contained one primer set to amplify a 94 bp region of the RSV polymerase gene from both types A and B, a 5' 6FAM-labeled probe specific for RSV type A, and a 5' VIC-labeled probe specific for RSV type B. Both probes were labeled on the 3' end with MGBNFQ.

2.5. Real-time RSV RT-PCR assays

Samples were analyzed without knowledge of the patient's FA result. For the quantitative assay, each 40 μ L RT-PCR mixture contained 1X Universal PCR Master Mix (Applied Biosystems), 1X Multiscribe and RNase Inhibitor (Applied Biosystems), 250 nM each of RSV forward primer and reverse primer A, 100 nM each of RSV reverse primer B, EXO forward and reverse primers, and RSV and EXO probes, and 10 μ L of extracted RNA, RNA transcripts for standard curves, or water for no template controls. For the RSV type-specific assay, the reaction components were the

Table 1
Real time RT-PCR primer and probe sequences designed to detect RSV and EXO RNA

Assay/targets	Function	Sequence	T _m (°C)	Amplicon size (bp)
Consensus RSV types A and B/RSV matrix gene and EXO external control (jellyfish)	RSV forward primer	GGA AAC ATA CGT GAA CAA GCT TCA	59.2	
	Reverse primer A	CAT CGT CTT TTT CTA AGA CAT TGT ATT GA	59.1	RSV A =80
	Reverse primer B	TCA TCA TCT TTT TCT AGA ACA TTG TAC TGA	58.8	RSV B =81
	RSV probe	6FAM-TGT GTA TGT GGA GCC TT-MGBNFQ	68.1	
	EXO forward primer	GCC TGG TGC AAA AAT TGC TT	58.8	130
Type-specific RSV A or B/polymerase gene	EXO reverse primer	TCG TTC ATT TGT TCT TTT GTG GAA	59.5	
	EXO probe	VIC-CAG CTA TTG CAA ACG CCA T-MGBNFQ	70.0	
	Forward primer	AAT ACA GCC AAA TCT AAC CAA CTT TAC A	58.7	94
	Reverse primer	GCC AAG GAA GCA TGC AAT AAA	58.8	
	Probe A	6FAM-TGC TAT TGT GCA CTA AAG-MGBNFQ	69.8	
	Probe B	VIC-CAC TAT TCC TTA CTA AAG ATG TC-MGBNFQ	69.7	

same as for the quantitative assay except that 250 nM each of the polymerase gene forward primer and reverse primers and 100 nM of each type specific probe (type A and type B) were used instead of the matrix gene and EXO primers and probes. Positive and negative controls were used in lieu of an RNA standard curve for the qualitative type-specific assay. The reactions were performed and analyzed in a 7000 Sequence Detection System (PRISM, Applied Biosystems) under the following conditions: 30 min at 48 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

For the quantitative assay, the threshold cycles of clinical samples were compared to a standard curve generated by amplification of known numbers of RSV and EXO RNA transcripts. Results were expressed as RSV copies per mL of original sample. All samples with negative RSV results required detection of at least 200 EXO RNA copies per reaction. RNA extraction (if sufficient sample volume was available) and RT-PCR were repeated on all samples that were negative for both RSV and EXO.

2.6. Preparation of RNA transcripts for quantitative RT-PCR standard curves

The RSV and EXO PCR amplicons synthesized from cDNA with High Fidelity Taq enzyme mix (Roche Applied Sciences, Indianapolis, IN) were cloned into vector pCR2.1 or pCRII-TOPO (Invitrogen, Carlsbad, CA). The plasmids were transformed into *Escherichia coli*, harvested, and the inserts were sequenced. RNA transcription of inserts was performed from the T7 or Sp6 promoter so that negative sense RNA transcripts were synthesized. The RNA was purified, confirmed for size and purity on a bioanalyzer (Center for Expression Arrays, University of Washington, Seattle, WA), and quantified by absorbance at 260 nm. Contaminating DNA was not detected by real-time PCR amplification. Ten-fold serial dilutions of 1×10^7 –10 copies of each RNA transcript were added to RSV real-time RT-PCR reactions in duplicate. The results were used to generate standard curves for quantification of RSV and EXO RNA in clinical samples.

2.7. Statistical analysis

To evaluate reproducibility, intra-assay and inter-assay standard deviations and coefficients of variation (CV) were calculated within and between RT-PCR runs. To compare results between different groups of patients, the Mann–Whitney *U*-test was used for non-parametric variables and student's *t*-test for parametric.

