Viral Etiology of Acute Respiratory Infections in Hospitalized and Outpatient Children in Buenos Aires, Argentina

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Objectives: To determine and compare the viral frequency, seasonality and clinical-demographic features in 2 groups of children (hospitalized versus outpatients) with acute respiratory infections.

Material and Methods: A cross-sectional, descriptive study was performed from 2008 to 2010 in 620 children <6 years of age with acute respiratory infection. Respiratory samples were studied for classical respiratory viruses by immunofluorescence and for human rhinoviruses (HRV) by real-time reverse transcription polymerase chain reaction. Clinical and demographic data were recorded.

Results: Viral detection by immunofluorescence was 48% in 434 inpatients and 37% in 186 outpatients. Viral diagnosis increased to 83% and 62%, respectively, when testing for HRV. HRV (41%) and respiratory syncytial virus (RSV) (27%) were most common viruses identified, followed by metapneumovirus (9%), influenza A and parainfluenza (3%), adenovirus and influenza B (2%). HRV frequency was significantly higher in hospitalized patients (47%) than in outpatients (27%) ($P < 0.001$). Coinfection was detected in 12% of hospitalized and 4% of outpatients ($P < 0.031$). HRV and adenovirus circulated throughout the entire year. RSV, influenza A and B predominated in winter, whereas metapneumovirus and parainfluenza predominated in spring. Of 362 patients with bronchiolitis, 84% had a virus identified; HRV (42%) and RSV (38%) were predominant. Of 77 patients with pneumonia, 84% had a virus detected with HRV (43%) and RSV (29%) predominating.

Conclusions: HRV were significant pathogens associated with bronchiolitis and pneumonia, especially in hospitalized patients. Both, HRV and coinfections, were risk factors for hospitalization. These findings support the importance of including HRV detection in children with acute respiratory infection.

Key Words: respiratory viruses, rhinovirus, acute respiratory infections, real-time reverse transcription polymerase chain reaction, coinfection

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Lower respiratory tract infections (LRTIs) are the most common cause of hospitalization in young children, whereas upper respiratory tract infections (URTIs) are the most frequent reason for pediatric emergency room visits. In children, most of these infections have a viral etiology.

The classical viruses such as respiratory syncytial virus (RSV), influenza (Flu) A and B, parainfluenza (PIV) and adenovirus (AdV) are traditionally diagnosed by conventional methods such as immunofluorescence (IF) and culture.

Viral detection using molecular methods has increased their diagnosis and has expanded the clinical knowledge of respiratory infections. Since the implementation of these methods, human rhinovirus (HRV) has gained a wider recognition as clinical relevant pathogens; classical viruses are diagnosed more efficiently, and new viruses, such as human metapneumovirus (hMPV), bocavirus (hBoV) and new coronavirus have been discovered.

Most studies on viral etiology of acute respiratory infections (ARIs) have been performed in hospitalized patients, and show that RSV is the most frequent pathogen in children under 2 years of age.[1,2] However, HRV is now detected more frequently than RSV.[3,4] HRV, the most frequent cause of the common cold, has also been associated with LRTI (bronchiolitis, pneumonia), asthma, chronic obstructive pulmonary disease exacerbations, and the presence of HRV has been identified as an important predictor of recurrent wheezing.[5,6]

In Argentina, LRTIs are the second leading cause of death in children under 5 years of age.[7] Most available data on respiratory viruses is based on the rapid antigen detection of classical respiratory viruses by IF from hospitalized patients.[8,9]

Data on viral frequencies and clinical features in outpatients are limited because viral diagnosis in this population is usually not considered standard of care.

The purpose of this study was to determine the frequency and seasonality of respiratory viruses, including classical (RSV, FluA, FluB, PIV and AdV) and 2 emergent viruses (HRV and hMPV) in hospitalized versus outpatient children with ARI under 6 years of age, to determine the presence of viral coinfections, and to analyze clinical characteristics and demographic data in both populations.

