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Journal of Clinical Virology 61 (2014) 138–144

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Comparison of virological profiles of respiratory syncytial virus and rhinovirus in acute lower tract respiratory infections in very young Chilean infants, according to their clinical outcome

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ARTICLE INFO

Article history:
Received 13 March 2014
Received in revised form 27 May 2014
Accepted 3 June 2014

Keywords:
Respiratory syncytial virus
Rhinovirus
ALTRI
Severe infantile respiratory infection

ABSTRACT

Background: Respiratory syncytial virus (RSV) and rhinovirus (HRV) are the main cause of acute lower respiratory tract infections (ALRTIs) in infants. Viral and host-related risk factors for severe disease have also not been clearly established.
Objective: To assess whether certain viral features of RSV and, or HRV are associated with severe ALRTI.
Study design: RSV and HRV were studied in nasopharyngeal samples of infants by immunofluorescence, Luminex® and/or real-time RT-PCR assays. Quantitation and genotyping of RSV and HRV by PCR were done.
Results: Of 124 virus positive specimens, 74 (59.7%) had RSV; 22 (17.7%) HRV and 28 (22.6%) RSV-HRV co-infection. Hospitalization was required in 57/74 RSV infants (77.0%); in 10/22 HRV cases (45.5%) ($p = 0.006$) and in 15/28 co-infected by both viruses (53.6%) ($p = 0.003$). Severe cases were 33/74 (44.6%) RSV infections, 2/22 HRV cases (9.1%), ($p < 0.002$) and 6/28 (21.4%) patients co-infected by RSV–HRV ($p < 0.026$). Three genotypes (NA1, B7, B9) of RSV circulated during the study. In 33 severe infants, NA1 was detected in 19 cases (57.6%); B7 in 13 (39.4%) and B9 in 1 (3.0%) ($p < 0.01$; OR = 10.0). RSV loads were similar between outpatients and hospitalized infants ($p = 0.7$) and among different severities ($p = 0.7$). NA1 loads were higher than other strains ($p = 0.049$). Three geno-groups of HRV circulated homogeneously.
Conclusion: In very young infants, RSV cause more severe disease than HRV. Co-infection does not increase the severity of illness. NA1 RSV genotype was associated with major frequency of hospitalization, severe respiratory disease and higher viral load.

1. Background

RSV is a leading cause of acute lower respiratory tract infections (ALRTI) in infants [1–3]. In Chile, RSV is the major cause of ALRTI causing yearly winter outbreaks with a fatality rate below 0.1% [4,5]. Rhinovirus (HRV) is the main agent of common cold and his prevalence in ALRI has been underestimated for many years. Recent studies have demonstrated that HRV is a cause of bronchiolitis, as common as RSV, with frequencies of 16 and 18% [6,7].

Clinical features for both viruses, RSV and HRV, may be indistinguishable. Some authors reported that children hospitalized with HRV were older than those infected with RSV and its association with atopy is higher than that recorded in RSV infections [6,8].

Viral and host-related risk factors for severe disease have not been clearly established. Infants younger than 6 months of age are the major risk group for RSV severe disease and some authors have suggested that co-infections with HRV can lead to severe illness [6–9], although anti-inflammatory response is different among HRV and RSV infection [10–13].

2. Objective

The aim of this study was to assess whether certain viral features of RSV and, or HRV are associated with severe ALRTI in

Abbreviations: RSV, respiratory syncytial virus; HRV, rhinovirus; ALTRI, acute low tract respiratory infection; RT-PCR, reverse transcription-polymerase chain reaction; hMPV, human metapneumovirus; NPA, nasopharyngeal aspirates; IFA, indirect immunofluorescence assay.

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http://dx.doi.org/10.1016/j.jcv.2014.06.004
1386-6532/

Chilean infants younger than 6 months of age, according to a clinical score.