3. Results

3.1. Specificity of the RSV RT-PCR assay

The specificity of the RSV RT-PCR assay was assessed by testing RNA or DNA purified from at least two isolates each of 19 viruses commonly found in respiratory specimens including parainfluenza types 1, 2, and 3, influenza virus types A and B, rhinovirus, coronavirus, metapneumovirus, enterovirus, coxsackie B virus, adenovirus, and herpes viruses 1–8. Nucleic acid extracted from all viral isolates other than RSV type A or B was not detected by the assay.

3.2. Sensitivity of the RSV RT-PCR assay

The assay was able to detect 10 copies of RSV or EXO RNA transcripts added to a reaction, and was linear to 10^8 copies per reaction. RSV subtype A and subtype B RNA transcripts generated standard curves with nearly identical characteristics. Amplification of EXO RNA in the same reaction did not affect amplification of RSV RNA. The minimum number of RSV stock virus seeded into uninfected HL cell culture harvests before specimen preparation that could consistently be detected was 2000 copies/mL.

3.3. Reproducibility

To assess intra-assay reproducibility, 10,000 or 100 copies of RSV A or RSV B were added to six RSV RT-PCR reactions in triplicate. The average CV for the six samples

was 0.9% (range = 0.2–1.7%). To further assess the reproducibility of the assay, RNA was extracted in duplicate from 34 nasal wash specimens. One of the two RNA samples from each specimen was analyzed for RSV by RT-PCR on three different days to determine interassay reproducibility. The duplicate samples for each specimen were run on the same assay to determine the reproducibility of the RNA extraction procedure. Eleven samples were RSV negative and 23 samples were RSV positive for all analyses. The number of RSV detected in the 23 positive samples ranged from 1.17×10^4 to 2.91×10^8 copies/mL. The average CV of 23 samples analyzed on three different runs was 3.7% (range = 0.2–19.7%). The average CV of 23 duplicate RNA extractions analyzed on the same run was 3.9% (range = 0.1–11.2%).

3.4. External control detection

Among 467 initially RSV-negative specimens, the yield of EXO RNA was >200 copies per reaction for 408 and <200 copies per reaction for 59 (12.7%). The average number of EXO RNA copies detected in the 408 samples with >200 copies per reaction was 698. After repeat RNA extraction and/or amplification of the 59 RSV negative specimens with low external control results, EXO amplification was satisfactory (>200 copies per reaction) for 58 and low again for one nasal wash specimen. Two of the 58 EXO positive specimens were also RSV positive on repeat testing. Thus, overall performance calculations were made based on 750 specimens.

3.5. Detection of RSV in clinical respiratory samples by RT-PCR and FA

RSV was detected in 267 (35.6%) and 286 (38.1%) of 750 respiratory samples by FA and RT-PCR, respectively (Table 2). Of 685 patients with at least one specimen tested, 283 (41.3%) were positive for RSV by PCR and 265 (38.7%) by FA. Among the specimen types tested, 271 (38.7%) of 701 nasal washes, 13 (50%) of 26 nasal swabs, 1 (6.7%) of 16 tracheal aspirates, and 1 (14.3%) of 7 BAL samples were RSV positive by RT-PCR. The sensitivity and specificity of the RSV RT-PCR assay compared to the FA were 99.3 and 95.7%, respectively. Among the 750 samples, 727 (96.9%) had concordant results by the two tests.

Table 2

Detection of RSV by real time RT-PCR compared to the indirect fluorescent antibody assay among 750 respiratory samples from children

Indirect fluorescent antibody results	Real time RT-PCR results		
	Positive	Negative	Total
Positive	265	2	267
Negative	21	462	483
Total	286	464	750

Twenty-one of 288 (7.3%) samples with RSV were positive only by RT-PCR. All 21 were nasal washes, all were positive again after re-extraction and re-testing by RT-PCR, and all were positive using the qualitative RSV type-specific RT-PCR assay. One discordant positive specimen was a repeat sample collected from a 25-month-old patient 7 days after an initially FA positive, PCR positive nasal wash had been collected. There were no differences between the 21 discordant samples and the 265 concordant positive samples in average patient age, gender, or ratio of RSV types detected. The two FA positive, RT-PCR negative samples were nasal washes from an 18-month-old male and a 17-day-old female. Both were negative again after repeat extraction of the original samples and retesting by RSV RT-PCR.

