Description of respiratory syncytial virus genotypes circulating in Colombia

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Abstract
Introduction: Respiratory syncytial virus (RSV) is the most common cause of acute respiratory infections in children younger than two years but also produces infection in older children and even reinfection in people of any age, a characteristic related to the existence of different infecting subtypes and genotypes. Although Colombia has established the surveillance of classical respiratory viruses, there is no information about the RSV genotypes circulating in Colombian patients.

Methodology: A subgroup of 227 previously RSV positive respiratory secretion samples were taken from a nationwide surveillance study, amplified and sequenced to define the circulation pattern of RSV subtypes and genotypes during 2000-2009 period in Colombia.

Results. RSV exhibited seasonal behavior with an A subtype more prevalent. Both RSV subtypes had low nucleotide variability. During the study period, the GA2 and GA5 genotypes from RSV subtype A and the BA genotype from RSV subtype B were found.

Conclusion. In this report, for the first time RSV genotypes circulating in Colombia were described, this information adds valuable information about virus epidemiology helping to understand the RSV epidemic and prepare our country for the introduction of new vaccines.

Key words: Respiratory syncytial virus; subtypes; genotypes; molecular epidemiology.


Introduction
Acute respiratory infections (ARI), one of the most important public health concerns in the world, are defined as a group of infections of the respiratory tract caused by both bacterial and viral pathogenic agents. Respiratory syncytial virus (RSV) is the most common cause of mild and severe respiratory infections in children younger than two years; however, the use of confirmatory laboratory diagnostic is infrequent, which leads to inadequate attention and antibiotic use and wider spreading of annual outbreaks [1].

RSV belongs to the Paramyxoviridae family and the Pneumovirus genus and has a lipid envelope and negative-sense non-segmented RNA that encodes 11 proteins [2]. The envelope glycoprotein (G) is a type II transmembrane protein involved in interactions with target cell membrane receptors the highly sulfated glycosaminoglycans such as heparin and chondroitin sulfate [3]. According to the G gene sequence, RSV could be differentiated into subtypes A and B as well as the genotypes between them [4,5]. Most of the genomic changes detected in clinical isolates are due to the lack of fidelity of polymerase activity, which defines the apparition of genotypes and subtypes [6]. In the RSV A subtype, the GA1 to GA7, SAA1, NA1, NA2 and ON1 genotypes have been identified, while in RSV subtype B, in addition to twelve BA genotypes, GB1 to GB4, SAB1 to SAB3, URU1 and URU2 genotypes have been described [7,8]. Subtypes A and B have 53% identity in the G protein gene, but this identity is approximately 80% among genotypes of subtype A and 91% among genotypes of subtype B [9,10]. It is unclear whether strain variations can be involved in reinfections or immune escape, and the importance of these genotypes in the development of a vaccine remains uncertain.

Recently, in a nationwide sentinel study, we reported that approximately 19% of respiratory samples tested positive by immunofluorescence to some of the main respiratory viruses. RSV was detected in a half of them, mainly subtype A (66% of PCR-confirmed samples), although there was an alternating annual pattern of the A and B subtypes and most of the RSV-confirmed patients were children under 5 years (85.3%) [11]. Despite these findings, there is no information about circulating genotypes in Colombia. Therefore, the purposes of this study were to describe the RSV genotypes and circulation patterns in a Colombian ten-year sample collection period.
**Methodology**

**Sample collection and RSV subtype detection**
This descriptive retrospective study was approved by the Ethics Committee of the Instituto Nacional de Salud of Colombia. During a nationwide respiratory infection sentinel study, more than 7300 patient secretion samples were collected in a ten-year period (2000-2009) and evaluated by immunofluorescence (Light Diagnostics Respiratory Viral Panel, Millipore, Livingston, UK) to the seven most frequent viruses (FluA, FluB, RSV, Parainfluenza 1, 2, 3 and AdV), revealing that 1092 samples (14.8%) were RSV positive. A subgroup of 227 samples, proportional to those sent from each Department which contributed with more than 1% of positive samples were randomly selected. Final samples number was calculated using the expected RSV prevalence at 95% confidence interval. Bogotá City, Caldas, Atlántico, Cundinamarca and Tolima Departments contributed more than 83 per cent of the collected samples (Figure 1), therefore these regions were selected for genotyping study. Then, RNA extraction (Qiamp Viral RNA Mini kit, Qiagen, Hilden, Germany) and amplified by one-step RT-PCR (Qiagen One-Step RT-PCR Kit, Valencia, CA, USA) following the protocol reported elsewhere [12].

