

**RSV ASSOCIATED DISEASE IN HOSPITALIZED CHILDREN IN SOUTHEAST
BRAZIL: HIGHER FREQUENCY IN OLDER CHILDREN THAN IN INFANTS**

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ABSTRACT

Respiratory tract infection can lead to diseases such as pneumonia, bronchospasm, bronchiolitis and pleural effusion, and consequently to hospitalization. The aim of this study was to investigate the presence of viruses in hospitalized children with respiratory tract infection and to assess the correlation between lower respiratory tract infection (LRTI) and the viruses studied. We screened children from 0 to 6 years of age with respiratory tract infection. From May 2004 to September 2005, a total of 272 nasopharyngeal aspirate samples were collected from hospitalized children in São José do Rio Preto, Brazil, and stored with trizol at -80°C until testing by RT-PCR. The distribution of LRTI in the study population was: 49.63% (135/272) pneumonia, 23.18% (63/272) bronchospasm, 16.17% (44/272) bronchiolitis, and 11.02% (30/272) pleural effusion. A viral infection was found in 54.41% (148/272) of the samples, as follows: in 79 (29%) RSV, in 63 (23%) HRV, in 14 (5.14%) PIV3, in 9 (5%) HMPV, in 8 (2.9%) PIV1, in 4 (1.4%) FLUB, in 3 (1.1%) FLUA and 1 (0.4%) PIV2. Furthermore, there was a significant correlation between viral infection and bronchiolitis (43/44 cases: 97.8%; $p \leq 0.001$) and RSV infection and bronchiolitis (30/44 cases: 68.2%; $p \leq 0.001$). Finally, this study has confirmed that not only is RSV the most important virus in association with bronchilitis in infants, but also can be frequently detected in children between 2 and 5 years of age hospitalized for ARI in this region. In addition, HRV was frequently detected in association with ARI requiring hospitalization.

INTRODUCTION

Acute respiratory infection (ARI) is a serious health problem worldwide, responsible for 18% of child mortality and an important cause of hospitalization in developing countries (Lindgren & Groggaard 1996, Williams et al. 2005). Children under five years of age frequently suffer from respiratory infections and human respiratory syncytial virus is the single most frequent viral agent of serious lower respiratory infection in this age group (Estrada et al. 2007).

Even though bacterial etiology can be involved, viruses are the most frequent agents detected in association with ARI (Forgie et al. 1991, John et al. 1991, Lin et al. 2004). The major symptoms of upper ARI include nasal stuffiness and discharge, sneezing, sore throat and cough. However, complications such as secondary bacterial infections, mainly acute otitis media and maxillary sinusitis, as well as exacerbations of chronic obstructive airway diseases occur frequently (Wat 2004). In developing countries the etiology of ARI is usually determined on the basis of clinical signs and symptoms. Thus, bronchial and bronchiolar syndromes are widely recognized as viral infections, whereas pneumonias are commonly attributed to bacterial infections (Berman 1991, Selwyn 1990).

Lower respiratory tract infections (LRTIs) are a leading cause of morbidity, hospitalization, and antibiotic use in patients with immunosuppression and/or chronic lung disease; however, their etiology remains undetermined in 48 to 70% of cases (File 2003, Garbino et al. 2002). This lack of etiologic diagnosis exists because appropriate lower respiratory tract samples are often not available and routine laboratory diagnostic procedures are limited both in their sensitivity and spectrum of agents usually targeted. Among the agents that go unrecognized, respiratory viruses are thought to contribute to a

substantial number of LRTIs in hospitalized patients, especially at times when their circulation in the community is seasonally increased (Garbino et al. 2004).