3.6. Quantification of RSV in clinical samples

The number of RSV copies detected in the 286 RT-PCR positive respiratory samples ranged from 840 to 4.2×10^9 copies/mL with a median value of 2.0×10^7 copies/mL. The median copy number of RSV in samples that were positive by both FA and RT-PCR was 2.5×10^7 copies/mL versus a median of 3.0×10^4 copies/mL for samples positive by RT-PCR only ($P < 0.001$) (Fig. 1).

3.7. RSV subtype analyses

The qualitative RSV type-specific assay was performed on 280 of the 286 respiratory specimens that were RSV positive by the quantitative RT-PCR assay. Among the 280 RSV positive specimens from 277 patients that were tested, 146 (52%) were found to be type A and 134 (48%) were type B. The median number of RSV copies/mL in type A positive samples was not statistically different from the number in type B positive samples, regardless of whether they were FA positive or negative (Fig. 1). There were no differences between type A and type B positive samples in average patient age, gender distribution, dates of specimen collection, or proportion of hospitalized patients.

3.8. Association of RSV detection and copy number with patient age

The number and proportion among the 750 samples tested that fall into one of four age groups, the proportion within

Table 3

Characteristics of 750 respiratory specimens by age group

Age group	Number (%) of 750 tested	Number (%) RSV positive	Median RSV (log ₁₀ copies/mL)
0 to 6 months	286 (38.1)	138 (48.3)	7.52
7 to 12 months	136 (18.1)	50 (36.8)	6.90
13 months to 5 years	249(33.2)	91 (36.5)	7.32
6 to 20 years	79 (10.5)	7 (8.9)	6.43

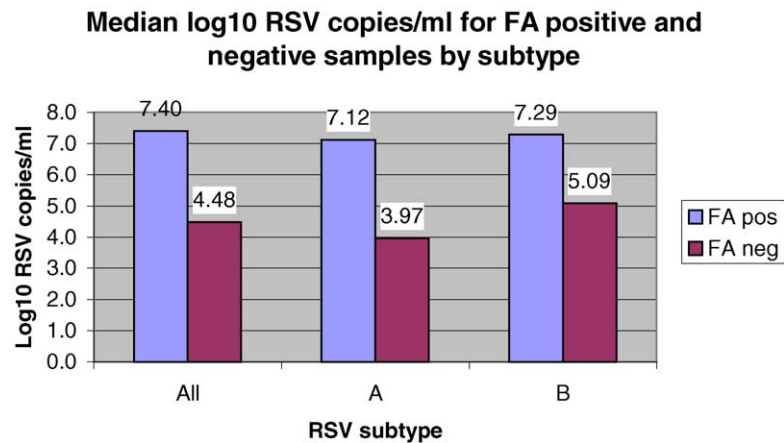


Fig. 1. The median number of RSV, expressed as log₁₀ copies/ml, quantified by real time RT-PCR, was determined for FA positive and FA negative respiratory specimens from children. Among 286 specimens positive by RT-PCR, 265 were FA positive and 21 were negative. Among 280 RSV positive specimens that were subtyped, 133 subtype A and 126 subtype B were FA positive; 13 subtype A and 8 subtype B were FA negative. The number of RSV detected in specimens that were FA positive was significantly higher than the number detected in FA negative specimens ($P < 0.001$) for all groups.

each group that were RSV RT-PCR positive, and the mean number of RSV copies/mL detected in positive samples for each age group are shown in Table 3. The average age of all patients with RSV positive samples was 13.0 months compared to 35.6 months for patients with negative samples ($P < 0.001$). The gender distribution and proportion of hospitalized patients were not different between patients with RSV positive and negative samples. The 280 RSV RT-PCR positive samples that were also tested by the type-specific assay, plotted by virus quantity and age, and sorted by RSV type and FA result, are shown in Fig. 2. Virus load was significantly higher in younger patients. The average age of the 16 patients with specimens containing 8×10^8 or more RSV copies/mL of specimen was 5.4 months compared to 13.4 months for patients with lower RSV loads ($P = 0.004$).