MATERIALS AND METHODS

Patients

Children with LRTI or URTI (n = 620), otherwise healthy, admitted at 2 hospitals (n = 434) (Centro de Educación Médica e Investigaciones Clínicas University Hospital and Mater Dei Hospital) or visiting the pediatric emergency room (n = 186) at Centro de Educación Médica e Investigaciones Clínicas University Hospital in Buenos Aires city, Argentina, were enrolled in a cross-sectional, descriptive study during 2 consecutive years (from June 1, 2008, to May 31, 2010). Institutional review board approval was obtained for all the study.

The inclusion criteria were: 1) children under 6 years of age with LRTI or URTI and symptoms onset <3 days before study entry; 2) informed consent form signed by parents or guardians; 3) a respiratory sample obtained at admission by pediatricians or

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respiratory therapists; 4) clinical and demographic data recorded by the attending physician using a specially designed form which included: demographic data, weeks of gestation, weight at birth, prematurity; vaccine schedule; personal or family history of asthma or allergy; breastfeeding, day-care attendance, school-age siblings, viral exposure and tobacco smoke exposure. "Viral exposure" was defined as contacts with another family member with ARI. Data on clinical course including days of hospitalization and oxygen therapy, admission to an intensive care unit and need for mechanical ventilation were later recorded.

The exclusion criteria were: cardiopathy, chronic pulmonary disease, metabolic diseases or immunosupression.

The power was 88% to detect a difference of 10% in the frequency of exposure between groups, and a 38% power to detect a difference of 5% between groups, given a baseline frequency of 10% and a confidence level of 95% ($\alpha = 0.05$).

Respiratory Virus Detection

Respiratory samples obtained at admission were nasopharyngeal aspirates in hospitalized patients and nasopharyngeal swabs (flocked swabs) (COPAN, CA) in outpatients. Samples obtained in viral transport media were immediately sent at 4°C to the Virology Laboratory, Centro de Educación Médica e Investigaciones Clínicas. Samples were processed for rapid antigen detection by IF. A minimum number of 10 cells/field was required for a sample to be considered adequate. RSV, AdV, FluA, FluB and PIV were detected by indirect IF with monoclonal antibodies (Chemicon/Millipore, CA), whereas hMPV was detected by direct IF (Argene, Verniolle, France).

An aliquot of the original sample was stored at –70°C for HRV detection by reverse transcription polymerase chain reaction (RT-PCR). Viral RNA/DNA extraction was manually performed using the QIAamp MinElute Virus Spin (Qiagen GmbH, Germany), according to the manufacturer´s recommendations. The extracted RNA was used to detect HRV by a real-time RT-PCR that amplifies a segment of the 5′ noncoding region of the genome, in a LightCycler 2.0 instrument (Roche Diagnostic, France). The RT-PCR reaction was performed using the 1-step RT-PCR Kit (Qiagen GmbH), a set of primers that amplifies a 207 bp area, and a Taqman probe labeled at the 5′ end with 6-carboxyfluorescein and the 3′ end with Black Hole Quencher 1. This RT-PCR was able to detect all 3 HRV species (A, B and C).[10,11] Samples were considered positive if the reaction growth curves crossed the threshold line within 40 cycles.

Statistical Analysis

Values were given as percentages for discrete variables or as median, first and third quartile for continuous variables. Associations between variables were assessed by $X2$ or Fisher exact test; P values were also adjusted by age. Continuous variables were compared using Wilcoxon tests. The odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Statistical significance was defined as P values <0.05. Statistical analyses were performed using STATA 7.0 (StataCorp, College Station, TX).

RESULTS

A total of 620 children with ARI under 6 years of age were enrolled: 358 (57.7%) in the first year and 262 (42.3%) in the second one. A total of 434 (70.0%) patients were hospitalized, whereas 186 (30.0%) were outpatients. Age ranged from 0 to 71 months, and the median age was significantly lower in hospitalized patients (9 months) compared with outpatients (18 months). Viral diagnosis, including both the classical viruses detected by IF and HRV by RT-PCR, was achieved in 361 (83.2%) hospitalized patients and 115 (61.8%) outpatients.

