Med Microbiol Immunol (2012) 201:311–317
DOI 10.1007/s00430-012-0233-6

ORIGINAL INVESTIGATION

Evaluation of viral load in infants hospitalized with bronchiolitis caused by respiratory syncytial virus

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Received: 20 July 2011 / Accepted: 24 February 2012 / Published online: 10 March 2012

Abstract The relationship between viral load, disease severity and antiviral immune activation in infants suffering from respiratory syncytial virus (RSV)-associated bronchiolitis has not been well identified. The main objective of this study was to determine the existence of a correlation between RSV load and disease severity and also between different clinical markers and mRNA levels of the interferon stimulated gene (ISG)56 in infants hospitalized for bronchiolitis. We also evaluated whether viral load tended to be persistent over the course of the RSV infection. The levels of RSV-RNA were quantified in nasopharyngeal washings, collected from 132 infants infected with RSV as a single (90.15%) or as a dual infection with other respiratory viruses (9.85%). Results indicated that viral load was positively related to the clinical severity of bronchiolitis, the length of hospital stay, the levels of glycemia and the relative gene expression of ISG56, whereas an inverse correlation was observed with the levels of hemoglobin. We also found that the RSV load significantly decreased between the first and second nasopharingeal washings sample in most subjects. These results suggest that infants with high RSV load on hospital admission are more likely to have both more severe bronchiolitis and a higher airway activation of antiviral immune response.

Keywords RSV · Viral load · ISG56 · Bronchiolitis

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Introduction

Bronchiolitis is an acute infection of the respiratory tract and is mainly associated with respiratory syncytial virus (RSV) [1]. The clinical spectrum of RSV bronchiolitis in previously healthy infants is extremely variable, ranging from mild upper respiratory symptoms to severe respiratory distress and, occasionally, death [2]. The factors determining severity of RSV-associated bronchiolitis have not been clearly established and are still unknown and are likely to be determined by a combination of host and viral factors. Host-derived risk factors associated with severe bronchiolitis include young age (<6 months), premature birth (<35 weeks of gestation), low birth weight, immunodeficiency or immunosuppression status, and congenital heart or chronic lung disease [2–4]. In addition single-nucleotide polymorphism in, and expression of, some genes codifying proteins involved especially in the control of innate immune response have been reported as being associated with the RSV disease severity [5–7]. On the other hand, the influence of viral factors such as the RSV-RNA levels on determining the bronchiolitis severity is not fully addressed. To this regard, some studies revealed significant association between disease severity and RSV load in nasopharyngeal secretion of infants with primary respiratory tract infection [8–11]. In contrast, other failed to found a correlation between bronchiolitis severity and viral load during primary RSV infection [12–14].

Furthermore, a recent study found that the correlation between respiratory disease severity and viral load was the highest in case of single RSV infection but was absent in RSV coinfection, suggesting that infants with RSV as the primary pathogen infection were more severely ill [15]. Further, the clinical significance of double infections of RSV and other respiratory viruses also appears to be

unclear, and there are conflicting studies regarding the effect of coinfection on disease severity [16–18].

In the framework of a study aimed at understanding the pathogenesis of RSV infection and at further characterizing viral and host factors involved in determining the severity of bronchiolitis, we addressed whether any differences in RSV-RNA levels in the airway tracts of infants with bronchiolitis might explain the broad clinical spectrum of RSV-associated bronchiolitis. In addition, we evaluated the airway innate immune response by measuring the mRNA levels of gene coding for a cytoplasmatic antiviral protein, strongly induced in the lung after interferon (IFN) production or virus challenge [19], namely the IFN stimulated gene 56 (ISG56). We, then, evaluated whether RSV load variations in bronchiolitis severity were correlated to level of ISG56-mRNA and whether viral load tended to be persistent over the course of the disease.

Materials and methods

Patients

A total of 132 infants with a clinical diagnosis of RSV-associated bronchiolitis, admitted over four successive winter seasons (2006–2010) to the Pediatric Department of Policlinico Umberto I Hospital, were included in this study. The institutional review board at Sapienza University of Rome approved the study. Written informed consent was obtained from the children's parents.