3. Methods

3.1. Subjects

Previously healthy term infants, younger than 6 months of age, with a normal weight at birth, having their first acquired-community ALRTI, were consecutively enrolled into the study during the winter seasons of 2010 and 2011 from the Cruz-Melo outpatient clinic and the Roberto del Rio Hospital, in the northern area of Santiago de Chile. Infants were enrolled during the first three days of respiratory symptoms (nasal discharge, cough or respiratory distress). ALRTI was confirmed by clinical signs of respiratory distress with crackles or wheezing, or hyperinflation in a chest radiograph. Exclusion criteria for patients were: (i) previous hospitalization for any cause, (ii) primary or secondary immunodeficiency, (iii) prematurity, (iv) bronchopulmonary dysplasia, (v) previous mechanical ventilatory support, (vi) congenital heart disease, and (vii) any previous respiratory disease, including common cold and otitis media.

3.2. Severity of ALRTI

The severity of ALRTI was classified according to a previously published scoring system [14]. A score of ≥7 points identifies severe infants; 4–6 points indicate a moderate disease and 0–3 points, a mild illness.

3.3. Samples collection

A nasopharyngeal sample (NPS) at enrollment was collected by Copan® swab in 5 ml of Hank's solution during the first three days of respiratory symptoms.

3.4. Viral studies

Immunofluorescent assay (IFA) for RSV, influenza, parainfluenza, metapneumovirus and adenovirus were conducted immediately as described elsewhere.

Nasopharyngeal samples were tested using xTAG RVP FAST by Luminex® and Abbott Molecular®, according to the manufacturer's instructions for RSV, enterovirus/rhinovirus, influenza, parainfluenza, metapneumovirus, adenovirus, coronavirus and bocavirus.

For real time PCR, total RNA was extracted from NPA by the guanidinium thiocyanate–phenol–chloroform method [15]. First-strand cDNA was synthesized using 5 μl of viral nucleic acid, random hexamer primers and MMLV-RT (Promega®), in a Perkin Elmer Gene Amp® PCR System 2400 during 1 h to 37 °C. A fragment of the N gene of RSV was amplified with specific primers [16] by real time PCR in a Light Cycler 1.5 (Roche®). The 5′NCR of HRV was amplified using primers and conditions previously described, resulting in a product of approximately 400 bp [17].

RSV infection was defined by positive IFA and, or PCR and, or Luminex and HRV infection by positive PCR and, or Luminex.

For sequencing of PCR products and phylogenetic analyses for RSV and HRV, the most variable region of the G gene of RSV was amplified with 10 pmol of each primer FV and GAB [18], 2.5 U Taq DNA polymerase (Promega®) and 10 μl cDNA. The reaction was carried out for 5 min at 94 °C and for 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and followed by 10 min of extension at 72 °C. Amplified products of 653 bp of RSV A and 656 bp of RSV B were visualized using ethidium bromide under UV light on a 1.5% agarose gel [16]. When required, semi nested PCR was performed with GAB and F1 primers [18] resulting in 489 bp and 492 bp products, depending on genotype.

PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN®) and FavorPrep™ Gel/PCR Purification Kit (Favorgen-Biotech Corp.). RSV and HRV amplicons were sequenced in both directions by Macrogen, Inc. Multiple sequence alignments were performed using the Clustal W program. Sequence alignment and phylogenetic analyses were performed with MEGA5 software [19], including reference sequences obtained from GenBank database (NCBI). The distance matrix analysis was conducted using the Kimura 2-parameter model and phylogenetic tree was constructed using neighbor joining method. Confidence of clustering of sequences was evaluated by bootstrapping (1000 replicates). Sequences were assigned to RSV genotype if they clustered with a significant bootstrap value >70% [20] and HRV with a value >50% [21]. RSV phylogenetic analyses were performed using 270 nucleotides sequences, including reference strains from all published RSV A and RSV B genotypes. HRV sequences comprised a 320 nucleotides region spanning the 5′ NCR, including 117 previously published sequences.

For quantitation of RSV genomes a quantitative real time RT-PCR was designed. Synthetic RNA was obtained by in vitro transcription after cloning of the N gene in pGEM-T-Easy vector (PROMEGA®). After purification, dilutions of RNA were used as quantitative standards during real time RT-PCR. The RT reaction was performed with specific oligonucleotide N1 (Table 1) and MMLV-RT during 1 h at 42 °C. Amplification was made with primers N1b and N2 and the N-Probe was included. An initial denaturation at 95 °C for 10 min was followed by 45 cycles of amplification (95 °C, 10 s; 58 °C, 10 s; 72 °C, 5 s) collecting fluorescence data on each cycle at 72 °C.

