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ARTICLE

Impact of rhinovirus nasopharyngeal viral load and viremia on severity of respiratory infections in children

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Abstract There are few and partially discordant data regarding nasopharyngeal rhinovirus (RV) load and viremia, and none of the published studies evaluated the two variables together. The aim of this study was to provide new information concerning the clinical relevance of determining nasopharyngeal viral load and viremia when characterising RV infection. Nasopharyngeal swabs were obtained from 251 children upon their admission to hospital because of fever and signs and symptoms of acute respiratory infection in order to identify the virus and determine its nasopharyngeal load, and a venous blood sample was taken in order to evaluate viremia. Fifty children (19.9 %) had RV-positive nasopharyngeal swabs, six (12 %) of whom also had RV viremia: RV-C in four cases (66.6 %), and RV-A and RV-B in one case each. The RV nasopharyngeal load was significantly higher in the children with RV viremia ($p<0.001$), who also had a higher respiratory rate ($p=0.02$), white blood cell counts ($p=0.008$) and C-reactive protein levels ($p=0.006$), lower blood O_2 saturation levels ($P=0.005$), and more often required O_2 therapy ($p=0.009$). The presence of RV viremia is associated with a significantly higher nasopharyngeal viral load and more severe disease, which suggests that a high nasopharyngeal viral load is a prerequisite for viremia, and that viremia is associated with considerable clinical involvement.

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Introduction

Rhinoviruses (RVs), which are frequently found in the nasopharynx of patients with respiratory disease [1], were identified some years ago and, although they have been widely studied, many aspects of their role in causing respiratory infections remain unclear. They were initially considered to have little medical significance because they were only isolated in subjects with upper respiratory tract infections (RTIs), mainly common colds [1]. However, the more recent availability of modern molecular methods of viral screening [2] has led to them being identified in the nasopharyngeal secretions of subjects with bronchitis, bronchiolitis and pneumonia, and it has been demonstrated that they are frequently associated with asthma exacerbations [3–8].

The real etiological role of RVs is difficult to ascertain. As they are shed in respiratory secretions for several days after the clinical resolution of an acute episode of RTI, their presence in the nasopharynx of a subject with acute RTI may only be due to prolonged shedding after a previous infection and is not proof that they caused the new disease [9–12]. Knowing the importance of RVs is critical for deciding the need for new preventive and therapeutic strategies, particularly in the case of lower RTIs. It has recently been suggested that simultaneously determining nasopharyngeal RV load and identifying RV viremic cases could differentiate the respiratory infections in which RV is the real causative agent [12]. However, the published data are few and partially discordant, and no study has yet evaluated the two variables together [13–20]. Moreover, there are no published data concerning the possible relationships between RV nasopharyngeal viral load, viremia and the severity of RV-associated disease.

The aim of this study was to provide new information concerning the clinical relevance of determining nasopharyngeal viral load and viremia when characterising RV infection.

Methods

Patient enrolment

The study was approved by the Institutional Review Board of the University of Milan, Milan, Italy, and was prospectively carried out at Pediatric Clinic 1 of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, between 1 November 2011 and 31 March 2012. The written informed consent of a parent or legal guardian was required, and the older children were asked to give their assent.

All of the children aged between 1 month and 14 years admitted to Pediatric Clinic 1 because of fever (i.e. an axillary temperature >38 °C) and signs and symptoms of acute RTI with an onset of less than 24 h before (i.e. rhinorrhea, cough, tachypnea, dyspnea or respiratory distress, and breathing with grunting or wheezing sounds with rales) were considered eligible for the study. The exclusion criteria were symptom onset more than 24 h before and chronic conditions increasing the risk of respiratory infections, including premature birth; chronic disorders of the pulmonary or cardiovascular systems; chronic metabolic diseases; neoplasia; kidney or liver dysfunction; hemoglobinopathies; immunosuppression; diseases requiring long-term aspirin therapy; and genetic or neurological disorders. The diagnoses of RTIs (upper RTI: common cold and pharyngitis; lower RTI: bronchitis, bronchiolitis, infectious wheezing and pneumonia) were based on well-defined clinical and laboratory criteria [21]. All of the children with lower RTI underwent chest radiography, and the radiograph was evaluated by an independent expert radiologist who confirmed pneumonia using the World Health Organisation (WHO) criteria for the standardised interpretation of pediatric chest radiographs for a diagnosis of pneumonia [22].

