Disease Severity and Viral Load Are Correlated in Infants With Primary Respiratory Syncytial Virus Infection in the Community

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Respiratory syncytial virus (RSV) is a major cause of respiratory tract infections in infants, with remarkable variability in disease severity. Factors determining severity of disease in previously healthy infants are still unclear. It was hypothesized that disease severity is correlated with viral load in primary RSV infection. Infants of a healthy birth cohort were included at signs of their first respiratory tract infection. Nasopharyngeal aspirate was obtained within 48–96 hr and disease severity was assessed with a previously published severity scoring model. PCR was applied to test the aspirates in a semi-quantitative way for the presence of 10 respiratory pathogens. In case of multiple infection, the pathogen with the highest load was defined as the primary pathogen. The correlation between disease severity and viral load was analyzed. A total of 82 infants were included over a period of 2 years. Median age at first respiratory tract infection was 3 months. Pathogens were detected in 77 (94%) infants; more than one pathogen was detected in 35 (43%) infants. RSV was present in aspirates of 30 infants; in 16 aspirates RSV was the primary pathogen. A negative correlation between RSV CT-value and disease severity was found in all RSV cases ($r = -0.52$, $P = 0.003$) and in cases with RSV as the primary pathogen ($r = -0.54$, $P = 0.03$). In conclusion, this is the first report on viral loads in previously healthy infants with RSV infection in the community. Disease severity correlated positively with viral load during primary RSV infection. J. Med. Virol. 82:1266–1271, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: healthy birth cohort; community; molecular diagnostics; nasopharyngeal aspirate; cycle threshold value

INTRODUCTION

Respiratory syncytial virus (RSV) is one of the most common causes of acute respiratory tract infection in infants. Approximately two-thirds of infants are infected during their first year of life [Collins and Graham, 2008]. There is large variability in the severity of RSV disease, ranging from insignificant clinical illness to severe respiratory distress. The overall hospital admission rate for RSV infection is estimated to be 0.5–2% [Karron et al., 1999; Collins and Graham, 2008]. Of infants admitted to hospital for RSV infection, about 10% require mechanical ventilation [Simoes, 1999; Bont and Kimpen, 2002].

The factors determining severity of RSV respiratory tract infection are still unclear and are likely to be determined by the host and viral factors [Collins and Graham, 2008]. Host derived risk factors for severe RSV infection include prematurity, low birth weight, young
age (<6 months), cardiopulmonary disease, and Down’s syndrome [MacDonald et al., 1982; Bloemers et al., 2007; Collins and Graham, 2008]. Despite these known risk factors, the majority of severe RSV infections occurs in healthy infants. The contribution to the pathogenesis of RSV infection of direct viral cytopathology versus the host immune response in these infants remains controversial [Collins and Graham, 2008]. Publications on viral factors that contribute to RSV disease severity are still sparse [Collins and Graham, 2008]. Published reports disagree as to whether or not there is a difference in the pathogenicity of the two RSV strains, A and B [Hall et al., 1990; McIntosh et al., 1993; Kneyber et al., 1996; Walsh et al., 1997; Hornsleth et al., 1998; Imaz et al., 2000].

Knowledge of the relationship between viral load and severity of disease will expand understanding of pathogenesis of RSV infection and is important for determining the potential benefit of antiviral treatment. Previous studies in infants treated in hospital disagree on the association between viral load in the nasopharynx and disease severity [Buckingham et al., 2000; Wright et al., 2002; Legg et al., 2003; DeVincenzo et al., 2005; Fodha et al., 2007]. Since the populations in these studies were very heterogeneous, and none of these studies did take into account possible co-infections, the level of evidence is limited. Analysis of RSV infection in the community in a healthy birth cohort is relevant, because of the high incidence resulting in a large impact on public health, and because it reveals mechanisms of pathogenesis that potentially apply to infants admitted to hospital. The current study is the first to investigate the correlation between viral load and disease severity during primary RSV infections in the community in previously healthy infants. We hypothesize that higher viral load leads to more severe disease.

**METHODS**

**Study Population**

This study is part of the Netherlands Amniotic Fluid (NAF) study (Wilhelmina Children’s Hospital, Utrecht University Medical Center), a birth cohort focusing on the role of perinatal inflammation in the pathogenesis of RSV respiratory tract infection [Houben et al., 2009]. The included infants were born at term delivery after an uncomplicated pregnancy. For the present observational study, a subgroup of these infants were included at signs of their first respiratory tract infection, in the period between April 2006 and February 2008. The Institutional Ethical Review Board approved the study protocol. Informed consent was obtained from parents of all participants.