Bronchiolitis was diagnosed from the presence of a history of upper respiratory tract infection followed by acute onset of respiratory distress with cough, tachypnea, retraction and diffuse crackles on auscultation (wheezing alone was not considered sufficient cause for inclusion in the study). The exclusion criteria were underlying chronic condition (such as premature birth, cystic fibrosis, chronic pulmonary disease, congenital heart disease, and immunodeficiency) and recurrent (more than one) wheezing episodes [17]. Disease severity and clinical evolution were evaluated using the clinical score index described by Midulla et al. [17]. In particular, on admission to hospital, a clinical severity score was assigned to each infant within the range 0–8, based on respiratory rate (<45/m = 0, 45–60/m = 1, >60/m = 2) arterial oxygen saturation in room air (>95% = 0, 95–90% = 1, <90% = 2), presence of retractions (none = 0, present = 1, present + nasal flare = 2), and ability to feed (normal = 0, reduced = 1, endovenous = 2).

Specimen collection

Nasopharingeal washings (NPW) were collected from 132 infants suffering from bronchiolitis in the first 24 h after admission to hospital and an aliquot was tested for viruses as previously described [20]. A subset of 56 NPW samples was centrifuged at 2,000 rpm for 10 min and each cell pellet was resuspended in 1 ml of phenol and guanidine isothiocyanate reagent (Trizol, Gibco BRL, NY) and frozen at −80°C for subsequent ISG56 gene expression analysis.

PCR assays for respiratory viruses

A panel of reverse transcription (RT) PCR or nested PCR assays, some in a multiplex format, was used for detection of fourteen respiratory viruses including: RSV, influenza A and B, coronaviruses, OC43, 229 E, NL63, HKU1, metapneumovirus, adenovirus, rhinovirus, parainfluenza 1–3, and bocavirus as previously reported [20, 21].

TaqMan-based real-time RT-PCR technique for RSV detection

A TaqMan-based real-time PCR technique for RSV-RNA quantification was performed on all NPW specimens with positive RT-PCR results for RSV. Briefly, viral RNA was extracted from NPW specimens that were positive for RSV using a QIAamp Viral RNA Mini Kit (Qiagen Spa, Milan, Italy). The RNA was dissolved in RNase-free water and the RSV quantification was performed by Taqman assay after generation of cDNA using a High Capacity cDNA Archive Kit (applied biosystems, Monza, Italy). Type-specific primers and probes for N gene of both RSV A and B [22] were added to the universal PCR master mix (applied biosystems) at 300 and 100 nM, respectively, in a final volume of 50 µl. The standards were obtained by cloning the 82 bp of viral N gene into the pCR2.1 plasmid using a TOPO TA cloning kit (In Vitrogen Corporation, San Diego, CA, USA). A linear distribution ($r = 0.99$) was obtained between 10^1 and 10^8 copies of RSV-DNA. Viral load values were Log transformed for analysis and data was expressed as the Log number of RSV copies per ml of NPW.

TaqMan-based real-time RT-PCR technique for ISG56 evaluation

The mRNA copy content of ISG56 was measured by a real-time 5′ exonuclease RT-PCR assay using the ABI 7000 sequence detector (applied biosystems). Briefly, the total cellular RNA was extracted from the cells using the Trizol reagent, following the manufacturer's instructions, and was retro transcribed as previously described [23]. Primers and probes for ISG56 gene were added to the universal PCR master mix (applied biosystems) at 300 and 200 nM, respectively, in a final volume of 50 µl [23]. Co-amplification of the beta-glucuronidase gene (assay-on-demand, Hs99999908_m1, applied biosystems) was used to normalize

the amount of total RNA present using the threshold cycle relative quantification according to the supplier's guidelines.

Statistical analysis

Descriptive analysis was made using median (range) or frequency (percentage). RSV load values were expressed as Log copy number of RSV-RNA/ml and related geometric mean. Differences between infants with RSV as single or dual infection, in terms of the level of viral load, were compared using the Mann–Whitney test. Spearman's rho coefficient was calculated in order to assess the correlation between the level of RSV load and the demographic and clinical parameters, and ISG56-mRNA levels measured in cells collected from NPW. The differences in the number of RSV–positive infants characterized by the presence or absence of retractions and/or nasal flare according to the RSV load levels were evaluated using the chi squared test for trend. Differences in the RSV load between the first and second NPW sample were compared using the Wilcoxon Test. Significance was fixed at the 5% level. The analysis was performed using SPSS v.13.0 for windows.