Demographic and Clinical Characteristics

Patients were Caucasian and well-nourished, and were of middle-high socioeconomic status. When analyzing the whole population, viral exposure was documented in 368 (59.4%) patients, day care attendance in 236 (38.1%), personal and familiar bronchial hyperreactivity in 265 (42.7%), passive smoking in 97 (15.6%), prematurity in 97 (15.6%) and breastfeeding in 563 (90.8%). Of all patients, 582 (93.8%) had received the vaccines included in the National Immunization Programme; of 451 patients aged 6 months or over, 117 (25.9%) had received influenza vaccine.

Demographic and clinical characteristics of hospitalized patients compared with outpatients and age adjustment are shown in Table 1. Univariate analysis revealed that day-care attendance, breastfeeding and up-to-date vaccination status were significantly more frequent in outpatients than in hospitalized patients. In contrast, bronchial hyperreactivity and viral exposure were significantly more frequent in hospitalized patients. Nevertheless, when the analysis was adjusted for age, these differences were no longer statistically significant with the exception of breastfeeding (OR: 0.47; 95% CI: 0.21–0.97).

Tachypnea, cough, wheezing and retraction were statistically more frequent in hospitalized patients, whereas URTI and bronchitis were statistically more frequent in outpatients, and remained significant even when adjusted for age. Bronchiolitis and pneumonia were more frequent in hospitalized children (72.6% and 15%, respectively), but this difference disappeared when the P value was adjusted for age.

Viral Diagnosis

A positive viral diagnosis was obtained in 476/620 (76.7%) patients. A respiratory virus was identified by IF in 208 (47.9%) hospitalized patients and 69 (37.1%) outpatients. However, when HRV detection by RT-PCR was added, viral detection increased to 361 (83.2%) of the hospitalized patients and 115 (61.8%) of the outpatients (Table 2).

Considering both populations, HRV was detected in 252 (40.6%) patients, RSV in 165 (26.6%), hMPV in 53 (8.5%), FluA in 21 (3.4%), PIV in 20 (3.2%), AdV in 15 (2.4%) and FluB in 12 (1.9%). Univariate analysis of viral distribution showed that HRV, RSV and AdV were significantly more frequent in hospitalized patients. On the other hand, FluB and PIV were significantly more frequent in outpatients (Table 2).

HRV was the only virus that remained significantly more frequent in hospitalized patients when P was adjusted for age. Therefore, the presence of HRV was a risk factor for hospitalization (OR: 2.47; 95% CI: 1.60–4.00). Furthermore, when HRV was detected as a single infection, the odds ratio for hospitalization was 2.23 (95% CI: 1.51–3.30).

Viral distribution in hospitalized and outpatients analyzed by age is shown in Figure 1A and B, respectively. Most hospitalized children (84%) and outpatients (65%) were <2 years old. Among RSV positive patients, 90% were <2 years old and 42% <6 months old. Among HRV positive patients, 77% were <2 years old and 25% <6 months old.

Seasonality

Most ARI occurred during the cold seasons: 206 (33.2%) in autumn and 249 (40.2%) in winter, compared with 116 (18.7%) in spring and 49 (7.9%) in summer.

Seasonal distribution varied according to the virus (Fig. 2). HRV was detected throughout the year, including spring and summer. RSV, FluA and FluB showed peaks at the end of autumn and during winter, but significantly diminished in summer. hMPV and

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TABLE 1. Demographic and Clinical Characteristics of 620 Hospitalized and Outpatient Children With ARI