**Collection of Data**

Baseline characteristics (gestational age, birth weight, apgar score, gender, breastfeeding, presence of siblings, parental smoking, parental atopy, day-care attendance) and clinical characteristics (age during infection, duration of illness, wheeze, fever (≥38°C), severity score) were collected prospectively. Breastfeeding was defined as being given mother milk beyond the age of 1 month. The presence of siblings was defined as one or more siblings under the age of 18 years living at least 3 days/week in the same house. Parental smoking was defined as smoking by one or both parents of at least one cigarette per day at the age of 1 month. Parental atopy was defined as the history of any atopic diagnosis (asthma, eczema, or hay fever) made by a physician in one or both parents. Day-care attendance was defined as attendance of any day-care during the first year of life.

Parents were instructed to notify the researchers on the second day of the (lifetime) first respiratory tract infection and a house visit was arranged within 36 hr. Disease severity was assessed by the researchers (M.L.H. or R.W.H.), using a previously published severity score by means of a standardized questionnaire and physical examination [Gern et al., 2002; Lemanske et al., 2005]. This scoring model is shown in Table I.

Nasopharyngeal aspirate was obtained using an infant mucus extractor (Vygon Pharmaceutiques, Escouen, France). The catheter was inserted in a nostril to a depth of 5–7 cm and drawn back while the researcher applied suction. Both nostrils were suctioned. The aspirate was directly and stored at −80°C until further work up.

**Pathogen Detection by Real-Time Semi-Quantitative Polymerase Chain Reaction (PCR)**

All samples were tested separately for influenza virus, parainfluenza virus, coronavirus, RSV, rhinovirus, human metapneumovirus (hMPV), bocavirus, and adenovirus. Because of the low incidence of Mycoplasma pneumoniae and Chlamydia pneumoniae, DNA was pooled before testing for these bacteria. Positive pool samples were tested separately to identify the positive sample(s).

**RNA extraction and cDNA synthesis.** RNA extraction was performed on aspirates, diluted in 2 ml viral transport medium, using a MagnaPure LC total nucleic acid kit (Roche Diagnostics, Mannheim, Germany). The isolated viral RNA was reverse transcribed using a MultiScribe reverse transcriptase.

| TABLE I. Scoring of Disease Severity [According to Gern et al., 2002, Lemanske et al., 2005] |
|-----------------|------------------|
| Item        | Point score |
| Fever (≥38°C) | 1               |
| Cough        | 1–2–3          |
| Rhinorrhea   | 1–2            |
| Hoarseness   | 1               |
| Duration of illness >4 days | 1 |
| Apnea        | 3               |
| Wheezing     | 5               |
| Cyanosis     | 5               |
| Retractions  | 5               |
| Tachypnea    | 5               |
| Severity score (sum)* | 0–31 |

* A higher severity score indicates more severe disease.
kit and random hexamers (Applied Biosystems, Foster City, CA), followed by RT inactivation for 5 min at 95°C. Both kits were used according to the manufacturer's guidelines. Murine encephalomyocarditis virus (RNA) or Phocine herpes virus (DNA) was used as internal control.

**Real-time TaqMan PCR.** Type-specific primers and probes for both RSV A and B were based on the highly conserved genomic regions of the N gene. The following primers were used:

RSR-1: 5’-AGATCAACTTCTGTCATCCAGCAA-3’
RSR-2: 5’-TTCTGCACATCATATAAGGAGTTATCCAA-T-3’
RSB-1: 5’-AAGATGCAAATCATAAATTACAGGATTCAGTT-3’
RSB-2: 5’-TGATATCAGCATCTTTAAGTATTTAT-AGTG-3’
RQA probe: 5’-CACCATCCACGAGACGACAGGAGAT-3’
RQA probe: 5’-TTCCCTTCCTAACCCTGGACATAGCATATACATACCT-3’

Primers and probes were tested for possible interactions to enable use in a multiplex assay. Samples were assayed in duplicate in a 25 μl reaction mixture containing 5 μl of cDNA, TaqMan universal PCR master mix (PE Applied Biosystems), primers (900 nM each), and fluorogenic probes (200 nM) labeled with the 5’ reporter dye 6-carboxy-fluorescein (FAM) and the 3’ quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). Amplification and detection were performed with an ABI Prism 7700 system for 2 min at 50°C to attain optimal AmpErase uracil-N-glycosylase activity, 10 min at 95°C, and 45 cycles of 15 sec at 95°C and 1 min at 60°C.