**RSV genotyping and phylogenetics**
From the subtype-confirmed samples, 37 samples to be genotyped were chosen randomly from different departments according their contribution, Atlántico 1, Caldas 7, Tolima 3, Cundinamarca 2, and Bogota 24), taking into account only the presence of at least one sample from each year and counting on 2 to12 samples selected from each one. RNA from the samples was processed using hemi-nested RT-PCR to amplify and analyze the second variable region of the G gene, as previously reported [13]. Briefly, 500 ng of RNA was amplified by RT-PCR using 0.2 μM of the ABG490 and F164 primers. The products were amplified using the F164 primer (reverse for both subtypes) and the AG655 and BG517 primers specific to the A and B subtypes, respectively, which produced amplicons of 450-585 bp for subtype A and 645 bp for subtype B. The purified products were sequenced, and the chromatograms were aligned in MEGA 6.06 software (Kumar, Stecher, and Tamura 2015) [14]. Evolutionary relationships were determined using Phylogeny.fr [15] to build phylogenetic trees and using MUSCLE algorithm with 2000 bootstrap iterations. The evolutionary history was inferred using the maximum likelihood method (ML), and the distance matrix was calculated with Kimura's 2-parameter evolution model. Comparisons to reported 19 RSV-A and 18 RSV-B, nucleotide polymorphisms and deduced amino acid sequence analysis were performed with MEGA 6.06. Finally, haplotype networking to each identified genotype was performed using Network 4.6.1.2 (http://www.fluxus-engineering.com) to evaluate the mutational steps and nucleotide differences between isolates.

![Figure 1: Geographic location of samples origin.](image1)

![Figure 2: Temporal co-circulation pattern of respiratory syncytial subtypes in Colombia by year (A) and cumulated by month (B) during the 2000 – 2009 period.](image2)
Figure 3. Phylogenetic tree of representative RSV subtype A and 19 sequences of Colombian virus studied (asterisks) which aligned with reference sequences of GA2 genotypes (n = 14) and GA5 genotype (n = 5). Confidence of the tree was verified by 2000 bootstrapping for 2000 times and values above 90%.

Figure 4. Clustering of 18 Colombian RSV-B isolates (asterisks) with reference strains where all of them grouped with BA genotype. The genotype distribution was validated after 2000 bootstrap iterations using MUSCLE algorithm.

Table 1. Respiratory syncytial virus subtypes circulating in Colombia provinces 2000 – 2009.

<table>
<thead>
<tr>
<th>Province</th>
<th>Genotyped samples</th>
<th>Year (n)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantico</td>
<td>RSV-A (1)</td>
<td>2006 (1)</td>
<td>MF372391</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2001 (1)</td>
<td>MF372401</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2002 (1)</td>
<td>MF372393</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2003 (1)</td>
<td>MF372402</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2004 (1)</td>
<td>MF173103</td>
</tr>
<tr>
<td></td>
<td>RSV-B (14)</td>
<td>2006 (1)</td>
<td>MF372394</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2007 (3)</td>
<td>MF372398, MF173104, MF372403, MF372395, MF372400</td>
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<tr>
<td></td>
<td></td>
<td>2005 (10)</td>
<td>MF372414, MF372404, MF372405</td>
</tr>
<tr>
<td></td>
<td>RSV-A (6)</td>
<td>2000 (2)</td>
<td>MF173101, MF173102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2001 (1)</td>
<td>MF372397</td>
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<tr>
<td></td>
<td></td>
<td>2006 (1)</td>
<td>MF372399</td>
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<tr>
<td></td>
<td></td>
<td>2007 (2)</td>
<td>MF372392, MF372390</td>
</tr>
<tr>
<td></td>
<td>RSV-B (2)</td>
<td>2008 (1)</td>
<td>MF372420</td>
</tr>
<tr>
<td></td>
<td>RSV-A (1)</td>
<td>2009 (1)</td>
<td>MF372396</td>
</tr>
<tr>
<td></td>
<td>RSV-B (1)</td>
<td>2008 (1)</td>
<td>MF372413</td>
</tr>
<tr>
<td></td>
<td>Tolima</td>
<td>2009 (1)</td>
<td>MF173105</td>
</tr>
<tr>
<td></td>
<td>RSV-B (2)</td>
<td>2009 (2)</td>
<td>MF372417, MF372409</td>
</tr>
</tbody>
</table>
Results

