Human respiratory syncytial virus and metapneumovirus in patients with acute respiratory infection in Colombia, 2000–2011

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Objective. To describe the epidemiology of respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) in Colombia from 2000–2011, including seasonal trends.

Methods. Nasopharyngeal aspirates and/or throat swabs from 14 870 patients with acute respiratory infections (ARI) were studied. Two subgroups were analyzed using molecular biology techniques. The first consisted of 264 RSV indirect fluorescence assay (IFA)-positive samples, the second of 264 RSV IFA-negative samples. RSV and hMPV were detected using reverse transcription polymerase chain reaction (RT-PCR).

Results. 2 799 samples (18.8%) contained a respiratory virus. RSV was detected by IFA in 1 333 samples (8.9%). RSV was detected by RT-PCR in 192 samples from the RSV IFA-positive subgroup and in 25 samples from the RSV IFA-negative subgroup. hMPV was detected in eight samples from the RSV IFA-positive subgroup and in 11 samples from the RSV IFA-negative subgroup. Among the RSV infections, subtype A was dominant in two-year intervals, subtype B was dominant in one-year intervals. 85.3% of RSV and 74% of hMPV infections occurred in children younger than 5 years old. RSV and hMPV infections were associated with rainy seasons. Co-infection with RSV A and RSV B was detected in two patients. Five cases of co-infection with RSV and hMPV were detected.

Conclusions. This report is the first to examine the epidemiology of ARIs in Colombia, with an emphasis on RSV and hMPV. The samples studied here were obtained over a 12-year period and represent all age groups and both genders.

Key words Respiratory syncytial viruses; metapneumovirus; polymerase chain reaction; respiratory tract infections; diagnostic techniques, respiratory system; Colombia.
ribonucleic acid (RNA) with a genomic organization similar to that of RSV.

hMPV and RSV infect similar human subpopulations and produce similar symptoms, although symptoms from the former are of lower severity than those from the latter (7, 8). Humans can become simultaneously infected with both viruses because of their overlapping seasonality—indeed, several studies have observed co-infection rates up to 10% (9). Children co-infected with RSV and hMPV have a 10-fold increased risk of suffering more severe symptoms and requiring mechanical ventilation compared with those infected with RSV alone (10). To date, only a few RSV and hMPV case studies have been reported in Colombia; consequently, there are no consistent data on local transmission and circulation patterns of these viruses (11, 12). The aim of this study was to describe seasonal and demographic patterns as well as annual and monthly dynamics of RSV and hMPV in Colombia from 2000 to 2011.

MATERIALS AND METHODS

Sample collection

This descriptive and retrospective study was based on the processing of nasopharyngeal aspirates and/or throat swabs from 14 870 patients with acute respiratory infection (ARI), displaying respiratory symptoms such as cough, sore throat, rhinorrhea and general feeling of discomfort (13). Swabs were collected in local health centers and hospitals in 28 departments and the capital city of Colombia from 2000 to 2011 (Figure 1). Samples were placed into 2 mL of viral transport medium—i.e. brain heart infusion medium (BHI) containing bovine serum albumin fraction V, 10 µg/mL gentamicin, and 250 µg/mL amphotericin B—and sent to the national reference laboratory, part of the respiratory infection surveillance system framework. 7 272 samples (48.9%) were collected from females and 7 598 samples (51.1%) from males. In total, 7 939 samples (53.4%) were obtained from children younger than five years of age (1 412 females and 1 022 males); 841 samples (5.6%) were obtained from adults older than 65 years of age (434 females and 407 males), and 565 samples (3.8%) were obtained from patients of unknown age (Figure 2). From 1997 to 2007, respiratory viruses in Colombia were monitored mainly

FIGURE 1. Map of Colombia showing geographical regions in which throat swab samples were collected

* Regions colored in white were not included in this study. Colombia’s location within South America is shown at upper left.

FIGURE 2. Infection by age and gender, 2000–2011

* The number of cases is shown in bars using the scale at the left, and the percentage positive are shown in lines using the scale at the right. Percentage positive of RSV = number of RSV cases x 100/number of viral ARI cases.
in children younger than 5 years and patients older than 65 years. From 2007 onwards, monitoring was expanded to all age groups.