Viral load was determined by the number of amplification cycles needed for a positive PCR test (cycle threshold, CT). Previous studies have shown a highly significant inverse linear relationship between viral load and CT-values [Borg et al., 2003]. For reference, calibration assays in our laboratory showed that a CT-value of 20 equals 2.9 × 10³ particles/ml and a CT-value of 30 equals 6.9 × 10⁶ particles/ml. A CT-value of 45 was chosen as cut-off value for sample positivity. Samples were controlled for the presence of possible inhibitors of the amplification reaction by the indicated internal control signals of which had to range within clear-cut intervals. In case of multiple pathogens the pathogen with the lowest CT-value was defined as the primary pathogen.

**Statistical Analysis**

Non-parametric tests were used, because of non-normally distributed data. Severity score was not normally distributed after logarithmic transformation. Mann–Whitney U-test, Kruskal–Wallis test, and Fisher's exact test were used to compare severity scores, CT-values and clinical variables between groups. Spearman’s correlation was calculated to analyze the correlation between continuous variables. A P-value below 0.05 was considered statistically significant. Analyses were performed with the aid of the Statistical Package for the Social Sciences version 15.0 (SPSS, Inc., Chicago, IL).

**RESULTS**

In this prospective study, 82 infants were enrolled during their first respiratory tract infection. All infants were born term, 61% was male, 61% had one or more siblings, and 70% attended day-care (Table II). The median age of the infants at their first respiratory tract infection was 3 months (interquartile range, IQR 2–5) and the median severity score was 3 (IQR 2–6) (Table III). For these 82 respiratory tract infection episodes, real-time PCR detected 125 pathogens: no pathogen was found in 5 infants, one pathogen in 42 infants, and multiple pathogens in 35 infants (Fig. 1). RSV was detected in 30 infants (37%); in 11 (13%) RSV was the single pathogen. The other 19 infants had aspirates that in addition to RSV contained rhinovirus (n = 17), coronavirus (5), bocavirus (3), or parainfluenza virus 2/4 (3), Mycoplasma pneumoniae (1), Chlamydia pneumoniae (1), or hMPV (1).

The median age of infants with RSV as the primary pathogen was 4 months (IQR 3–5); the median severity score was 5 (IQR 4–11) (Table III). The severity score was higher in infants with RSV as the primary pathogen (median 5, IQR 4–11) than in infants with RSV co-infection in which another pathogen was the primary pathogen (median 3, IQR 2–4, P = 0.007). In addition, RSV CT-values were lower in infants with RSV as the primary pathogen (median 21, IQR 20–23) than in infants with RSV co-infection (median 36, IQR 34–38, P < 0.001). In infants with RSV being the primary pathogen, there was no association between the duration of illness and the RSV CT-value (r = 0.16, P = 0.56), age and severity score (r = −0.14, P = 0.61), or any of the baseline characteristics and severity score (all P-values >0.05) (data not shown).

**TABLE II. Baseline Characteristics of Infants With Primary Respiratory Tract Infection (n = 82)**

| Item                      | Value                        |
|---------------------------|------------------------------|
| Infant Gestational age, wk| 39.9 (39.3–40.6)             |
| Birth weight, kg          | 3.6 (3.3–4.0)                |
| Apgar score, 5 min        | 10 (10–10)                   |
| Male gender               | 50/82 (61)                   |
| Breastfed                 | 48/81 (59)                   |
| Parents/environment       |                              |
| Siblings in household     | 50/82 (61)                   |
| Parental smoking          | 15/82 (18)                   |
| Parental atopy            | 37/80 (46)                   |
| Day-care attendance       | 57/81 (70)                   |

Wk, weeks.
Values represent median (interquartile range) or frequency (percent-age).
Data on breastfeeding, parental atopy, and day-care attendance were missing from 1, 2, and 1 infants, respectively.

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Correlation Between Viral Load and Disease Severity

A positive correlation between RSV viral load and disease severity was observed in infants with RSV in their aspirate during first respiratory tract infection (CT-value and severity score $r = -0.52$, $n = 30$, $P = 0.003$, Fig. 2A). Similar results were found in the 16 cases in which RSV was the primary pathogen (Fig. 2B). In infants in which RSV was the only detected pathogen, the correlation between RSV CT-value and disease severity was even stronger ($r = -0.68$, $n = 11$, $P = 0.02$, Fig. 2C), while there was no correlation in the remaining 19 infants ($r = -0.06$, $P = 0.81$, not shown).

No association was found between rhinovirus CT-value and disease severity in infants with rhinovirus as the primary pathogen ($n = 43$, $r = 0.06$, $P = 0.71$, Fig. 2D) or in infants in which rhinovirus was the only detected pathogen ($n = 22$, $r = -0.23$, $P = 0.31$, not shown).