3.5. Statistical analysis

Qualitative and quantitative data were compared between infants groups according to viral results by chi-square and Mann–Whitney Rank Sum test, respectively. Chi-square was also used for comparison of patients according to viral results, genotypes and severity of illness. Statistical analyses were performed using the SigmaStat (3.5) and EPI-Info-7 programs; $p < 0.05$ was considered significant.

4. Results

4.1. Subjects, severity and viral detection

A total of 139 infants were enrolled in the study and none of them died. Fifteen patients were excluded because they were negative for viruses or infected by other viruses. Of 124 included

Table 1
Oligonucleotides used for Respiratory syncytial virus genomes quantitation.

Oligo	Sequence	Purpose
Primer N1	5′-GGAACAAGTTGTTGAGGTTTATGAATATGC-3′	Reverse transcription
Primer N1b	5′-CTACCATATATTGAAYAAYCCAAARGCATC-3′	Amplification
Primer N2	5′-CTTCTGCTGTCAAGTCTAGTACACTGTAGT-3′	Amplification
N-Probe	5′-6FAM-CT + AGGC + AT + A + ATGGG + AGAATA-BBQ-3′	Fluorescent detection

Table 2
Demographic features of the study subjects.

Subject group	Number of cases	Inpatients	Outpatients	Gender		Age (month)	*p*-Value[a]
		n	*n*	M	F	Mean ± SE	
RSV group	74	57	17	31	43	2.236 ± 0.186	
Severe RSV group	33	33	0	16	17	2.379 ± 0.322	
Moderate RSV group	19	17	2	7	12	2.184 ± 0.377	0.9
Mild RSV group	22	7	15	8	14	2.068 ± 0.242	
HRV group	22	10	12	15	7	2.750 ± 0.42	
Severe HRV group	2	2	0	0	2	1.000 ± 0.00	
Moderate HRV group	4	4	0	3	1	4.000 ± 0.577	0.2
Mild HRV group	16	4	12	12	4	2.656 ± 0.53	
RSV–HRV group	28	13	15	14	14	3.071 ± 0.381	
Severe RSV-HRV group	6	6	0	4	2	3.167 ± 0.703	
Moderate RSV-HRV group	10	6	4	5	5	2.200 ± 0.359	0.3
Mild RSV-HRV group	12	1	11	5	7	3.750 ± 0.730	
Total	124	80	44	60	64	2.609 ± 0.149	

[a] *p*-Values were obtained comparing age among severe, moderate and mild subgroups for each viral group (RSV, HRV and RSV–HRV) by Mann–Whitney Rank Sum test. RSV: Respiratory syncytial virus; HRV: Rhinovirus.

patients, 74 (59.7%) were infected by RSV as a single infection; 22 (17.7%) had HRV and 28 (22.6%) both viruses (Table 2); 80 (64.5%) were hospitalized; 41 (33.1%) had severe, 33 (26.6%) moderate and 50 (40.3%) mild illness.

Among 74 RSV cases, 17 (23.0%) were outpatients and 57 (77.0%) required hospitalization. Of 22 infants with HRV, 12 (54.5%) were outpatients and 10 (45.5%) hospitalized. Thirteen of 28 children (46.4%) co-infected by both RSV and HRV were outpatients and 15

Table 3
Clinical features and maximal clinical requirement of the study subjects.