On the day of enrolment, a nasopharyngeal swab of respiratory secretions was taken using a pernasal flocked swab, and stored in a tube of UTM-RT (Kit Cat. No. 360c, Copan Italia, Brescia, Italy), and a blood sample was drawn using standard procedures. Nasopharyngeal swab was used because it is the simplest sampling method for the identification of respiratory viruses without any reduction in diagnostic performance in comparison to nasopharyngeal aspiration and nasal washes. Moreover, it has the advantages of improved standardization in different age groups and between operators, and better acceptability in a wider range of settings [23]. One swab per patient was collected. Plasma was obtained by centrifuging an aliquot of 10 mL of whole blood at 2,500 rpm for 15 min, some of which was used to determine RV viremia, and the rest for the other laboratory variables, including a white blood cell (WBC) count, the measurement of C-reactive protein (CRP) levels, and the preparation of blood cultures.

The viral tests were performed for study purposes and were always available after discharge. Upon enrolment, detailed information was recorded concerning the patients' demographics and history, and the clinical characteristics of the disease. The children with pneumonia received antibiotic therapy in accordance with the guidelines of the Expert Committee of the European Society for Paediatric Infectious Diseases [24]. All of the children with mild disease returned home and were followed up by means of a bi-weekly telephone call until clinical cure. The children with more severe disease were hospitalised and monitored daily until discharge.

Respiratory viral analyses of nasopharyngeal swabs

Viral RNA or DNA was extracted from the nasopharyngeal swabs by means of a Nuclisens EasyMAG automated extraction system (Biomeriéux, Craponne, France), and the extracts were tested using the RVP Fast assay (Luminex Molecular Diagnostics Inc., Toronto, Canada) in accordance with the manufacturer's instructions. The RVP Fast assay simultaneously detects influenza A virus (subtyped H1 or H3), influenza B virus, RSV-A and -B, parainfluenza-1, -2, -3, and -4, adenovirus, human metapneumovirus, coronaviruses 229E, NL63, OC43, and HKU1, enterovirus/RV, and human bocavirus [25, 26].

The enterovirus/RV-positive nasopharyngeal samples were retested in order to identify the rhinovirus and determine viral load. The real-time RT-PCR assay was performed using the iAg-Path-ID one-step RT-PCR Kit (Applied Biosystems, Foster City, CA), the primers and probe sequences reported by Lu et al. [27], and previously described methods [6].

The hypervariable part of the 5′ NCR, the entire VP4 gene, and the 5′ terminus of the VP2 gene were amplified using an RT-PCR assay as previously described [28], with minor modifications. The eluted RNA was transcribed into cDNA using Moloney Murine Reverse Trascriptase (MMLV-RT, Invitrogen, Monza, Italy) and random hexamers for 1 h at 37 °C, and the MMLV-RT was subsequently denatured at 70 °C. The PCR was carried out in a final volume of 50 μL containing the virus-specific oligonucleotide primers (0.2 mM each), 2 U AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems), 1x reaction buffer, 0.2 mM each of dNTP, 2 mM $MgCl_2$ and 2 mL of c-DNA template. The 40 PCR cycles consisted of a denaturation step (45 s at 95 °C), an annealing step (45 s at 61 °C), and a DNA extension step (1 min at 72 °C). The presence of 549 bp PCR products was visualised after electrophoresis on an ethidium bromide-stained 2 % agarose gel.

