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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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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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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