	Hospitalized (n = 434)		Outpatient (n = 186)			
	n	(%)	n	(%)	*P**	Adjusted *P*†
Age (mo)	9	(0–71)	18	(0–71)	<0.001	—
Gender (male)	254	(58.5)	104	(55.9)	0.546	0.666
Viral exposure	272	(62.7)	96	(51.6)	0.010	0.520
Day care	146	(33.6)	90	(48.4)	<0.001	0.634
Bronchial hyperreactivity	198	(45.6)	67	(36.0)	0.027	0.641
Familiar bronchial hyperreactivity	209	(48.2)	73	(39.2)	0.041	0.143
Passive smoking	67	(15.4)	30	(16.1)	0.828	0.851
Prematurity	72	(16.6)	25	(13.4)	0.323	0.326
Breastfeeding	387	(89.2)	176	(94.6)	0.031	0.031
Mandatory vaccines up-to-date	401	(92.4)	181	(97.3)	0.019	0.257
Fever	287	(66.1)	142	(76.3)	0.012	0.219
Tachypnea	345	(79.5)	85	(45.7)	<0.001	<0.001
Cough	401	(92.4)	161	(86.6)	0.022	0.022
Wheezing	285	(65.7)	66	(35.5)	<0.001	<0.001
Retraction	298	(68.7)	42	(22.6)	<0.001	<0.001
Bronchiolitis	315	(72.6)	47	(25.3)	<0.001	0.068
Pneumonia	65	(15.0)	12	(6.5)	0.003	0.270
Upper ARI	47	(10.8)	95	(51.1)	<0.001	<0.001
Bronchitis	9	(2.1)	19	(10.2)	<0.001	0.029

Age (mo) are given as median (range).
*X^2 test or median test was used.
†Statistical analysis was adjusted by age.

PIV predominated by the end of winter and during spring. AdV was observed at low frequency throughout the year.

Clinical Features

Respiratory symptoms were more frequent, and severity was worse in positive patients compared with patients with no virus detection (Table 3). Of 362 patients with bronchiolitis, 84% had 1 or more viruses detected; 151 (42%) patients had HRV and 139 (38%) had RSV. Of 77 patients with pneumonia, 84% had 1 or more viruses detected; 33 (43%) patients had HRV and 22 (29%) had RSV.

Of 476 virus-positive patients, 76% required hospitalization and 58% oxygen supplementation, compared with negative patients, for whom the corresponding percentages were 51% and 30%, respectively ($P < 0.001$; $P = 0.002$). Bronchiolitis was significantly more frequent in virus positive patients compared with virus negative patients even when *P* was adjusted for age. In contrast, URTI was significantly more frequent in virus negative patients.

Mechanical ventilation was required only in virus-positive patients. None of the patients died.

Coinfection

Coinfection with 2 or more respiratory viruses was observed in 61 of 361 (16.8%) positive patients. Coinfection was significantly more frequent in hospitalized patients (12.0%) than in outpatients (4.3%) ($P = 0.003$). Of 61 patients with coinfection, 53 (87%) were hospitalized. In contrast, of 415 with a single infection, 308 (74%) were hospitalized. This difference was statistically significant ($P = 0.031$).

The most frequent pattern of coinfection was HRV with RSV, followed by HRV with hMPV. Only 1 case of triple infection (HRV, hMPV and AdV) was observed in a hospitalized patient.

All coinfected patients were <3 years old, and bronchiolitis was the most frequent diagnosis among these patients (79%).

DISCUSSION

Studies on the epidemiology of respiratory viruses are usually performed in hospitalized patients, and data in outpatient children with ARI are limited.

In this study, we compared children from both populations and found that the frequency of respiratory viruses was significantly higher in hospitalized children (83%) than in outpatients (62%). This difference cannot be attributed to the different type of respiratory sample collected in each population (nasopharyngeal aspirates versus nasopharyngeal swabs) because it has been previously documented that the sensitivity for viral detection is similar for both type of samples.[12,13]

Viral antigen detection by IF with monoclonal antibodies has been a traditional method for the rapid diagnosis of classical respiratory viruses. This method is still widely used in many clinical laboratories due to its validity, rapidity and low cost and complexity. In our study, the frequency of viral detection by IF for RSV, AdV, Flu, PIV and hMPV was similar to that observed in previous studies using the same procedure.[14–16] The total viral frequency by IF was significantly higher (48%) in hospitalized patients than in outpatients (37%). However, this difference lost significance when the analysis was adjusted for age, as hospitalized patients had a lower mean age compared with outpatients.