The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Milan, Italy), and the purified products were sequenced in both directions using the same forward and reverse primers as those used in the PCR. The nucleotide sequences were obtained by means of automated DNA sequencing using an ABI PRISM 3730 genetic analyser (Applied Biosystems). In order to identify the RV species (A, B and C), the newly determined sequences were checked and aligned using the BioEdit program, and the resultant consensus sequences were compared with sequences

from GenBank using the nucleotide-nucleotide BLAST algorithm (http://www.ncbi.nlm.nih.gov). The analysed fragment was 400 nt in the VP4/VP2 region.

A plasmid containing the corresponding target viral sequence (kindly provided by Dr Baldanti, Molecular Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy) was then used to quantify the positive samples. Ten-fold plasmid serial dilutions ranging from 5 to 5×10^7 input copies were prepared to generate calibration curves and then run in parallel with the tested samples. The cycle threshold (Ct) values of each dilution were measured in duplicate, and plotted against the logarithm of their initial quantities. The copy numbers present in each clinical sample were derived from the regression line. The quantitative results were expressed as $\log_{10}$ RNA copy numbers/mL nasopharyngeal swab following data multiplication by the appropriate dilution factor. In order to evaluate reproducibility, intra-assay and interassay standard deviations (SDs) and coefficients of variation (CVs) were calculated for each standard concentration within and between the individual PCR runs. Each run included template and non-template controls.

Determination of RV viremia

RV viremia was only analysed in the plasma samples of patients with RV-positive nasopharyngeal swabs. RNA was extracted using a Nuclisens EasyMAG automated extraction system in accordance with the manufacturer's instructions, and the extracts were tested by means of quantitative real-time RT-PCR using the same primers and methods as those used to measure the viral load of the nasopharyngeal specimens. The nasopharyngeal and plasma samples were handled separately in order to prevent cross-contamination.

Statistical analysis

The continuous variables are given as mean values ± standard deviation (SD), and were analysed using a non-parametric Wilcoxon rank-sum test or the Kruskal-Wallis test as appropriate. The categorical variables are given as numbers and percentages, and were analysed by means of contingency tables and the chi-squared or Fisher's exact test as appropriate. All of the tests were two-sided and a p value of <0.05 was considered statistically significant. The data were analysed using SAS version 9.1 statistical software (SAS Institute, Cary, NC, USA).

Results

Fifty (19.9 %) of the 251 children enrolled (157 males, 62.5 %; mean age±SD, 2.9±3.7 years) had RV-positive nasopharyngeal swabs. RV was the only virus identified in 31 cases (62.0 %), whereas it was detected with two other viruses in 18 cases (36.0 %) and with three other viruses in one (2.0 %). The RV was RV-A in 18 cases (36.0 %), RV-B in five (10.0 %), RV-C in 22 (44.0 %), and untypeable in five (10.0 %). Viremia was identified in six cases (12 %): RV-C in four (66.6 %), and RV-A and RV-B in one case each.

Table 1 shows that nasopharyngeal RV load was independent of all of the studied variables, including the demographic data, disease diagnosis, respiratory rate, O_2 saturation, the need for O_2 therapy, hospitalisation, admission to an intensive care unit, and the laboratory findings. The blood cultures of all of the patients were negative. The only significant relationship seemed to be the presence of RV viremia: the plasma-positive children had a significantly higher nasopharyngeal RV load (7.90 ± 1.07 $\log_{10}$ cp/mL vs 5.15 ± 1.53 $\log_{10}$ cp/mL; $p<0.001$).

Table 2 summarises the characteristics of the patients with RV-positive nasopharyngeal swabs on the basis of their plasma RV results. In comparison with the patients without RV viremia, the children with RV viremia were significantly more frequently female ($p=0.03$) and, at the time of enrolment, had a significantly higher respiratory frequency ($p=0.02$), significantly lower O_2 blood saturation levels ($p=0.005$), required O_2 therapy significantly more often ($p=0.009$), and had significantly higher WBC counts ($p=0.008$) and C reactive protein levels ($p=0.006$). Moreover, nasopharyngeal viral load was significantly higher in the children with RV viremia ($p=0.02$).