**Virus identification**

Influenza A, influenza B, parainfluenzae 1, 2, and 3, adenovirus and RSV were detected in a first screening by fluorescent microscopy using a commercial polyclonal antibody conjugate (Respiratory Panel Viral Screening and Identification IFA Kit. Light Diagnostics™; cat. # 3105) (Millipore Corporation. Livingston, United Kingdom) (i.e., indirect fluorescence assay, IFA) according to the manufacturer’s instructions. The samples were stored at −70°C and thawed at 4°C before reverse transcription polymerase chain reaction (RT-PCR). Nucleic acid quality was tested by quantitative RT-PCR (qRT-PCR) amplification of the human RNAse P gene.

**Study subsample**

To investigate hMPV and RSV subtype A and B positivity using RT-PCR, a required study subsample size of 528 (Table 1) was calculated using standardized statistical methods, assuming the following parameters: estimated prevalence of hMPV infection in the RSV-negative population with ARI = 10% (14), estimated prevalence of RSV A or B subtype infection in the population with ARI = 7.4%, significance level = 90%, and p = 0.05. The study subsample was selected by convenience from among selected samples were collected from patients aged 18 months to 86 years (47.5% female and 52.5% male) in the Colombian provinces with higher RSV frequencies: Bogotá D.C. (capital region, 241 samples), Huila, Cundinamarca and Tolima (central eastern region, 56 samples), Atlántico (Caribbean region, 23 samples), Caldas and Valle del Cauca (western region, 156 samples), and Guaviare (Orinoquia region, 52 samples). These provinces accounted for 94.8% of all the RSV infections in Colombia from 2000 to 2011. The distribution of these samples by age, geographical region, and gender is shown in Table 1.

**Nucleic acid extraction and multiplex nested RT-PCR**

To detect hMPV and RSV A or B subtypes in the study subsample, nucleic acids from respiratory secretions were extracted using the QIAamp Viral RNA Mini Kit (cat. # 52906) (Qiagen. Hilden, Germany) according to the manufacturer’s instructions. A one-step conventional RT-PCR amplification reaction was performed followed by nested conventional PCR amplifications according to previously standardized methodologies (15–17). Briefly, the samples were subjected to an initial cycle of 48°C for 45 min and 95°C for 15 min. The PCR cycling conditions were 45 cycles of 95°C for 30 sec, 53°C for 2 min and 72°C for 30 sec, and a final incubation of 72°C for 5 min. For the nested multiplex PCR, 2 µL of the first reaction was added to 48 µL of a PCR mixture for a final volume of 50 µL. The mixture was heated to 94°C for 2 min. The cycling conditions were as follows: 35 cycles of 94°C for 30 sec, 53°C for 1 min, 72°C for 30 sec, and a final incubation of 72°C for 5 min. The positive controls were RSV 737-base pair (bp) genome fragments cloned into pGEM bacterial plasmid vectors and hMPV 739-bp genome fragments cloned into pCR4 bacterial plasmid vectors. To avoid cross-contamination, safety cabinets located in separate laboratories were used for manipulating respiratory specimens, preparing reagents, processing samples, and performing PCR amplifications. The nested PCR products were analyzed using electrophoresis on 2% molten point agarose gel (cat. # A9539-1006) (Sigma-Aldrich. Munich, Germany) containing 5 mg/ml of ethidium bromide (cat. # 15585011) (Invitrogen. Grand Island, New York, USA) in 0.5 x Tris-borate EDTA buffer (TBE 10X; cat. # 1001-605) (QBiogene MP Biomedicals. Santa Ana, California, USA). The sizes of the PCR amplicons were 737 bp and 790 bp for generic RSV and hMPV, respectively, and 376 bp for RSV subtype A, 615 bp for RSV subtype B and 487 bp for hMPV after the nested PCR. The properties of the oligonucleotide primers and amplicons are shown in Table 2.

**Determination of ARI, viral ARI, RSV and hMPV seasonal and geo-demographic dynamics**

Rainfall levels across Colombia from 2000 to 2011 were acquired from the Colombian National Institute of Hydrology, Meteorology and Environmental Studies (IDEAM) for those departments and years for which samples were obtained. The correlation of rainfall with incidence of ARI and viral ARI was measured for the overall sample. Demographic variables such as patient age range and gender, as well as geophysical and meteorological variables such as the place of origin of samples and rainfall levels, were analyzed and correlated with the incidence of RSV and hMPV infections in the study subsample.