4. Discussion

The quantitative real-time RT-PCR assay described here for the detection of both A and B subtypes of RSV exhibited excellent sensitivity, specificity, and reproducibility. Several features combine to make this a unique assay, including consensus primers targeting the RSV matrix gene, reverse transcription and PCR in a one-step reaction, absolute quantification based on an RNA standard curve, and amplification of a control molecule multiplexed with the RSV reaction. Typing of the RSV positive specimens was performed with a second real-time RT-PCR assay that used primers to the RSV polymerase gene and two type-specific probes labeled with different fluorescent reporter dyes. Absolute quantification of RSV was achieved using standard curves that were generated with negative sense RNA transcripts of the RSV PCR amplicons, providing targets for reverse transcription and amplification that were similar to the RSV genomic RNA present in the clinical specimens. Although the one-step RT-PCR reaction could detect as few as

10 viral RNA copies, the lower limit of sensitivity for clinical specimens (1000 RSV copies/mL) was affected by the amount of respiratory specimen that could be added to a reaction (10 μ l) without producing inhibition. An external control RNA (EXO) was added to each specimen prior to RNA extraction and amplified using primers and probe included in the RSV reaction to prevent false negative RSV results due to poor RNA extraction or to inhibitors of the RT-PCR reaction. Initially low EXO amplification prompted repeat testing of 12.7% of the RSV negative specimens. One sample was subsequently eliminated from analysis due to lack of amplification and two additional RSV positive specimens were identified among the 58 samples that became satisfactory for EXO amplification on repeat testing. Simultaneous amplification of the external control in the RSV reaction provides greater confidence that the negative specimens are truly negative for RSV RNA.

The correlation between the real-time RT-PCR assay and FA for the detection of RSV in respiratory specimens from children was excellent. The prevalence of RSV was only slightly higher by PCR than by FA. Others have reported the prevalence of RSV to be 2–19% higher by PCR than by conventional methods, including FA (Gueudin et al., 2003; Hu et al., 2003; Ong et al., 2001; Whiley et al., 2002) and culture and enzyme immunoassay (Henkel et al., 1997; Kehl et al., 2001). Discrepant results in our study were likely due to the higher sensitivity of the RT-PCR assay. Nineteen (90%) of the 21 PCR positive, FA negative specimens had low viral loads (less than 10^6 copies/mL) compared to only 13% of samples positive by both methods. Fifty percent of the 28 samples with fewer than 10^5 RSV copies/mL were FA negative, suggesting a lower limit of sensitivity for FA in this range. All 21 discrepant RT-PCR positive samples were again positive when retested with the quantitative assay and also with the RSV type specific RT-PCR assay, providing evidence that these were not false positive results.