Clinical features	Number of cases	Clinical outcome at discharge			*p*-Value[a]
Groups	124	Severe	Moderate	Mild	
RSV group	74	*n* = 33	*n* = 19	*n* = 22	
Supplemental oxygen requirement	52	33	19	0	<0.001.
Critical care unit	18	18	0	0	<0.001
Mechanical ventilation	16	16	0	0	<0.001
HRV group	22	*n* = 2	*n* = 4	*n* = 16	
Supplemental oxygen requirement	2	2	0	0	<0.001
Critical care unit	0	0	0	0	–
Mechanical ventilation	0	0	0	0	–
RSV–HRV group	28	*n* = 6	*n* = 10	*n* = 12	
Supplemental oxygen requirement	16	6	10	0	<0.001.
Critical care unit	4	4	0	0	<0.001
Mechanical ventilation	4	4	0	0	<0.001
Clinical requirement[b]	Number of cases	Severe	Moderate	Mild	*p*-Value[c]
RSV group	74	*n* = 33	*n* = 19	*n* = 22	
Length of hospital stay (days)		11.09 ± 0.93	4.32 ± 0.34	2.35 ± 0.35	<0.001
Length of supplemental oxygen (days)		8.94 ± 0.86	2.47 ± 0.23	0.00 ± 0.00	<0.001
Maximal FIO_2 administrated (%)		45.14 ± 3.76	27.47 ± 0.32	21.00 ± 0.00	<0.001
HRV group	22	*n* = 2	*n* = 4	*n* = 16	
Length of hospital stay (days)		11.50 ± 4.50	3.50 ± 0.50	1.12 ± 0.47	<0.006
Length of supplemental oxygen (days)		8.60 ± 4.50	2.00 ± 0.41	0.00 ± 0.00	<0.001
Maximal FIO_2 administrated (%)		28.00 ± 0.00	27.25 ± 0.75	21.00 ± 0.00	<0.001
RSV–HRV group	28	*n* = 6	*n* = 10	*n* = 12	
Length of hospital stay (days)		17.17 ± 4.36	4.00 ± 0.42	1.83 ± 0.62	<0.001
Length of supplemental oxygen (days)		15.67 ± 4.41	2.30 ± 0.33	0.00 ± 0.00	<0.001
Maximal FIO_2 administrated (%)		54.75 ± 16.93	28.30 ± 0.88	21.00 ± 0.00	<0.001

[a] *p*-Values were obtained comparing clinical features among severe, moderate and mild patients for each group by Chi-square.
[b] Data are geometric mean ± SE value.
[c] Comparison of clinical requirement among severe, moderate and mild patients for each group by Mann–Whitney Rank Sum test. RSV: Respiratory syncytial virus; HRV: Rhinovirus.

Table 4
Genotypes of respiratory syncytial virus in outpatient and hospitalized infants, according to its clinical outcome.

Clinical outcome	RSV genotypes			
	Inpatients		Outpatients	
	NA1[a]	B7/B9	NA1[a]	B7/B9
Severe	10	2	–	–
Moderate	4	0	–	2
Mild	2	1	3	9
Total	19		14	

[a] NA1strains were more frequent in hospitalized infants ($p < 0.001$), and with severe disease ($p = 0.01$; OR = 10.0). Chi-square.

(53.6%) were hospitalized. Significantly more children were hospitalized with RSV infection than those infants infected with HRV ($p < 0.007$) or co-infected with both viruses ($p < 0.04$). No differences were observed in gender ($p > 0.09$) nor age ($p > 0.1$) between RSV, HRV and co-infected infants.

Severe cases were significantly more frequent in RSV infected patients (33/74, 44.6%) than HRV infants (2/22 = 9.1%) ($p = 0.002$) and cases with RSV and HRV (6/28, 21.4%) ($p = 0.04$). By contrast, infants infected with HRV showed a milder disease (16/22, 72.7%) than those with RSV (22/74, 29.7%) ($p < 0.001$) and co-infected patients 12/28 (42.9%) ($p = 0.04$). Moderate disease cases were similar among groups: 19/74 (25.7%) infected by RSV; 4/22 (18.2%) with HRV and 10/28 (35.7%) cases with both viruses ($p > 0.3$) (Tables 2 and 3).

Clinical features and requirements for each viral group were distributed significantly according to clinical outcome (Table 3).

4.2. RSV genotypes

Typification was performed in 33 RSV cases (Table 4); 14 (42.4%) of them were from outpatients and 19 (57.6%) in-patients. Among 33 genotipified RSV, 19 were NA1 genotype (57.6%) belonging to the group A; 13 were B7 (39.4%) and 1 was B9 (3.0%). Both genotypes belong to the group B. The NA1 strains were significantly more frequent in hospitalized infants ($p < 0.001$) and in severe disease ($p = 0.01$; OR = 10.0). This difference is mainly supported among infants with severe and mild disease ($p < 0.03$; OR = 10.0). There were no differences between RSV genotypes and the type of infection, i.e. single infection or co-infection ($p = 0.5$).