In this study, viral RNA was successfully amplified from 181 out of 227 evaluated samples. The technique allowed the simultaneous confirmation of RSV and subtype. In 93 respiratory samples, the A subtype was detected (51.4%), while the B subtype was less frequent (45/181). Interestingly, 23.8% of the samples had both A and B subtypes simultaneously, which was even more frequent during the years 2000 and 2001, thus hampering the genotype frequency evaluation (Figure 2A). The RSV A subtype was detected in all departments, but the B subtype was not detected in the Huila and Guaviare departments. RSV subtype circulation showed alternating behavior, meaning that when the frequency of the A subtype increased, that of the B subtype decreased, as reported in many studies. Independently of the viral subtype, March to May were the months when RSV was most frequently detected in samples; this is the rainy season in most Colombian regions (Figure 2B).

RSV circulating genotypes

Nineteen RSV-A and eighteen RSV-B viral subtype (n = 37) isolates were sequenced to determine the circulating genotypes. In Table 1 are showed the isolates and accession numbers of strains reported here, which were genotyped based on clustering with reference sequences of known genotypes. The RSV-A subtype virus was grouped in the GA2 (n = 14) and GA5 (n = 5) genotypes (Figure 3). All the viral isolates belonging to the B subtype were grouped in the BA genotype (Figure 4). These genotype distributions were validated with bootstrap values above 90%.

The nucleotide identity between RSV-A subtype sequences was 94.8% (25 nucleotide substitutions), while that of RSV-B subtypes was 93% (49 substitutions), showing low genetic variability in both cases. Evaluating the nucleotide diversity, there were more transitions (17 changes) than transversions in A subtype isolates, and the number of AG+GA and TC+CT transitions were the same. In the case of the RSV B subtype, transversions were more frequent (30 changes), AC+CA being the most frequent (Figure 5A).

The genotype of the RSV-A subtypes varied by region and year. The GA2 genotype was found in all the sampled regions, but not in years 2002, 2003, 2005 and 2008, while the GA5 genotype was mainly found in Caldas and Bogota during 2006 and 2007. The BA genotype of the B subtype was mainly found in Bogota (14/18) and in the year 2008, when it was the only virus detected; this also occurred in the year 2005 (Figure 5B). Although we showed that multiple genotypes co-circulated within the same year, the BA genotype entirely replaced the other genotypes when it appeared.

Deducing amino acid sequences in MEGA 6.06 software allowed us to identify in 2006-2009 RSV-A subtypes the stop codon in the protein position 1857, the same observed in those previously reported (reference sequence JX015493.1) and different from that found in 2000-2004 isolates with one additional amino acid (position 1858) (Figure 6A). The results showed that nucleotide differences were lower than amino acid sequence differences, as has been described in other studies [16,17]. A similar finding was observed in genotype BA of RSV-B since late circulating strains (2008-2009 period) had a premature stop codon at position 1849 which generates a six amino acid shorter protein than those found in 2005-2007 isolates that had its stop codon at 1857 position, the same reported in reference strains (Figure 6B).

Discussion

Most developing countries do not have data on circulating RSV subtypes and genotypes; therefore,
monitoring is important to obtain the clinical and molecular information necessary to help in control of RSV respiratory infections and in the forecast of the vaccine introduction. In this study, we reported, for the first time, the RSV genotypes circulating in Colombia and evidence of the circulation of multiple genotypes with an annual pattern. Although, the majority of samples were received from cities located in central region and the capital City, we were able to find different genotypes during the years of the study. Bogotá City hosts one fifth of Colombian population and has a large respiratory illness surveillance program, referring more than half of national samples, explaining the large number of analyzed genotypes from this City.