Results

Demographic, clinical and virological characteristics of infants suffering from bronchiolitis

One hundred thirty-two infants diagnosed with bronchiolitis over a period of 4 years were included (Table 1). The median age of the infants was 2.20 months, of which 78.69% had <6 months, and the sex ratio was 1. A total of 119 (90.15%) infants carried a single RSV infection whereas 13 (9.85%) had a coinfection with other respiratory viruses. The median clinical score level was 4; 22 (16.7%) infants had a severe bronchiolitis with a clinical score ≥7; 48 infants (36.4%) required oxygen supplementation and 37 infants (28.0%) had retraction with nasal flaring.

Table 1 Demographic and clinical characteristics of 132 infants hospitalized with respiratory syncytial virus (RSV) associated bronchiolitis

Item	Value
Birth weight (kg)	3.05 (1.46–4.50)
Male gender	66/132 (50%)
Weight on admission (kg)	4.6 (2.9–9.1)
Age (months)	2.20 (0.23–32)
Caucasian race	123/132 (93%)
Hemoglobin (g/dl)	11.2 (5.3–17.7)
C-reactive protein (mg/dl)	0.615 (0–38.64)
C-reactive protein >0.8 mg/dl	71/132 (53.79%)
White blood cells (cells/µl)	9,420 (3,630–35,020)
Neutrophils (cells/µl)	3,238 (57–26,600)
Lymphocytes (cells/µl)	3,888 (368–10,136)
Eosinophilis (cells/µl)	58 (0.68–1,081)
Platelets (10^6 cells/µl)	0.47 (0.09–1.12)
Glycemia (mg/dl) (mean ± SD)	112.75 ± 44.15
Sodium (mEq/l) (mean ± SD)	136.90 ± 3.64
Presence of fever[a]	72/129 (55.81%)
Clinical score index[b]	4 (0–8)
Days in hospital (days)	5 (0–27)
Infants with RSV single infection	119/132 (90.15%)
Infants with RSV coinfection	13/132 (9.85%)
RSV + rhinovirus	3/132 (2.27%)
RSV + metapneumovirus	3/132 (2.27%)
RSV + bocavirus	7/132 (5.30%)

Data are expressed as median (range) or frequency (percentage), unless otherwise indicated

[a] Data on presence of fever were missing from 3 infants

[b] Disease severity were evaluated using the clinical score index described by Midulla et al. [17]

Evaluation of RSV viral load

An RT-Taqman assay was used to determine RSV load (Log copy number of RSV-RNA/ml) in NPW collected from all 132 infants with bronchiolitis. There was a distribution of viral load values of several orders of magnitude in infants with bronchiolitis (Fig. 1). The Log copies of RSV-RNA per ml ranged from 1.68 to 8.93 and the median viral load values was 5.47. The distribution of RSV load showed that half of infants have a copy number of RSV-RNA per ml within the range of 5.47–8.93 Log. When the RSV-positive infants were divided on the basis of RSV detection as a single infection or as a coinfection, the viral load was not significantly different between groups ($p = 0.49$).

Relationship between RSV viral load and demographic, clinical and immunological characteristics of infants with bronchiolitis

The relationship between patient data as independent variables and RSV-RNA levels measured in NPW was analyzed (Table 2). A positive correlation between Log number of RSV-RNA copies/ml and clinical score index was found in infants with RSV infection ($r = 0.17$, $p = 0.024$). In particular, RSV load seems to influence the severity of respiratory disease. Indeed, when RSV loads were categorized into three groups ranked from lowest to highest values and analyzed in function of the presence or absence of retractions and/or nasal flaring, we observed that the number of infants who have both the highest RSV load values (>6.84 Log) and the presence of intercostal retractions