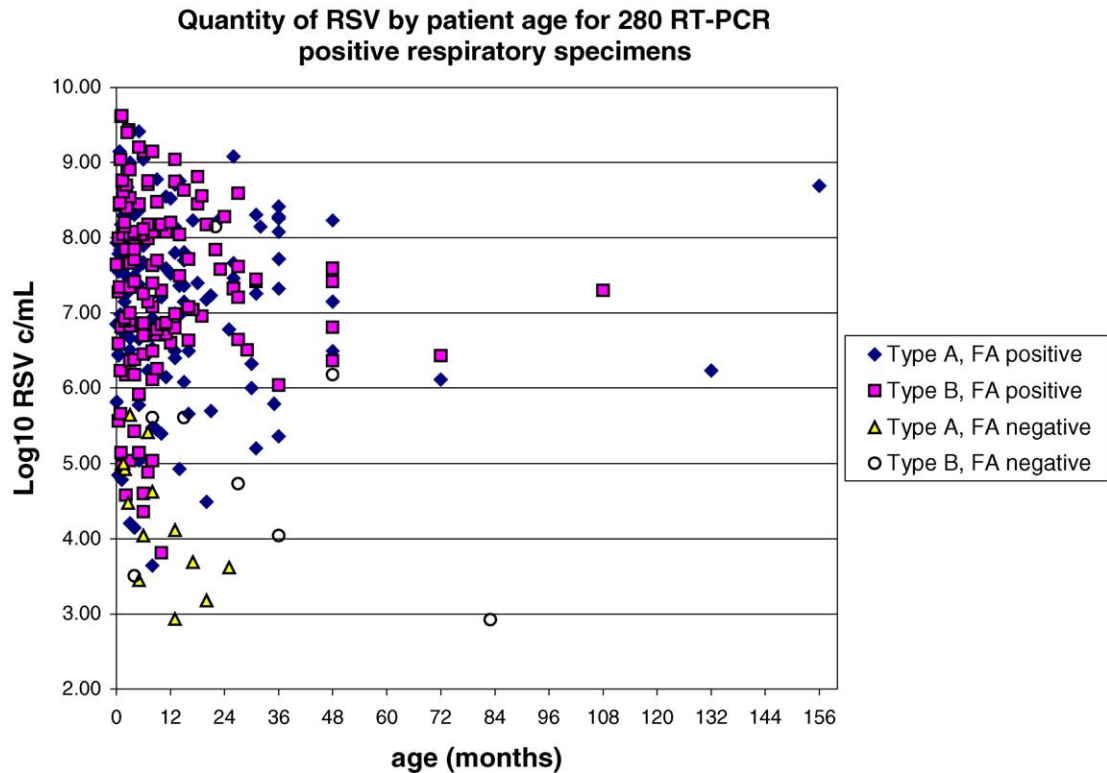


Fig. 2. The number of RSV, expressed as log 10 copies/mL, quantified in 280 samples by real-time RT-PCR is plotted against the patient's age in months. Specimens are sorted by the RSV subtype and the FA result. Six RSV RT-PCR positive specimens were not tested with the type-specific assay.

The pediatric specimens analyzed in this study contained high RSV copy numbers. The median number of 2.0×10^7 RSV copies/mL among all RSV positive specimens was very similar to that found by Borg et al. (2003) and Gueudin et al. (2003) who used quantitative real-time PCR assays to detect RSV type A only, and types A and B, respectively, in pediatric respiratory specimens. In contrast, other investigators, using tissue cell cultures to quantify the virus, detected approximately 100-fold lower titers of RSV in specimens from children than are apparent by PCR (Buckingham et al., 2000; DeVincenzo et al., 2003; Englund et al., 1996; Hall et al., 1976; Wright et al., 2002). RSV subtypes A and B were detected with equal frequency in our study. In contrast, the ratio of RSV subtypes detected has varied greatly among different populations tested as reported by others. The proportion of RSV positive samples that were subtype A has ranged from 15 to 100%, with type A samples more prevalent in most years (Freymuth et al., 1991; Hall et al., 1990; Hu et al., 2003; Kehl et al., 2001; Ong et al., 2001; Tang et al., 1999; Walsh et al., 1997; Whiley et al., 2002). In this population of mostly hospitalized children in Seattle, the presence and quantity of RSV in respiratory specimens was associated with younger age, but not with gender or hospitalization. These results are in agreement with those of Hall et al. (1976) who reported that the quantity of RSV shed was significantly greater in infants less than 1 month of age.

This quantitative real-time RT-PCR assay provides a rapid, specific, reliable, and highly sensitive diagnostic tool

for investigating RSV in clinical specimens. It offers several advantages over viral culture including its potential for high throughput processing, objective endpoint, simpler specimen handling requirements, and ability to quantify a wide range of viral loads. Advantages over FA include increased sensitivity and the objective endpoint. Although the time required to process and test small numbers of samples is similar for both real-time RT-PCR and FA (approximately 4 h to complete 10 specimens), analysis of larger numbers of specimens is more quickly accomplished using RT-PCR because as many as 40 clinical samples can be simultaneously amplified in a 96-well reaction plate, while slides for FA must be read individually. However, the per specimen cost of reagents and supplies required for the RT-PCR assays is about three times higher than for FA and the cost of equipment for the real-time PCR assays is about four times higher than for FA (PRISM 7000 Sequence Detection System versus fluorescent microscope).

The sensitivity of RT-PCR will be especially useful for detection of RSV in specimens from adults, which reportedly contain about 100 to 1000-fold less RSV than specimens from children (DeVincenzo et al., 2003; Englund et al., 1996; van Elden et al., 2003). The diagnostic value of knowing an individual's RSV viral load or subtype is unclear. Some studies suggest that viral load (Buckingham et al., 2000; Hall et al., 1976) and RSV subtype (Hall et al., 1990; Walsh et al., 1997) are associated with severity of illness, while others report no associations (Gueudin et al., 2003;

Martinello et al., 2002; Wright et al., 2002). Combined with the RSV subtyping assay, the quantitative assay described here will provide information to better understand the relationship between illness severity and the quantity and subtype of virus being shed.

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