Surprisingly, when HRV detection by real-time RT-PCR was added, viral detection significantly increased to 83% in hospitalized and 62% in outpatients. Furthermore, the high HRV frequency detected in hospitalized patients (47%) compared with outpatients (27%) remained significantly different even when adjusted for age.

Of all respiratory viruses studied, HRV was the most frequent in both populations. In the literature, HRV frequencies range from 17% to 44% in children with ARI.[17–19] Frequencies are even higher (45–58%) when children with wheezing or asthma are enrolled.[20,21] Although our study enrolled children from the general population, with no chronic diseases, HRV frequency in hospitalized patients (47%) was higher than expected. Interestingly, HRV was detected in all age groups, even in the youngest age group (0 to 6 months old).

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TABLE 2. Respiratory Viral Detection by Immunofluorescence and RT-PCR for HRV in 620 Hospitalized and Outpatient Children With Acute Respiratory Infection

	Hospitalized (n = 434)		Outpatient (n = 186)			
	n	(%)	n	(%)	*P**	Adjusted *P*†
Positive patients	361	(83.2)	115	(61.8)	<0.001	<0.001
Negative patients	73	(16.8)	71	(38.2)		
Identified viruses						
HRV	202	(46.5)	50	(26.9)	<0.001	<0.001
RSV	136	(31.3)	29	(15.6)	<0.001	0.251
hMPV	40	(9.2)	13	(7.0)	0.292	0.391
AdV	14	(3.2)	1	(0.5)	0.047	
FluA	14	(3.2)	7	(3.8)	0.735	
FluB	3	(0.7)	9	(4.8)	0.002	
PIV	6	(1.4)	14	(7.5)	<0.001	
Single infection	308	(71.0)	107	(57.5)	0.001	
HRV	153	(35.3)	46	(24.7)	0.010	<0.001
RSV	103	(23.7)	27	(14.5)	0.010	0.292
hMPV	30	(6.9)	9	(4.8)	0.330	
AdV	8	(1.8)	1	(0.5)	0.291	
FluA	12	(2.8)	6	(3.2)	0.754	
FluB	0	(0.0)	7	(3.8)	<0.001	
PIV	2	(0.5)	11	(5.9)	<0.001	
Dual infection	52	(12.0)	8	(4.3)	0.003	
HRV-RSV	30	(6.9)	1	(0.5)	<0.001	§
HRV-hMPV	8	(1.8)	1	(0.5)	0.291	
HRV-AdV	5	(1.2)	0	(0.0)	0.329	
HRV-PIV	3	(0.7)	2	(1.1)	0.639	
RSV-FluB	2	(0.5)	1	(0.5)	0.999	
Others‡	4	(0.9)	3	(1.6)	0.434	
Triple infection	1	(0.2)	0	(0.0)	§	§
HRV-hMPV-AdV						

HRV was detected by a real-time RT-PCR; the other respiratory viruses were detected by immunofluorescence. Only the analysis of HRV, RSV and hMPV was adjusted for age.
*Data were examined using a X^2 test or a Fisher exact test when where appropriate.
†Statistical analysis was adjusted for age.
‡Others dual infection: in hospitalized children: HRV-FluA, HRV-FluB, RSV-FluA and hMPV-PIV; in outpatients: hMPV-FluA, hMPV-FluB and hMPV-PIV.
§Not possible to calculate this value.