Table 3 shows nasopharyngeal RV load in the children with and without viremia by type of RV. RV was the only infectious agent identified in the nasopharynx of RV viremic patients, who had higher mean nasopharyngeal loads of all of the RV types. However, given the limited number of viremic patients with RV-A or RV-B infection, the only significant difference in nasopharyngeal viral load was observed in the case of the children infected by RV-C ($p=0.03$). RV detected in blood was always of the same type of RV detected in nasopharyngeal secretions.

Discussion

Our findings confirm that RVs are frequently found in the nasopharynx of children with respiratory diseases (regardless of the site of infection), sometimes together with one or more other respiratory viruses [6, 8]. In terms of clinical relevance, we did not find any positive correlation between nasopharyngeal RV load and the type of respiratory infection diagnosed, but the children who were positive for RV viremia simultaneously had a significantly higher viral load and more severe disease, thus suggesting that the former is a prerequisite for viremia and is more frequently associated with greater clinical involvement. The lack of a correlation between nasopharyngeal

Table 1 Nasopharyngeal rhinovirus (RV) load by demographic and clinical characteristics upon enrolment

Characteristics	*n*	Mean nasopharyngeal RV load (log_{10} cp/mL)	Standard deviation	*P* value[a]
Age				
<1 year	17	5.80	1.78	
≥1 year	33	5.32	1.70	0.23
Gender				
Male	30	5.33	1.67	
Female	20	5.70	1.82	0.61
Diagnosis				
Upper respiratory tract infection	25	5.37	1.81	
Bronchiolitis/bronchitis	9	5.63	1.21	
Pneumonia/bronchopneumonia	16	5.30	1.89	0.76
Respiratory rate upon enrolment[b]				
Normal	16	5.01	1.76	
Higher than normal	7	6.02	2.34	0.37
O_2 saturation upon enrolment				
<93 %	8	6.49	2.36	
≥93 %	40	5.30	1.56	0.13
O_2 therapy				
No	34	5.30	1.63	
Yes	16	5.85	1.92	0.24
No. of days of O_2 therapy				
<3	3	6.71	1.45	
≥3	13	5.66	2.01	0.35
Hospitalisation				
No	20	5.60	1.77	
Yes	30	5.40	1.72	0.62
No. of days of hospitalisation				
<7	12	5.22	1.54	
≥7	18	5.75	1.83	0.44
Intensive care (upon enrolment or during hospitalisation)				
No	46	5.47	1.78	
Yes	4	5.59	0.92	0.71
White blood cells, cells/μL				
<15,000	26	5.41	1.73	
≥15,000	24	5.66	1.97	0.90
PCR, mg/dL				
<7	27	5.36	1.76	0.85
≥7	23	5,69	1.82	
RV viremia				
Negative	44	5.15	1.53	
Positive	6	7.90	1.07	<0.001
Type of RV				
A	18	6.46	1.73	
B	5	6.83	1.09	
C	22	5.96	1.76	
Not specified	5	4.48	1.32	0.01
Overall	50	5.48	1.72	

SD standard deviation

[a] Calculated using Wilcoxon's non-parametric rank-sum test or the Kruskal-Wallis test as appropriate

[b] Higher than normal was defined as >60 in children aged <1 year, >50 in children aged 1–3 years, and >40 in children aged >3 years

Table 2 Characteristics of patients with rhinovirus (RV)-positive nasopharyngeal swabs by the presence or absence of RV viremia