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**Table 1. Geographical and demographic distribution of the study subsample. Number of samples per age group, geographical region and gender**

<table>
<thead>
<tr>
<th>Age group (Years)</th>
<th>Capital Region</th>
<th>Central-East</th>
<th>Caribbean</th>
<th>West</th>
<th>Orinoquia</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>0–5</td>
<td>98</td>
<td>77</td>
<td>15</td>
<td>12</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>6–30</td>
<td>19</td>
<td>8</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>31–65</td>
<td>9</td>
<td>9</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&gt; 65</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>7</td>
<td>9</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>135</td>
<td>106</td>
<td>32</td>
<td>24</td>
<td>7</td>
<td>16</td>
</tr>
</tbody>
</table>
TABLE 2. Primers used for respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) gene amplification in the multiplex conventional nested reverse transcription polymerase chain reaction (RT-PCR)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’–3’)</th>
<th>Virus</th>
<th>Gene</th>
<th>Gene position</th>
<th>Melting temp (°C)</th>
<th>G+C content (%)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSVB1F</td>
<td>ATGGAGYGTGCRATCWCARRCAARTGCWAT</td>
<td>RSV</td>
<td>F</td>
<td>1–31</td>
<td>62</td>
<td>45</td>
<td>737</td>
</tr>
<tr>
<td>RSVB1R</td>
<td>AGGTGTGWTACCTGATTRACACTRRAATTCC</td>
<td>RSV</td>
<td>F</td>
<td>705–737</td>
<td>60</td>
<td>39</td>
<td>737</td>
</tr>
<tr>
<td>HNMV1F</td>
<td>AGTCCAYCTAGTAGACAC</td>
<td>hMPV</td>
<td>M</td>
<td>2181–2200</td>
<td>41</td>
<td>42</td>
<td>790</td>
</tr>
<tr>
<td>HNMV1R</td>
<td>TGTGYYCCTTGRGTCCTCAA</td>
<td>hMPV</td>
<td>M</td>
<td>2863–2881</td>
<td>41</td>
<td>42</td>
<td>790</td>
</tr>
<tr>
<td>RSVB2R</td>
<td>AAATTCCTGGTATACTCTAGTAGCTGT</td>
<td>RSV</td>
<td>F</td>
<td>682–710</td>
<td>57</td>
<td>38</td>
<td>376 (RSV-A)</td>
</tr>
<tr>
<td>RSVB2F</td>
<td>AAGGTATGTAATTACACTCAACATT</td>
<td>RSV</td>
<td>F</td>
<td>335–362</td>
<td>55</td>
<td>25</td>
<td>376 (RSV-A)</td>
</tr>
<tr>
<td>RSVB2R</td>
<td>GGAGATGCGAGCTCTGGATTACT</td>
<td>RSV</td>
<td>F</td>
<td>617–644</td>
<td>57</td>
<td>46</td>
<td>615 (RSV-B)</td>
</tr>
<tr>
<td>RSVB2F</td>
<td>ATCTTCTAAGCTCTGGTTAATAGCATT</td>
<td>RSV</td>
<td>F</td>
<td>30–59</td>
<td>57</td>
<td>35</td>
<td>615 (RSV-B)</td>
</tr>
<tr>
<td>HNMV2R</td>
<td>TCTTGCAKATYTRCTKATGCT</td>
<td>hMPV</td>
<td>M</td>
<td>687–700</td>
<td>53</td>
<td>35</td>
<td>487</td>
</tr>
<tr>
<td>HNMV2F</td>
<td>GCRCGATGTCTGTACTTCC</td>
<td>hMPV</td>
<td>M</td>
<td>222–241</td>
<td>53</td>
<td>50</td>
<td>487</td>
</tr>
</tbody>
</table>

Statistical Analysis

The proportion of viruses detected by geographical region, month, gender and age group were analyzed. The Chi-squared test was used for univariate analysis, and P values of < 0.05 were considered statistically significant. Analysis was performed using Stata 11 software (Stata Corp LP, College Station, Texas, USA).