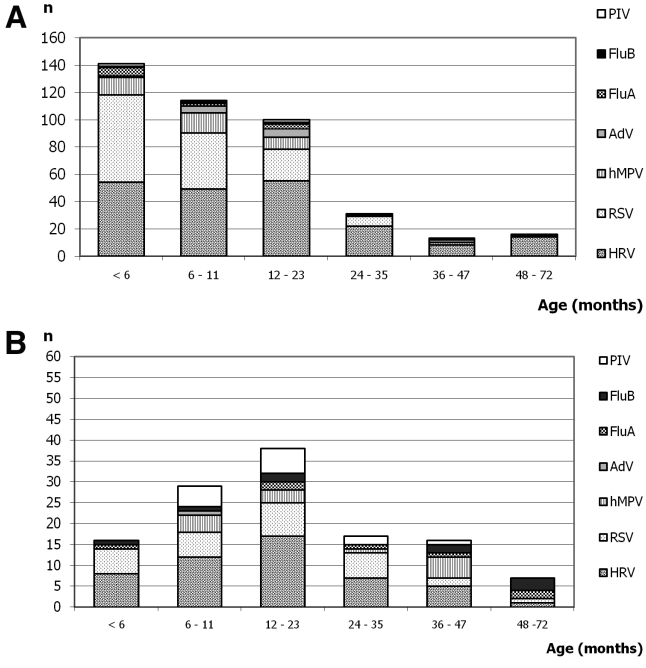

FIGURE 1. Distribution of respiratory viruses detected in hospitalized (A) and outpatient (B) children with ARI by age group. The breakdown of each bar by respiratory virus is indicated.

Nowadays, HRV is considered significant pathogens because its presence has been associated not only with URTI but also with severe LRTI,[22,23] recurrent episodes of wheezing and asthma.[5,24,25] In our study, HRV was the most frequent detected viruses in patients with bronchiolitis and pneumonia. Furthermore, the presence of HRV, even as a single infection, was a risk factor for hospitalization (OR: 2.23). This association remained significant even when adjusted for age.

RSV was the second most frequently detected virus, but the most common one detected by IF. It is widely known that molecular assays are more sensitive than IF for RSV detection. However, the sensitivity of IF for RSV compared with a molecular method is among the highest for all respiratory viruses.[26] In our laboratory, the sensitivity of IF for RSV in children was 80%, compared with an RT-PCR (data not shown). Therefore, RSV would still have been the second most frequently detected virus, even accounting for the different sensitivity.

hMPV was the third most frequent virus, and similar frequencies were observed in both populations, comparable to those reported in hospitalized patients in Argentina.[27–29]

The frequency of Flu viruses in our population (FluA: 3.4% and FluB: 1.9%) was comparable to that previously reported in nonpandemic years.[16,30,31] Since the pandemic FluA H1N1 (2009) occurred at the beginning of winter 2009 in Argentina—the second year of our study—a higher frequency of FluA would have been expected. During winter 2009, at the pandemic peak, the number of patients enrolled in our study diminished by half compared with 2008. However, the frequency of the other respiratory viruses did not change compared with the first year of the study. Most patients

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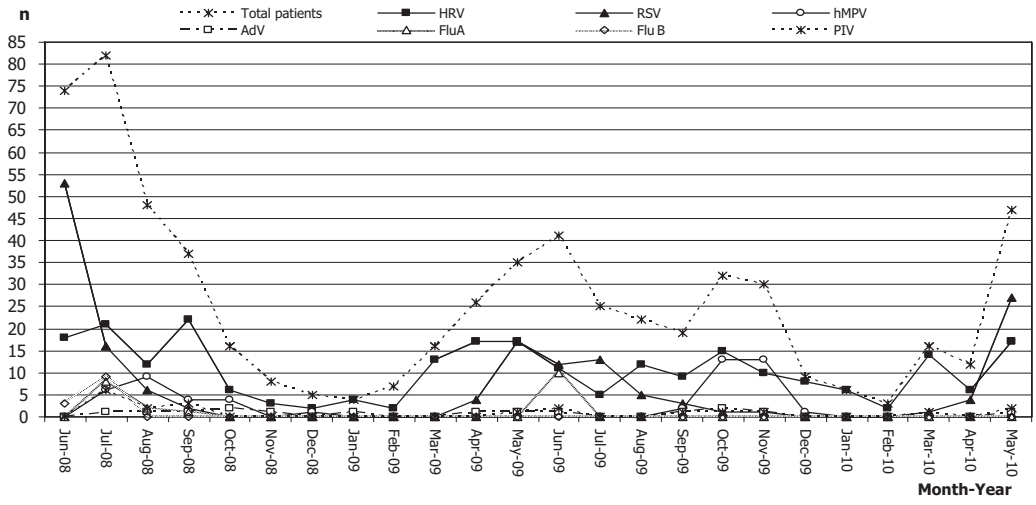