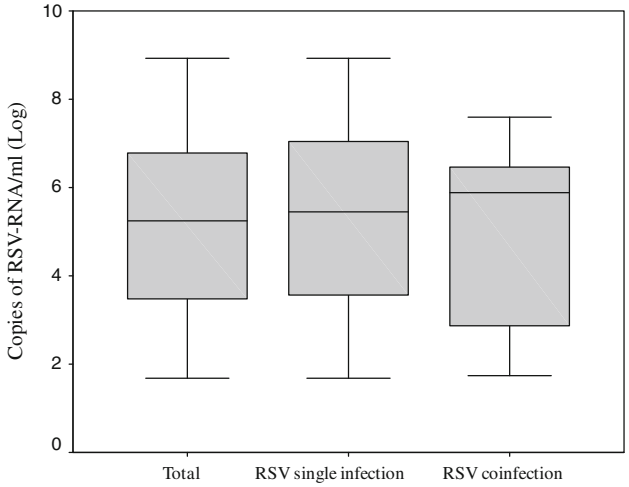

Fig. 1 Level of respiratory syncytial (RSV) load in 132 infants suffering from the first episode of acute bronchiolitis. Log values of RSV-RNA/ml measured in nasopharingeal washings are shown for total RSV-positive infants ($n = 132$) and for those with a single RSV infection ($n = 119$) or a RSV infection with other respiratory viruses ($n = 13$)

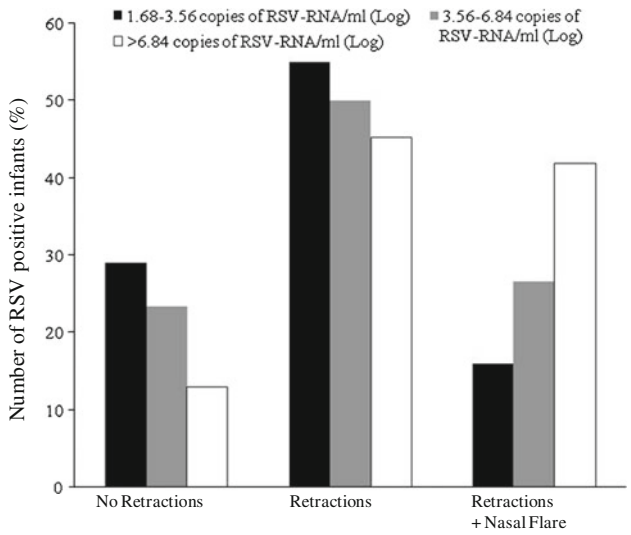

Fig. 2 Number of respiratory syncytial (RSV)—positive infants characterized by the presence or absence of retractions and/or nasal flaring according to the RSV load levels stratified in three groups from lowest to highest values. $p = 0.020$ by using Chi squared test for trend

Table 2 Relationship between respiratory syncytial virus (RSV) load (Log RSV-RNA copies/ml) measured in nasopharyngeal washes and physical and clinical parameters in 132 infants hospitalized with bronchiolitis caused by RSV

Host factors	r*	p
Age (days)	0.15	0.47
Weight (kg)	0.20	0.24
Clinical score index	**0.17**	**0.024**
Days in hospital (days)	**0.16**	**0.038**
Neutrophils (cells/μl)	0.42	0.31
Lymphocytes (cells/μl)	0.11	0.11
Eosinophils (cells/μl)	0.007	0.47
Platelets (cells/μl)	0.005	0.47
Sodium (mEq/l)	0.09	0.23
C-reactive protein (mg/dl)	−0.05	0.29
Hemoglobin (g/dl)	**−0.18**	**0.024**
Glycemia (mg/dl)	**0.24**	**0.028**

* Spearman's rho test; significant correlations are highlighted in boldface

and nasal flaring was higher with respect to the other groups ($p = 0.020$; Fig. 2).

Furthermore, we found a significant correlation between RSV viral load and the length of hospital stay ($r = 0.16$, $p = 0.038$), and levels of hemoglobin ($r = -0.18$, $p = 0.024$) or glycemia ($r = 0.24$, $p = 0.028$). In contrast, we failed to detect any correlation between the RSV-RNA levels and age or weight of tested infants, numbers of neutrophils, lymphocytes, eosinophils or platelets, and levels of sodium, or C-reactive protein (Table 2). Also no difference was detected in viral load for male and female and between infants with fever or without fever (data not shown).