Approval by an ethics committee

This research was approved by the ethics committee of the Colombian National Institute of Health on October 7, 2010.

RESULTS

Virus identification

2,799 samples (18.8% of the study sample) were viral infections that tested positive for either influenza A, influenza B, parainfluenza 1, 2, or 3, adenovirus, or RSV (henceforth referred to as viral ARI). In total, 1,333 samples (8.9% of the study sample) were RSV IFA positive. RSV was detected by RT-PCR in 192 samples (72.7%) of the IFA-positive subgroup and 25 samples (9.47%) in the IFA-negative subgroup. Subtype A RSV accounted for 66% of all RSV cases in the study subsample, whereas subtype B RSV accounted for 34%. hMPV was detected by RT-PCR in a total of 19 samples: eight samples (3%) in the RSV IFA-positive subgroup and 11 samples (4.2%) in the RSV IFA-negative subgroup. In addition, 14 cases involved single infections while five involved RSV/hMPV co-infection.

Distribution of RSV by age group

In total, 85.3% of all RSV infections occurred in children younger than 5 years old. In this age group, 58% of RSV infections occurred in males and 42% in females. The proportion of RSV infections among viral ARI by age group was calculated. As expected, the majority (57%) of viral ARI in children younger than 5 years were RSV IFA positive. This proportion decreased with increasing age: 28.1% for people between 6 and 30 years of age, 22.5% between 31 and 65, and just 9.2% in patients over 65. The proportion of RSV infections among viral ARI cases by gender and age was also determined. In children younger than 5 years, RSV infections accounted for an identical proportion (57%) of all viral ARIs for males and females. In the 6 to 30-year-old age group, RSV infection rates were 32.6% for males and 23.0% for females; among 31 to 65-year-olds, the equivalent proportions were 22.0% for males and 22.8% for females; and for those over 65, observed rates were 6.5% for males and 12.2% for females. The number of cases of ARI, viral ARI, RSV and the percentage of positive RSV cases by gender and age group are shown in Figure 2.

Seasonal trends for RSV infections

During the 12-year study period, two peaks of respiratory infections occurred annually, the first during the rainy season in April, May, and June, when 29.3%, 37.2%, and 43.8% of ARIs, viral ARIs and RSV infections occurred, respectively, and the second during the secondary rainy season in September, October and November, which saw equivalent rates of 24.8%, 22.0% and 16.3% for ARI, viral ARI and RSV infections. The percentage of positive viral ARI samples among all ARI samples varied widely from 2000–2011. The highest positive percentage was reported in 2001 (50.1%) followed by 2000 (37.1%) and 2002 (32.9%). The lowest positive percentage was reported in 2010 (20.5%) and 2011 (21.3%) and 2009 (15.2%) (Figure 3). The percentage of viral ARI samples that was positive for RSV infections also varied widely over the period of observation, with the highest percentage reported in 2001 (82.6%), followed by 2000 (80.7%) and 2002 (78.7%). The lowest percentage was reported in 2010 (23.6%), followed by 2004 (29.6%) and 2005 (34.0%). The percentage of samples that was posi-
hMPV infections, 68% (13 cases) occurred in females, and 32% (6 cases) in males. However, only two co-infections of RSV subtypes A and B were detected in children younger than five years of age (one female and one male). In addition, 5 RSV co-infections with hMPV were detected in children younger than five years (four females and one male). Subtype A RSV was involved in four of these co-infections. In this study, co-infections did not display a different pattern than single infections. Furthermore, this analysis does not determine whether disease severity was associated with co-infections.

DISCUSSION

It is well known that RSV and hMPV primarily infect infants (3, 5). This phenomenon has been related to the low level of immune response in that age group, typical within the first two years in humans. In fact, more than 95% of children under two years have been infected with RSV, and 50% of these children subsequently become re-infected (18). RSV re-infection often occurs throughout life because complete immunity against the virus is never developed (18–20).