4.3. RSV viral load

Sixty-seven NPS were evaluated for viral load of RSV; 46 (68.7%) were from inpatients and 21 (31.3%) from outpatients. Inpatients' median viral load was 417.300 copies/μl/sample (Standard Error (SE): 1341.2), compared to 133.200 copies/μl/sample (SE: 6569.3) from outpatients; ($p = 0.7$). Twenty-six samples corresponded to severe children, 20 to moderate and 21 to mild infants. Median viral load was 293.100 copies/μl/sample (SE: 2063.76) for severe patients, 286.200 copies/μl/sample (SE: 1277.04) for moderate disease and 360.600 copies/μl/sample (SE: 6577.52) for mild disease ($p = 0.711$). The median viral load of NA1 genotype was 963.000 copies/μl/sample (SE: 7435.02), while for B genotype was 33.525 copies/μl/sample (SE: 1571.546) ($p = 0.04$).

4.4. Rhinovirus genotypes

Fifty children were infected with HRV: 22 as a single infection and 28 as co-infection with RSV. We could typify 21/50 strains of HRV (42%); 13 outpatients and 8 hospitalized infants (Fig. 1). Seven strains belong to genogroup A, seven to the genogroup B and seven to genogroup C. There were no differences among genogroups of HRV, as a single infection or as co-infection whit RSV ($p = 0.8$), neither according to severity of illness.

5. Discussion

During two consecutive winter seasons it was confirmed the high frequency of RSV infections in infants younger than 6 months, but also the high frequency of rhinovirus infections. The need for hospitalization of infants infected with RSV was significantly higher compared with those infected by HRV or co-infected with both viruses.