FIGURE 2. Monthly distribution of respiratory viruses, from June, 2008, to May, 2010. Total patients represent the number of patients enrolled in each month.

with flu-like illness were enrolled in a different and specific protocol for studying FluA H1N1 by RT-PCR. Results from our laboratory showed 59% positivity rate for H1N1 in children <5 years old.[32]

According to the literature, the frequency of coinfection with 2 or more respiratory viruses ranges from 11% to 33%, when hBoV is not included.[18,20,33] In our study, coinfection was 17%, significantly more frequent in hospitalized patients than in outpatients. The relationship between coinfection and severity is still under discussion as discrepant results have been observed.[34] In our study, a significant association between coinfection and hospitalization has been observed, with HRV and RSV standing out as the most frequent association.

In this study, neither hBoV nor coronavirus were included. The clinical association of hBoV with ARI is still controversial, and available data cannot determine whether this virus is a pathogen or a colonizer.[35]

TABLE 3. Demographic and Clinical Characteristics of 620 Children With Viruses Detected vs. Children With No Virus Detected

Characteristics	Virus Detected (n = 476)		Virus Not Detected (n = 144)			
	n	(%)	n	(%)	*P**	Adjusted *P*†
Age (mo)	10	(0–71)	13	(0–71)	0.116	—
Gender (male)	274	(57.6)	84	(58.3)	0.870	0.870
Hospitalized	361	(75.8)	73	(50.7)	<0.001	<0.001
Outpatient	115	(24.2)	71	(49.3)		
Fever	323	(68.0)	106	(73.1)	0.190	0.523
Tachypnea	348	(73.3)	82	(56.6)	<0.001	0.005
Cough	440	(92.6)	122	(84.1)	0.002	0.005
Wheezing	300	(63.2)	51	(35.2)	<0.001	0.005
Retraction	292	(61.5)	48	(33.1)	<0.001	0.035
Bronchiolitis	304	(64.0)	58	(40.0)	<0.001	<0.001
Pneumonia	64	(13.5)	13	(9.0)	0.149	0.605
Upper ARI	86	(18.1)	56	(38.6)	<0.001	0.043
Bronchitis	18	(3.8)	10	(6.9)	0.109	0.434
Oxygen supplementation	275	(57.7)	43	(29.9)	0.002	0.033

Age (mo) is given as median (range).
*X^2 test was used.
†Statistical analysis was adjusted for age.

Regarding seasonality, HRV was detected throughout the year, including spring and summer. RSV epidemic seasons started in April or May (autumn) and peaked in June or July (winter), as previously observed in Argentina.[36] Influenza exhibited the typical peak pattern during the winter months.[8] As previously described, AdV was observed at low frequency throughout the year,[9] and hMPV circulated in winter and spring, from July to December.[27]

Among the demographic characteristics studied, breastfeeding was the only one that remained significantly higher in outpatients compared with hospitalized patients, even when adjusted by age. These data may support breastfeeding as a protective factor of hospitalization in children with ARI.

Clinical findings were significantly different between hospitalized and outpatients: pneumonia and bronchiolitis predominated in inpatients, whereas URTI and bronchitis were predominant in outpatients. Interestingly, HRV was the most frequently detected viruses in bronchiolitis and pneumonia cases, followed by RSV.

CONCLUSIONS

This study contributes to the knowledge of viral epidemiology in hospitalized and outpatient children with ARI in Buenos Aires, Argentina. HRV was the most frequent detected virus in both populations. HRV was significantly higher in hospitalized patients and was the most frequent detected viruses in LRTI.

Further studies analyzing the molecular epidemiology of HRV, as well as the presence of coinfection as an independent risk factor for higher hospitalization, are ongoing.

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