In this study, 85.3% and 74% of RSV and hMPV infections, respectively, occurred in children younger than 5 years old. More RSV infections were observed in boys and more hMPV infections in girls. However, low numbers of cases prevented statistically significant conclusions from being drawn. Similar trends have been reported in previous studies (21). In this study, the frequency of both RSV and hMPV infection decreased with increasing age, as only 5.6% and 0% of RSV and hMPV infections, respectively, occurred in patients more than 31 years old.

Apart from age, other previously-identified risk factors for RSV infection include (20): (i) increased exposure to infectious agents because of day care attendance, birth season, hospitalization, or low socioeconomic status; (ii) decreased body size because of gestational age, malnutrition, or low birth weight; (iii) increased susceptibility because of the absence of breastfeeding or low IgG levels in breast milk; and (iv) factors affecting lung function, such as exposure to smoke and air pollution.

In tropical regions RSV activity varies, from no seasonality to multiple annual peaks. RSV activity has been associated with excessive rainfall, higher environmental temperatures, warm rainy seasons, lower relative humidity and higher magnitude of day-to-day temperature variations (21–25). Regional climate diversity contributes to a corresponding diversity of RSV activity. In South America, RSV infections have been associated with winter in some countries, including Argentina, Brazil, Chile, and Uruguay, and with rainy seasons in other countries, including Colombia, Puerto Rico and Venezuela (26–28). High ambient humidity may increase the lifetime of respiratory viruses outside the host, thereby increasing transmission rates during rainy seasons.

Rainfall is the major relevant climatic variable in Colombia, where temperature varies within a narrow range. This study documented year-round ARI, viral ARI and RSV activity in Colombia from 2000 to 2011. Nevertheless, an association was observed between ARI, viral ARI, and RSV activity and rainfall (Figure 4 and Figure 5). In particular, 54.1%, 59.2% and 60.1% of ARIs, viral ARIs and RSV infections occurred, respectively, during the six rainiest months in Colombia during the period of observation. 10% of ARIs and 48.4% of viral ARIs were caused by
RSV during the rainy seasons, whereas the corresponding numbers for dry seasons were only 7.8% of ARIs and 46.5% of viral ARIs. Thus, RSV cases increased slightly among ARI and viral ARI cases during rainy seasons. The association of RSV epidemic periods with the wet seasons validates previous observations in tropical and subtropical countries with seasonal rainfall. Previous studies have shown similar trends in other tropical areas with year-round RSV activity (26, 27, 29–32).

RSV subtype A was dominant among RSV infections and accounted for 66% of all RSV cases in the study subsample, while subtype B accounted for 34% of cases. No demographic variable served to distinguish subtype A from subtype B among the RSV infections, however, subtype A RSV infections were dominant over two-year intervals (i.e., in 2000–2001, 2003–2004, 2006–2007, 2009–2010) and subtype B RSV infections were dominant over one-year intervals (i.e., in 2002, 2005, 2008 and 2011) (Figure 6). Previous studies have revealed similar temporal dynamics for RSV subtypes in Cuba, Uruguay, Hawaii and Argentina where RSV A is dominant and accounts for 62–91% of all RSV infections (26, 31, 33, 34).

During 1997 to 2007, respiratory viruses in Colombia were primarily monitored in children younger than 5 years of age and elderly patients older than 65 years of age. From 2007 onwards, monitoring was increased to cover all age groups. For this reason, the number of ARI samples increased by 2-fold beginning in 2007 (approximately). This is a limitation of this study that may influence the analysis of distribution of RSV and hMPV among age groups. However, because RSV primarily infects children under 5 years old, the number of RSV cases remained stable before and after 2007 (35). In 2009, pandemic H1N1 influenza A emergence resulted in an approximately 4-fold increase in the number of samples collected, primarily from young adults, for ARI monitoring (35). Again, this sudden increase may have influenced the analysis performed on distribution of viruses among the population. However the number of RSV cases remained stable before and after the pandemic, likely because RSV primarily infects children under 5 years old.

An IFA false-positive rate of 27.3% and an IFA false-negative rate of 9.4% were observed when compared to RT-PCR. Diagnostic techniques based on fluorescent microscopy are often dependent upon antigen quality and subjective criteria determined by the technician which are limitations when used for clinical diagnosis.

Because hMPV prevalence is low, the study subsample was not large enough to draw statistically significant conclusions. This became a limitation when...
investigating the demographic patterns and monthly and annual dynamics of hMPV infections.