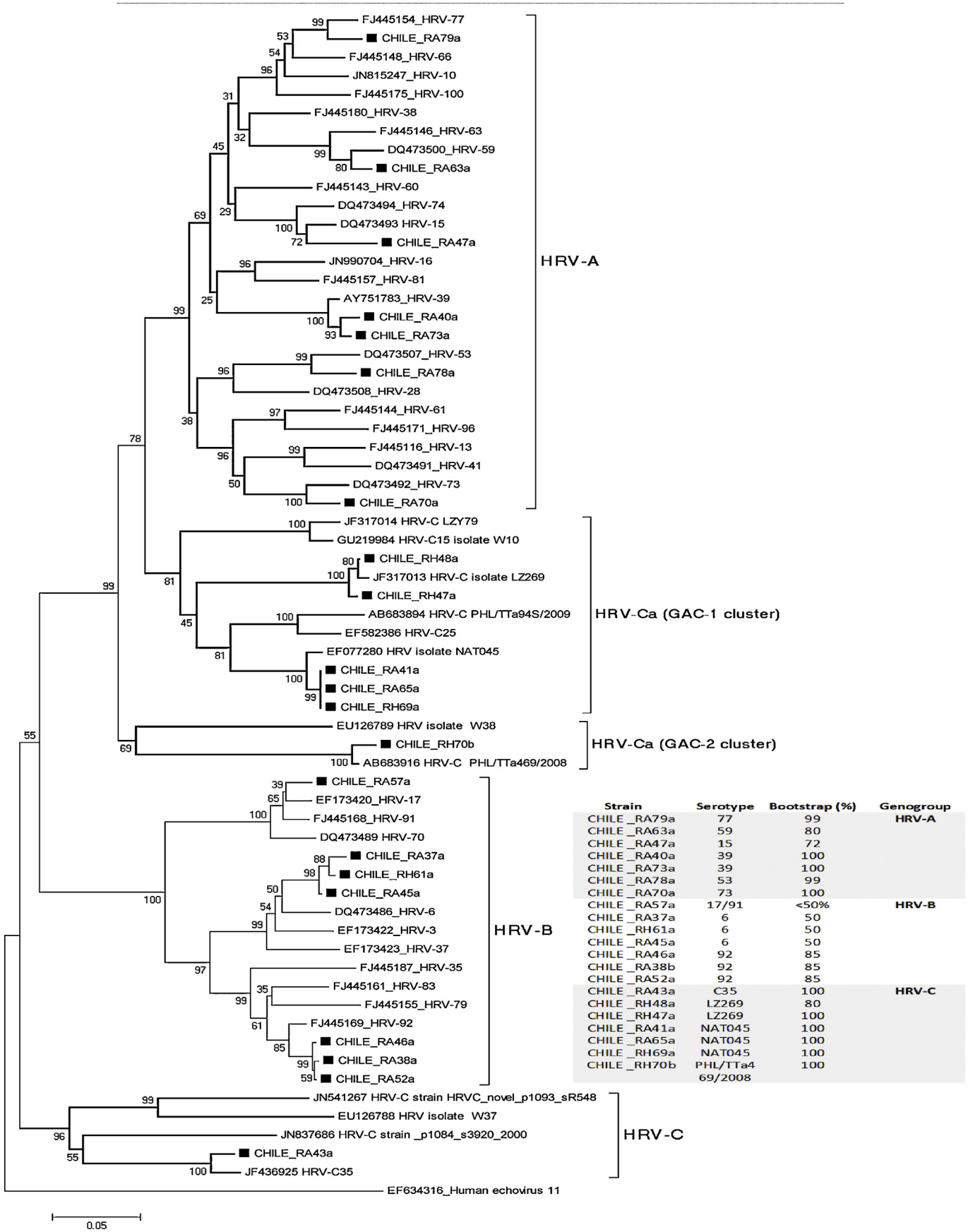
Unlike other authors, who observed that infants with ALRTI caused by HRV can be as serious as those infected with RSV [6,8,22,23], our results indicate that RSV infection was significantly more severe than rhinovirus infection and also than co-infection with both RSV and HRV viruses. One possible explanation is that in our study patients were infants younger than 6 months of age, with a median of 2.6 months, which were attended in their first episode of respiratory infection. Similar results were found by Marguet et al. [9]. It is known that severe RSV infections mainly affect very young infants [2,3], while severe HRV infections principally could affect children older than 6 months of age, or infants with previous morbidity [6,8,24].

Certain clinical conditions increase the risk of developing severe RSV disease. Likewise the impaired immune response, such as low counts of lymphocytes subpopulations and poor blood proinflammatory cytokine production, elicited by the virus plays a leading role in the severity of respiratory disease [1,14,25]. In recent years, new studies indicate that genetic factors linked to the host could have an important role in the phenotype of RSV disease [1,26–29]. Additionally, controversial results have been obtained regarding the link between genotypic variations of the virus and disease severity [18,20,30].

Previous studies have demonstrated the co-circulation of multiple RSV genotypes during annual outbreaks and the most prevalent strain depends on geographical and seasonal factors [31,32]. No connection has been well established among circulating genotypes and the size of the epidemic, nor with their clinical impact, since results are contradictory [20,32,33]. In Chile, it has been detected more frequently epidemics with predominance of group A [34]. Previously, we found no differences between virus genogroup and clinical outcome. Until now, NA1 genotype had not been detected in Chile. This genotype was significantly more related to hospitalized infants and severe disease, especially during the winter season of 2010 and regardless of single RSV infection or a co-infection with HRV.

NA1 genotype was previously described and we believe that its introduction in Chile occurred in recent years [35–37]. This would explain why most of NA1 strains were found during 2010: 14 of 19 strains (73.7%) which also were significantly associated with severe disease. In fact, during 2011, although there was also circulation of NA1, B7 strain was the most prevalent, reducing significantly the severity of disease. Additionally, from 5 infants infected with NA1 in 2011, only one had a severe disease. So, we hypothesized that the emergence of new RSV strains may be associated with severe disease in infants when the general population is still not immune to these new genotypes.