Interestingly, in the first three years of the study, in one-fourth of respiratory infections in which RSV was confirmed, there was co-infection with the two RSV subtypes, although the A subtype was the most frequent in the studied samples from 2003 onward, reaching the half of confirmed samples where one subtype was detected. As has been reported, most RSV cases and laboratory confirmations occur during the rainy season in tropical countries such as Colombia [11]. The findings show that there was an annual alternating pattern of RSV subtypes, with one subtype predominating and compelling the other to reduce its frequency, due to the reduction of susceptible populations or the absence of an immune-specific response.

Eleven genotypes of RSV-A have been described, including the recently reported ON1, and 23 RSV-B genotypes have been described based on nucleotide sequence analysis of the glycoprotein gene [7,8,18]. The A and B subtypes have approximately 53% identity, but the identity between genotypes can be higher than 80% in the A subtype virus and higher than 91% in the B subtype virus [9,10]. The findings in the present work are consistent with this description; nucleotide identity levels of 95% and 93% were found to the RSV-A and RSV-B sequenced strains, respectively.

The GA2 and GA5 genotypes belonging to the A subtype were the predominant strains circulating in Colombia, as is the case in most countries [19-21]. Similarly, we detected only the BA genotype of the RSV-B subtype. The BA genotype is the most frequently detected in the world since its discovery in 1999, even displacing the other RSV-B genotypes, as previously reported [4].

Circulating RSV strains in Colombia also have alternative stop codons in the second variable region of G, as evidenced in other studies [16,22]. Both viral subtypes have stop codons, a characteristic putatively involved in immune evasion or the annual re-infection by RSV frequently detected in the same host [23]. The alternative stop codons are one of the major mechanisms used by RSV to avoid being recognized by antibodies and generating escape variants. Due to a premature stop codon 14 out of 18 BA genotypes are

**Figure 6.** Alignment of (A) 19 RSV-A and (B) 18 RSV-B deduced amino acid sequences by year. Conserved residues appear as black squares with a dot. Non-synonymous changes are pointed out with the appropriate letter. Nucleotide changes leading to the apparition of stop codons are presented with asterisks.
six amino acids shorter than those circulating earlier (2005-2007). In the work by Rueda et al., [24] the isolated strains had a shift in the open reading frame due to an adenine deletion near the C-terminus of the glycoprotein, resulting in a second hypervariable domain that was 11 to 42 amino acids shorter.

Many studies have confirmed that the premature stop codons affect the antigenicity of the G protein without modifying its entire function or its role in infectivity. Truncated genes have been described in both A subtypes [16] and with greater frequency in B subtype viruses [25], evidencing that this mechanism is important for generating genetic and antigenic diversity [4,26] and contributing to shaping the immune response in a defined population when changing the selective pressure on each subtype. For example, this stop codon apparition was used by the influenza A virus, which modified the length of NS1 without affecting its function [27] in a similar way to that used by RSV.

There is little information about the molecular epidemiology of circulating RSV in South America. This study, in conjunction with those conducted in Uruguay and Argentina, showed a wide degree of variability in viral nucleotide and amino acid sequences between the A and B subtypes. It is possible that these differences result from selection pressure by the immune system in our population, as previously described [16,28]. This is the first report on RSV genotypes circulating in Colombia and might help elucidate seasonal epidemics and prepare our country for the introduction of new vaccines.

Conclusion

The RSV circulation pattern had an annual alternating behavior between A and B subtypes, most frequently detected during the Colombian rainy season. Based on nucleotide sequence analysis of the glycoprotein gene, we found that GA2 and GA5 genotypes of RSV A were the predominant strains and out of eighteen RSV-B analyzed strains, the BA genotype was the only one found. In this report, RSV circulating genotypes were described for the first time in Colombia, adding valuable information about virus epidemiology helping to improve the understanding of the RSV epidemic and preparing our country for the introduction of new vaccines.

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