In an attempt to determine whether RSV load influences gene expression of a well-known antiviral protein such as ISG56, levels of RSV-RNA were examined for any significant correlation with expression of ISG56-mRNA. An RT-Taqman assay specific for ISG56 transcript was performed on the NPW samples from a subset of infants with single RSV infection ($n = 56$), for which we collected cells. A significant positive correlation ($r = 0.35$, $p = 0.008$) was observed between viral load and levels of ISG56-mRNA (Fig. 3).

Changing of RSV viral load after the time of hospitalization

We also evaluated changes in RSV load in a subset of NPW samples collected from 17 infants at the time of admission and at hospital discharge, after a median of 2 days (range 1–15 days). Fourteen had single RSV infection and 3 had double infection (2 metapneumovirus; 1 rhinovirus). Results indicated that RSV load decreased significantly ($p = 0.012$) in most infants (71%) between the first (median Log copies of RSV-RNA/ml: 5.95) and second NPW samples (median Log copies of RSV-RNA/ml: 3.22) independently of the presence of RSV as single or dual infection. In particular, a decline of RSV-RNA levels of at least 2.7 Log was observed in half of infants who have a viral load decrease, whereas in the remaining infants the Log copy number of RSV/RNA per ml decreased within the range of 1.76–0.40. In contrast no change in viral load, and an

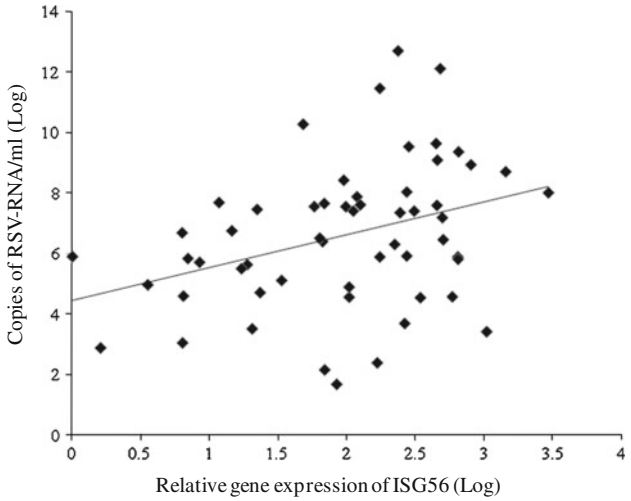

Fig. 3 Positive correlation between respiratory syncytial (RSV) load and gene expression of interferon stimulated gene 56 (ISG56) measured in nasopharingeal washings collected from a subset of 56 infants suffering from bronchiolitis. Rho spearman coefficient ($r = 0.35$); $p = 0.008$

increase in copy number of RVS-RNA copies/ml within the range of 0.18–4.65 Log, were observed respectively in one and four infants.

Discussion

This study showed that level of RSV load, measured shortly after hospital admission, was highly variable among infants suffering from bronchiolitis in agreement to several other studies [9, 10, 12]. In addition we found that coinfection of RSV and other respiratory viruses did not change significantly RSV load although it has been reported that RSV levels were higher in infants with RSV as the primary pathogen that in infants with RSV coinfection [15, 18].

Interestingly, our results demonstrated a positive correlation between RSV load and the clinical score index used for determining the severity of bronchiolitis. In particular, we found that in infants with high levels of RSV-RNA in their NPW, there is a tendency to have more signs of severe respiratory distress. An association between high RSV load and the respiratory disease severity has been reported in previous papers [8–11], whereas other studies have failed to detect such an association [12–14]. Studies that did not find this correlation may have been confounded by the presence in children of risk factors such as cardiopulmonary disease or of previous RSV infections, or by the use of different molecular or virological techniques for RSV level evaluation [12–14]. The conflicting results could be also explained considering the different bronchiolitis diagnostic criteria and/or clinical scores used in the various studies [8–10, 12].