Association between RSV loads and disease severity is still controversial. Although some authors have not founded correlation between severity of illness with titer of RSV [38], other studies have correlated significantly higher viral load with severe RSV bronchiolitis in infants [33,39]. In our study, viral loads were similar between severe and mild patients except for the NA1 strain,



Strain	Serotype	Bootstrap (%)	Genogroup
CHILE _RA79a	77	99	HRV-A
CHILE _RA63a	59	80	
CHILE _RA47a	15	72	
CHILE _RA40a	39	100	
CHILE _RA73a	39	100	
CHILE _RA78a	53	99	
CHILE _RA70a	73	100	
CHILE _RA57a	17/91	<50%	HRV-B
CHILE _RA37a	6	50	
CHILE _RH61a	6	50	
CHILE _RA45a	6	50	
CHILE _RA46a	92	85	
CHILE _RA38b	92	85	
CHILE _RA52a	92	85	
CHILE _RA43a	C35	100	HRV-C
CHILE _RH48a	LZ269	80	
CHILE _RH47a	LZ269	100	
CHILE _RA41a	NAT045	100	
CHILE _RA65a	NAT045	100	
CHILE _RH69a	NAT045	100	
CHILE _RH70b	PHL/TTa4 69/2008	100	

Fig. 1. Comparison of rhinovirus clinical isolates phylogeny using Neighbor-joining method. A total of 117 reference sequences obtained from Genbank were used for alignment and 43 of them are shown in the figure. Genbank accession numbers are indicated. Isolates were assigned to genogroup to which they clustered in the phylogenetic tree with a significant bootstrap value >50% [21]. HRV genogroups are indicated in the right side of each branch. HRV-Ca belongs to genogroup C but genetic similarity in the 5′NCR region forces clustering with HRV-A [17,21,41,42,43].

whose loads were significantly higher than B7/B9 strains, probably because of the viral emergency in a susceptible population.

Although viral loads in respiratory samples could be affected by multiples variables such as time or amount of sample collected [30,39], in our study sampling performed by trained personnel following a specific protocol, minimize the differences. Furthermore, these differences would not affect the observed results since would cause random effects rather than systematic bias.

Rhinovirus strains are grouped into three genogroups, A, B and C [17,21,40]. A few epidemiologic data exist on the relationship between HRV genogroups and illness severity. Kiang et al. found children hospitalized with severe respiratory illnesses, which were infected with genogroup C. In a prospective multicenter study, bronchiolitis in African American infants were more often associated with HRV than other children [6]. In our work, HRV infection was concentrated significantly in patients with mild disease, where strains were distributed homogeneously among the three known genogroups. The co-infection with both RSV and HRV viruses did not correlate with a more severe disease. The rate observed in HRV infection is similar to that reported by other authors. However, the clinical behavior of patients differs depending on the design of the studies. In this study, we have included very young infants, without previous morbidity and in their first episode of ALRTI. In our opinion, these characteristics influence the clinical behavior of studied patients.

In conclusion, severe RSV infections were in part influenced by the emergence of strain NA1. However, in our experience and according to other authors, we postulate that in infants younger than 6 months of age, the clinical severity of RSV infection is mainly due to the profile of immune response that is induced by the virus in each individual child [1,14,25]. In contrast, in very young infants infected by HRV, the relative immaturity of the immune system may avoid the induction of an intense inflammatory response, as it is seen in older infants or in atopic individuals throughout life. In the future, it will be necessary to compare meticulously the profile of the immune response on different cohorts with rhinovirus infection.

Funding

All funding was provided by the National Fund of Science and Technology; FONDECYT 1100477.

Competing interests

The authors declare that there are no conflicts of interest to disclose.

Ethical approval

Study protocol was approved by each of the Institutional Review Boards from the Roberto del Río Hospital, the Faculty of Medicine of the University of Chile and the National Fund of Science and Technology (FONDECYT 1100477). Informed and signed consent was obtained from the parents of all study participants as is required.

Acknowledgments

The authors thank to Mrs. Dina Silva, Rosa Corvalán, Mónica Peña and to Mr. Cristian Moreno for their technical assistance. Also we thank to Dr. Roberto Vidal for their help in phylogenetic analysis of rhinovirus and Dr. Bettina Von Dessauer and the nurses Mariel Bolvarán, Grisel Navarrete, Macarena Monsalve and Paulina Cifuentes who helped with patient's recruitment.

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