Characteristics	Absence of RV viremia, *n* (%)	Presence of RV viremia, *n* (%)	*P* value[a]
Age			
<1 year	15 (34.1)	2 (33.3)	
≥1 year	29 (65.9)	4 (66.7)	1.00
Gender			
Male	29 (65.9)	1 (16.7)	
Female	15 (34.1)	5 (83.3)	0.03
Diagnosis			
Upper respiratory tract infection	23 (52.2)	2 (33.3)	
Bronchiolitis/bronchitis	9 (20.5)	0 (0.0)	
Pneumonia/bronchopneumonia	12 (27.3)	4 (66.7)	0.45
Respiratory rate upon enrolment[b]			
Normal	16 (80.0)	0 (0.0)	
Higher than normal	4 (20.0)	3 (100.0)	0.02
O_2 saturation upon enrolment			
<93 %	4 (9.5)	4 (66.7)	
≥93 %	38 (90.5)	2 (33.3)	0.005
O_2 therapy			
No	33 (75.0)	1 (16.7)	
Yes	11 (25.0)	5 (83.3)	0.009
No. of days of O_2 therapy			
<3	2 (16.7)	1 (25.0)	
≥3	10 (83.3)	3 (75.0)	1.00
Hospitalisation			
No	18 (40.9)	2 (33.3)	
Yes	26 (59.1)	4 (66.7)	1.00
No. of days of hospitalisation			
<7	10 (41.7)	2 (50.0)	
≥7	14 (58.3)	2 (50.0)	1.00
Intensive care (upon enrolment or during hospitalisation)			
No	43 (97.7)	3 (50.0)	
Yes	1 (2.3)	3 (50.0)	1.00
White blood cells, cells/μL			
<15,000	26 (59.1)	0 (0.0)	
≥15,000	18 (40.9)	6 (100.0)	0.008
C reactive protein, mg/dL			
<7	27 (61.4)	0 (0.0)	
≥7	17 (38.6)	6 (100.0)	0.006
Nasopharyngeal RV load			
<median (i.e., 5.5 $\log_{10}$ cp/mL)	25 (56.8)	0 (0.0)	
≥median	19 (43.2)	6 (100.0)	0.02
Range	2.44–8.59	5.94–8.85	
Type of RV			
A	17 (38.6)	1 (16.7)	
B	4 (9.1)	1 (16.7)	
C	18 (40.9)	4 (66.6)	
Not specified	5 (11.4)	0 (0.0)	0.09
Overall	44 (88.0)	6 (12.0)	

[a] Calculated using the chi-squared or Fisher's exact test

[b] Higher than normal was defined as >60 in children aged <1 year, >50 in children aged 1–3 years, and >40 in children aged >3 years

Table 3 Nasopharyngeal rhinovirus (RV) load in children with presence or absence of viremia by RV type

Type of rhinovirus	Absence of RV viremia	Presence of RV viremia	P value[a]
RV-A load (n=18)			
<median (i.e., 6.16 $\log_{10}$ cp/mL), n (%)	9 (52.9)	0 (0.0)	
≥median, n (%)	8 (47.1)	1 (100.0)	1.00
Range	3.90–8.59	8.83	
Mean (SD)	5.83 (1.57)	8.83 (−)	0.15
RV-B load (n=5)			
<median (i.e., 7.26 $\log_{10}$ cp/mL), n (%)	3 (75.0)	0 (0.0)	
≥median, n (%)	1 (25.0)	1 (100.0)	0.33
Range	5.59–7.26	7.64	
Mean (SD)	6.42 (1.18)	7.64 (−)	0.54
RV-C load (n=22)			
<median (i.e., 5.91 $\log_{10}$ cp/mL), n (%)	12 (66.7)	0 (0.0)	
≥median, n (%)	6 (33.3)	4 (100.0)	0.08
Range	2.44–8.38	5.94–8.85	
Mean (SD)	5.38 (1.51)	7.73 (1.25)	0.03
RV-unspecified load (n=5)			
<median (i.e., 4.48 $\log_{10}$ cp/mL), n (%)	2 (40.0)	0 (0.0)	
≥median, n (%)	3 (60.0)	0 (0.0)	–
Range	2.44–7.50	–	
Mean (SD)	4.48 (1.32)	–	–