Respiratory infections caused by respiratory syncytial virus (RSV), influenzaviruses types A and B (FLUA and FLUB), parainfluenza viruses types 1, 2 and 3 (PIV1, PIV2 and PIV3), human metapneumovirus (HMPV) and rhinovirus (HRV) are major causes of ARI in infants and young children, causing bronchiolitis, brochospasm, pneumonia and pleural effusion (Hall 2001). As these respiratory viral pathogens cause very similar clinical symptoms, laboratory tests are required for etiologic diagnosis (Templeton et al. 2004).

In this study we investigated the presence of viruses in samples from hospitalized children one month to six years of age with respiratory tract infections manifested as pneumonia, bronchospasm, bronchiolitis and pleural effusion.

MATERIAL AND METHODS

Patients.

This study was conducted at the Genomic Studies Laboratory of the São Paulo State University, in São José do Rio Preto city, State of São Paulo, with 398,079 million inhabitants. Samples were collected at the “Hospital de Base” (HB), São José do Rio Preto, a tertiary health care facility with 130 pediatric beds. Patients were children one month to six years of age seen between May 2004 and September 2005, with ARI. Only patients with ARI symptoms starting within five days prior to admission were enrolled. The ARI symptoms considered were runny nose, cough, wheezing, difficulty to breath, and fever. Diagnosis of lower respiratory tract infection was clinical and radiological, according to the

World Health Organization (WHO) criteria. The lower respiratory diseases were classified as pneumonia, bronchospasm, bronchiolitis or pleural effusion.

A single nurse was responsible for collecting the samples during the whole study. Nasopharyngeal washes were obtained after instillation of 0.5mL of sterile PBS into each nostril with immediate aspiration through a sterile neonatal canula inserted into the child's nasopharynx. The sample was transferred to a sterile vial and immediately transported to the laboratory, processed and frozen at -80°C in Trizol LS (Invitrogen, Carlsbad, CA) for later RNA extraction.

RNA extraction and cDNA synthesis.

Viral RNA was extracted from 250 μl of clinical specimen following the Trizol LS manufacturer's instructions and suspended in 20 μl of MiliQ water treated with DEPC (diethylpyrocarbonate, Sigma, St Louis, USA), cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) utilizing random primer according to the manufacturer's protocol, and stored at -20°C .

PCR amplification.

The amplification reaction of HMPV, FLUA and FLUB, PIV1, PIV2 and PIV3 and HRV was performed in a final volume of 25 μl , with 3 μl of cDNA, 5 μl of buffer (75mM Tris-HCl pH 9.0, 50mM KCl, 20mM $(\text{NH}_4)_2\text{SO}_4$), 3 μl of 50 mM MgCl_2 , 1 μl of 10 mM dNTPs, 2.5 μl of each primer (Table 1) at 10 pmol (Falsey et al. 2003, van Woensel et al. 2003), 0.5 μl (2 U) of DNA polymerase (Biotools, Jupter, USA), and MilliQ water treated with DEPC (diethylpyrocarbonate, Sigma, St Louis, USA). The reaction consisted of 40 amplification cycles with denaturation at 94°C for 45 seconds, annealing at 54°C for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 7 minutes. For

rhinovirus identification, the amplicons produced by PCR were confirmed through hybridization performed with OLP and OLE oligonucleotide probes as previously published (Arruda et al. 1997).

RSV amplification was done by a semi-nested PCR. The first round was done in a final volume of 50µl, with 5µl of cDNA and reaction composition identical to the one describe above for the other viruses. The reaction started with a denaturation step at 95°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and a final extension step at 72°C for 7 minutes. The samples which tested negative in this step were submitted to a second round of semi-nested PCR. For the second round, 5 µl of product from the previous reaction were mixed with 5µl of buffer and the same remaining components as the 1st round, except for the use of 1.5µl of each primer at 10pmol (Table 1) (Estrada et al. 2007, Peret et al. 2000, Peret et al. 1998). The reaction was preceded by a denaturation step at 95°C for 5 minutes, followed by 40 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and an extension step at 72°C for 7 minutes.

The amplification products were analyzed on a 1.2 % agarose gel stained with ethidium bromide, visualized under UV light.