For future studies, the authors recommend the selection of a time period during which the surveillance of respiratory viruses is performed uniformly without major deviations in target population groups. The molecular methods described in this study are suitable for detecting RSV and hMPV. However, the results clearly show the need for extreme care when using and interpreting IFA. Although IFA is a good option for screening and surveillance purposes, a recommendation derived from this study is that IFA should not be used as a confirmatory test for clinical diagnosis.

Future studies on the epidemiology of respiratory viruses should calculate required sample size based on prevalence data for the country or area of study, in order to guarantee that results are statistically significant and conclusions are solid.

Conclusions

Although RSV showed year-round activity and infected all age groups, it clearly increased during rainy seasons and infected mainly children under 5 years old. RSV infections were more frequent in boys than in girls. During the 12-year period, subtype A RSV dominance occurred in periods of two years followed by a year of subtype B RSV dominance.

Acknowledgements. This study was sponsored by the Instituto Nacional de Salud (INS) in Bogotá D.C., Colombia and the Departamento Administrativo de Ciencia, Tecnología e Innovación (COLCIENCIAS) in Bogotá D.C., Colombia (Project 210 451 928 989). The authors wish to thank the Instituto de Hidrología, Meteorología y Estudios Ambientales (IDEAM) in Colombia, for providing the rainfall data across Colombia. Authors are thankful to the Instituto de Salud Carlos III in Madrid, Spain and the Red Latinoamericana de Virología (VIRORED) for the donation and shipment of bacterial plasmid vectors.

Conflict of interests. The authors of this paper declare no conflict of interests in this research.

REFERENCES

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Original research

Barbosa Ramirez et al. • Human respiratory syncytial virus and metapneumovirus in Colombia


Manuscript received on 24 May 2013. Revised version accepted for publication on 14 June 2014.
**Resumen**

**Objetivo.** Describir la epidemiología y tendencias estacionales del virus sincitial respiratorio (VSR) y metapneumovirus humano (MPVh) en Colombia durante el periodo 2000–2011.

**Métodos.** Se recolectaron aspirados nasofaríngeos o hisopados faríngeos de 14 870 pacientes con infección respiratoria aguda. Se analizaron dos subgrupos por técnicas de biología molecular. El primero estuvo compuesto por 264 muestras positivas para el VSR por inmunofluorescencia indirecta (IFI), y el segundo estuvo compuesto por 264 muestras negativas para el VSR por IFI. La técnica utilizada para la detección del VSR y el MPVh en ambos subgrupos fue la transcripción inversa asociada a la reacción en cadena de la polimerasa (RT-PCR).

**Resultados.** 2 799 muestras (18,8%) contenían algún virus respiratorio. Se detectó VSR por IFI en 1 333 muestras (8,9%), e igualmente fue detectado por RT-PCR en 192 muestras en el subgrupo de muestras positivas para el VSR por IFI y en 25 muestras en el subgrupo de muestras negativas para el VSR por IFI. Se detectó MPVh en 8 muestras en el subgrupo de muestras positivas para el VSR por IFI, y en 11 muestras en el subgrupo de muestras negativas para el VSR por IFI. De las infecciones causadas por el VSR, el subtipo A fue dominante en períodos bianuales; en contraste, el subtipo B fue dominante en períodos anuales. El 85,3% de las infecciones por el VSR y 74% de las infecciones por el MPVh ocurrieron en niños menores de 5 años de edad. Las infecciones causadas por el VSR y el MPVh se asociaron con las estaciones de lluvia. Se encontró coinfección con VSR A y VSR B en 2 pacientes, y coinfección con VSR y MPVh en 5 pacientes.

**Conclusiones.** Esta investigación es la primera en estudiar la epidemiología de las infecciones respiratorias agudas en Colombia, con énfasis en las causadas por el VSR y el MPVh. Las muestras estudiadas fueron recolectadas durante un periodo de 12 años y representan todos los grupos etarios de ambos sexos.

**Palabras clave**

Virus sincitial respiratorio, metapneumovirus, reacción en cadena de la polimerasa, infecciones del sistema respiratorio, técnicas de diagnóstico del sistema respiratorio, Colombia.