SD standard deviation

[a] Calculated using Fisher's exact test

RV load and the severity of lower RTIs has been previously reported by van Elden et al. in adults with asthma [13]. However, these data and those collected by us are different from the data published by other authors [14, 16]. Gerna et al. found that RVs were mainly associated with lower RTIs (in the absence of other viral agents) and wheezing in immunocompetent pediatric patients with nasopharyngeal loads of ≥6 $\log_{10}$ RNA copies/mL, whereas lower values represented a probable carrier state in immunocompromised asymptomatic adult patients [14]. Similarly, Takeyama et al. found a significant positive correlation between nasopharyngeal RV load and disease severity in patients aged ≥11 months, but not in younger patients [16]. Finally, Kieninger et al. studied the bronchoalveolar lavages of patients with severe chronic respiratory disease and healthy controls, and found that patients with cystic fibrosis or bronchiectasis had higher RV loads in respiratory secretions than controls, particularly during pulmonary exacerbations, whereas asthmatic patients had similar loads to those measured in healthy subjects [19]. On the basis of our findings, it is possible that these discrepancies may be at least partially due to the different number of viremic patients enrolled in each study, although none of the previous studies simultaneously evaluated nasopharyngeal RV load and RV viremia.

RVs have been traditionally associated with RTIs, and extrarespiratory localisations have been considered relatively unimportant. However, they have recently been linked to pericarditis [28], and RVs have been found in stool samples of children with gastroenteritis, thus suggesting that they may be the cause of diarrhea [15].

Viremia during respiratory RV infection has been studied over recent years but, when RVs could only be identified by means of culture, it was only found in a very small number of children who died suddenly and unexpectedly [29, 30], or because of an acute RTI [31]. Current molecular methods are significantly more sensitive and, contrary to expectations, have revealed that viremia is relatively common in children with RV infection and is usually associated with a more severe course of RV disease. Xatzipsalti et al. detected RV in the blood of 11.4 % of 88 children with upper and lower RTIs, particularly during the early course of disease, and found that it was mainly associated with a significant increase in moderate or severe exacerbations of acute asthma [17]. Similarly, Fuji et al. found that 12.3 % of 243 subjects with RV infection and severe respiratory symptoms were viremic, most of whom had reduced O_2 blood saturation levels [20]. They also found that RV-C was more frequently associated with viremia than RV-A and RV-B, thus suggesting that it may be more pathogenic.

Our findings confirm that viremia is relatively common in RV-positive patients and that it seems to be a marker of more severe RV disease, particularly when the infection is caused by RV-C. The prevalence of viremia in our study was 12 %, which is practically identical to previously reported prevalence, and most of the viremic patients had RV-C infection [20]. Interestingly, the children who were viremic upon admission not only had a worse clinical presentation and a higher

incidence of low O_2 saturation levels, but also had significantly higher WBC counts, CRP levels and nasopharyngeal RV loads. Blood culture for bacteria was negative in all the cases. This finding, although it does not exclude the possible coexistence of a bacterial co-infection due to the poor sensitivity of culture in the identification of bacteremic cases [32], supports the hypothesis that RV was the cause of the disease. On the other hand, in viremic cases RV was the only virus identified both in the nasopharyngeal swab and in the blood.

In conclusion, the findings of this study suggest that nasopharyngeal viral load and viremia influence the severity of RV infection. However, further studies are needed to verify the relationships between viremia and duration of signs and symptoms of disease, duration of viral shedding and risk of superimposed bacterial infections. This information could be useful to decide characteristics of RV-related disease and its outcome.

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Conflict of interest The authors declare that they have no conflict of interest.

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