Table 1. Oligonucleotides virus-specific used in PCR reactions.

<i>Virus</i>	<i>Primers</i>	<i>Sequence</i>	<i>bp</i>
RSV	sense (+)	5'- GTT ATG ACA CTG GTA TAC CAA CC-3'	650
	anti-sense (-)	5'- YCA YTT TGA AGT GTT CAA CTT-3'	
	anti-sense (-)	5'-CAA CTC CAT TGT TAT TTG CC-3'	480
HMPV	sense (+)	5'- GAG CCA ATT GAA AAT CCC AGA CA -'3	343
	anti-sense (-)	5'- GAA AAC TGC CGC ACA ACA TTT AG-3	
FLUA	sense (+)	5'- CTA AGG GCT TTC ACC GAA GA -3'	191
	anti-sense (-)	5'- CCC ATT CTC ATT ACT GCT TC-3',	
FLUB	sense (+)	5'- ATG GCC ATC GGA TCC TCA AC -3'	240
	anti-sense (-)	5'- TGT CAG CTA TTA TGG AGC TC-3'	
PIV-1	sense (+)	5'-CCG GTA ATT TCT CAT ACC TAT G -3'	317
	anti-sense (-)	5'- CCT TGC AGC GGA GTT GTT AAG -3'	
PIV -2	sense (+)	5'- CCA TTT ACC TAA GTG ATG GAA T -3'	204
	anti-sense (-)	5'- GCC CTG TTG TAT TTG GAA GAG A -3'	
PIV -3	sense (+)	5'- ACT CCC AAA GTT GAT GAA AGA T-3'	102
	anti-sense (-)	5'- TAA ATC TTG TTG TTG AGA TTG A -3'	
RSV	sense (+)	5'- CGG ACA CCC AAA GTA -3'	350
	anti-sense (-)	5'- biotin GCA CTT CTG TTT CCC C -3'	

RSV: Respiratory Syncytial Virus; HMPV: Human Metapneumovirus; FLUA: Influenzavirus A; FLUB: Influenzavirus B; PIV1: Parainfluenzavirus 1; PIV2: Parainfluenzavirus 2; PIV3: Parainfluenzavirus 3; HRV: Human Rhinovirus.

Statistical Analysis.

Statistical analysis was performed using the Minitab Statistical Software for Windows, version 12.22, and differences were considered significant if $p < 0.05$.

RESULTS

During the study period, 272 nasopharyngeal washes were collected from 272 children 1 to 68 months old (mean age 29.5 months), 44.5% (121/272) younger than 24 months, 57% of boys. Clinical diagnosis at admission were pneumonia (135/272, 49.64%), bronchospasm (63/272, 23.16%), bronchiolitis (44/272, 16.18%) and pleural effusion (30/272, 11.03%).

Of the 272 samples tested, 148 (54.41%) were positive for at least a virus. RSV was the most frequently detected, in 29% (79/272) of the samples, followed by HRV in 23% (63/272), PIV3 in 5.1% (14/272), HMPV in 3.3% (9/272), PIV1 in 2.9% (8/272), FLUB in 1.4% (4/272), FLUA in 1.1% (3/272) and PIV2 in 0.4% (1/272), (Table 2). Forty-five percent of the samples were negative for all the viruses tested.

The analysis of age distribution according to viral etiology showed that the largest numbers of virus positive cases occurred in children older than 24 months (65.82%) as compared to infants (Table 2). Likewise, RSV was more frequently found in association with ARI in children older than 24 months ($p=0.029$) (Table 2). Viral co-infections were observed in 8.0% (22/272) of the ARI episodes with highest rates being caused by an association of rhinovirus with RSV (5.88% of all positive samples). There was no obvious

association of virus positive ARI episodes in general, or of specific viral etiologies, with gender: 55% the patients were males.