DISCUSSION

In this prospective birth cohort study, it was found that disease severity during primary RSV infection in the community in previously healthy infants is associated positively with viral load. In infants with RSV as the primary pathogen, viral load was high and moderately related to disease severity.

RSV viral load was found to determine disease severity, which is consistent with conclusions drawn by others stating that RSV disease severity in children treated in hospital depends on viral load [Buckingham et al., 2000; DeVincenzo et al., 2005; Fodha et al., 2007]. Studies that did not find this relation may have been confounded by the presence of risk factors such as cardiopulmonary disease, by RSV immunization, or by the occurrence of co-infections, since none of the before-mentioned studies considered co-pathogens during RSV infection [Wright et al., 2002; Legg et al., 2003]. In the current study, two or more pathogens were found in 43% of the first respiratory tract infections in previously healthy children, strongly suggesting asymptomatic acquisition of respiratory viruses in early infancy. However, the possibility that a previous mild respiratory tract infection was not noticed by the parents or not reported to the researchers cannot be excluded. The correlation between disease severity and viral load was highest in case of single RSV infection and was absent in RSV co-infections. Infants with RSV as the primary pathogen infection were more severely ill. Rhinovirus disease severity was not correlated to viral load or the presence of co-infections. Apparently, the contribution of viral load to disease severity cannot be extrapolated to other viruses, implying distinct pathophysiology of RSV and rhinovirus respiratory tract infection.

The high viral loads found in children with RSV as the primary pathogen in this cohort are in the range of viral loads in infants admitted to hospital with severe RSV lower respiratory tract infection. Similar CT-values (15–25) were found in infants with RSV infection that were ventilated mechanically, using a similar sampling

### TABLE III. Clinical Characteristics of Infants With Primary Respiratory Tract Infection

| Item                  | All infants (n = 82) | No RSV infection (n = 52) | RSV co-infectiona (n = 14) | RSV as the primary pathogena (n = 16) | P-value |
|-----------------------|----------------------|--------------------------|-----------------------------|--------------------------------------|---------|
| Age, mo               | 3.3 (2.2–4.6)        | 3.0 (1.9–4.4)            | 3.8 (2.6–5.6)               | 3.9 (3.1–4.7)                       | 0.04a   |
| Duration of illness, d| 3 (3–4)              | 3 (3–4)                  | 3 (3–4)                     | 3 (3–4)                             | 0.86a   |
| Wheeze                | 5 (6)                | 4 (8)                    | 0 (0)                       | 1 (6)                               | 0.57    |
| Fever                 | 16 (20)              | 8 (15)                   | 1 (7)                       | 7 (44)                              | 0.02    |
| Severity score        | 3 (2–6)              | 3 (2–6)                  | 3 (2–4)                     | 5 (4–11)                            | 0.007a  |

RSV, respiratory syncytial virus; mo, months; d, days.
Values represent median (interquartile range) or frequency (percentage).
Comparison between the three groups by Kruskal–Wallis test and Fisher’s exact test.

aIn case of multiple infection the pathogen with the lowest CT-value was defined as the primary pathogen.

Fig. 1. Frequency of number of pathogens detected during primary respiratory tract infection. A: Based on the total of 82 episodes. B: Based on the total of 125 pathogens detected. Detection frequencies of the most prevalent viruses were: Rhinovirus 53 (65%), respiratory syncytial virus (RSV) 30 (37%), coronavirus 12 (15%), parainfluenzavirus 2/4 8 (10%), bocavirus 7 (9%), adenovirus 6 (7%).

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technique (own unpublished data). Therefore, the conclusion must be that viral load is not a dominant or conditional factor in the pathogenesis of RSV infection. However, this study confirms that, even in relatively mild RSV infections, disease severity depends on viral load. Apparently, RSV viral load modifies the severity in all forms of the disease.

In this study, unique clinical and virological data were collected from otherwise healthy children during their first respiratory tract infection in life. Studying respiratory tract infections in the community in a healthy birth cohort has the advantage of a high incidence, homogeneity of participants, absence of co-morbidity and a known and short interval between onset of symptoms and sampling. Home visits and a validated severity instrument made it possible to assess disease severity in a reliable manner [Gern et al., 2002; Lemanske et al., 2005]. In order to prevent selection bias home visits were performed, rather than inviting infants and their parents to the hospital during illnesses. The broad panel of pathogens included in the quantitative PCR analysis enabled accurate determination of the primary pathogen. Although strong correlations were observed, the study is limited by the relatively small sample size and the absence of data on the immunological response against the RSV infection.

In conclusion, this is the first study showing that disease severity in primary RSV respiratory tract infection in the community in previously healthy infants depends on RSV molecular viral load.

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