Furthermore, we demonstrated that RSV load was positively correlated with the duration of hospital stay, an indirect indicator of the severity of RSV-associated illness. In agreement, other studies have reported higher nasal RSV load as a significant independent predictor of longer hospitalization [9, 24]. However, in 23% of the infants in which we could measure RSV-RNA level at hospital discharge, an increase of viral load has been recorded.

In addition, our results indicate that viral load correlates with glycemic levels and hemoglobin values but not with other clinical characteristics such as C-reactive protein levels or leukocytes count as reported recently by Franz A and coauthors [25]. The mechanism by which RSV load affects levels of glycemia and hemoglobin is currently unknown. However it has been reported that hyperglycemia is frequent in children with RSV infection [26]. Furthermore, recently anemia has been found to affect the higher rate of thrombocytosis observed in infants with RSV-associated bronchiolitis [27].

The apparent discrepancies along our data on the dynamic of RSV load in bronchiolitis, make more evident the complexity of the RSV-host interactions and suggest the need of evaluating also host factors for fully understanding the pathogenesis of RSV infection. Directly related to this, it is well established that the clinical spectrum of bronchiolitis might reflect differences in the profile and extent of immunological and/or inflammatory mediators produced by RSV infected respiratory epithelial cells [6, 28, 29].

Here, we demonstrated for the first time to our knowledge that a high RSV-RNA level correlates with high levels of ISG56-mRNA in the airways of infants suffering from bronchiolitis. This protein has been implicated in the suppression of viral replication and protein translation and it seems also to be a mediator of negative-feedback regulation of virus-triggered induction of type I IFN and cellular antiviral response [30]. Interestingly it has been demonstrated that the nonstructural NS1 and NS2 proteins of RSV appear to antagonize both the cellular antiviral response as well as the induction of IFN transcription [31–33]. In addition Ling and coauthors have showed that NS2 inhibited ISG56 promoter activity induced by RNA-activated RIG-I in a dose-dependent manner [34]. However, although RSV has evolved accessory gene products that neutralize or inhibit various steps of the IFN pathway, in our previous study we found a strong and a coordinated induction of IFN stimulated genes in the airway tract of infants with RSV infection [28]. Furthermore recently Pichlmair and coauthors have proposed that ISG56 protein antagonizes viruses by sequestering specific viral nucleic acids [35].

Therefore our results suggest that quantitative differences in the replication of RSV may influence the magnitude of innate immune activation which in turn can

determine the clinical course of bronchiolitis in either beneficial or detrimental direction as previously suggested [5, 28].

A potential limitation of this study was that we did not perform the evaluation of known pathogenic bacteria to explore whether the presence of bacterial superinfection affects levels of RSV-RNA. Indeed, considering that infants hospitalized for bronchiolitis have low reported rates of serious bacterial illness [36], we have collected only NPW and not well established respiratory samples such as those from lower respiratory tract, which are likely most suitable for performing isolation and identification of bacteria. In addition, although the marked elevation of C-reactive protein observed in some of the RSV-positive infants could hints to bacterial superinfection, no significance difference in leukocyte count as well as in chest X rays signs was found among infants who have elevated C-reactive protein levels and those with normal C-reactive protein values suggesting that globally there is no concomitant bacterial superinfection (data not shown). Furthermore, recently it has been demonstrated that C-reactive protein levels could be affected by the presence of respiratory viral infection [25].

In conclusion, our results showed that RSV load has a significant impact on the clinical and immunological parameters of hospitalized infants suffering from bronchiolitis and underline the importance of implementing diagnostic tools for a rapid, sensitive and quantitative RSV detection in order to improve the clinical management of bronchiolitis**.** Further larger scale studies are needed to support the predictive value of measuring RSV load on the clinical course of bronchiolitis and to establish the actual "cutoff value" of RSV load associated to disease severity.

Acknowledgments This work was supported by grants to G.A from Pasteur Institute (Cenci Bolognetti Foundation; title of the project: "Molecular characterization of viruses causing bronchiolitis and study of viral and host factors affecting Type I IFN antiviral response induced by respiratory viruses"), and to A.P. from "Sapienza" Università di Roma (Fondi ricerche Universitarie) and Italian Ministry of Health (Ricerca Finalizzata Conv. No. 88).

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