Viruses were detected in 97.8% (43/44) of the cases of bronchiolitis, in 73% (46/63) of the cases of bronchospasm, in 68.9% (93/135) of the cases of pneumonia and 44.4% (16/30) of the cases with pleural effusion. The distribution of specific viral etiologies by clinical diagnosis is summarized in Table 3. Remarkably, rhinovirus was detected in 28.8% (39/135) and RSV in 23% (31/135) of the patients with pneumonia. Also, rhinovirus was the agent most frequently detected in association with bronchospasm (31.7%, 20/63) followed by RSV (17.5%, 11/63). As expected, bronchiolitis was significantly associated with RSV ($p \leq 0.001$), detected in 68.2% (30/44) of the patients with this diagnosis.

Table 2. Distribution of viruses detected hospitalized children with acute respiratory infection during the May 2004 at September 2005 according to gender and age.

		RSV	HMPV	FLUA	FLUB	PIV1	PIV2	PIV3	HRV	Total
N positive (%)		79 (29)	9 (3.3)	3 (1.1)	4 (1.4)	8 (2.9)	1 (0.4)	14 (5.1)	63 (23)	181
Age	0-24 months	27 (34.18)	5 (55.56)	1 (33.33)	0	4 (50)	1 (100)	8 (57.14)	45 (71.4)	90
	>24 months	52 (65.82)*	4 (44.44)	2 (66.67)	4 (100)	4 (50)	0	6 (42.86)	18 (28.5)	98
gender	Male	38 (48.10)	7 (77.77)	2 (66.66)	2 (50)	4 (50)	1 (100)	8 (57.14)	31 (49.2)	93
	Female	41 (51.89)	2 (22.22)	1 (33.33)	2 (50)	4 (50)	0	6 (42.85)	32 (50.7)	88

* $p = 0.029$; RSV: Respiratory Syncytial Virus; HMPV: Human Metapneumovirus; FLUA: Influenzavirus A; FLUB: Influenzavirus B; PIV1: Parainfluenzavirus 1; PIV2: Parainfluenzavirus 2; PIV3: Parainfluenzavirus 3; HRV: Human Rhinovirus.

Table 3. Distribution of hospitalized children with acute respiratory infections during May 2004 at September 2005 according to clinical diagnosis at admission and found virus.

		RSV	HMPV	FLUA	FLUB	PIV1	PIV2	PIV3	HRV	Total
LRTI	Pneumonia	31 (23)	5 (3.7)	2 (1.5)	2 (1.5)	6 (4.4)	0	6 (4.4)	39 (28.8)	135
	Pleural Effusion	7 (23.3)	0	0	0	0	0	1 (4.4)	6 (20)	30
	Bronchospasm	11 (17.5)	4 (6.3)	1 (1.6)	1 (1.6)	1 (1.6)	1 (1.6)	7 (11.1)	20 (31.7)	63
	Bronchiolitis	30 (68.2)*	0	0	1 (2.3)	1 (2.3)	0	0	10 (22.7)	44

* $p \leq 0.001$; RSV: Respiratory Syncytial Virus; HMPV: Human Metapneumovirus; FLUA: Influenzavirus A; FLUB: Influenzavirus B; PIV1: Parainfluenzavirus 1; PIV2: Parainfluenzavirus 2; PIV3: Parainfluenzavirus 3; HRV: Human Rhinovirus. LRTI: Lower Respiratory Tract Infection.

DISCUSSION

The most frequent clinical diagnosis of the patients in this study was pneumonia, followed by bronchiolitis, bronchospasm and pleural effusion. The high frequency of pneumonia and bronchiolitis are in accordance with most published studies that show those to be the most frequent causes of hospitalization in children with ARI (van Woensel et al. 2003).

This study revealed that RSV and HRV were the most frequent viruses detected in hospitalized children with ARI, mainly of the lower respiratory tract, in the city of São José do Rio Preto, State of São Paulo, Brazil. The detection of RSV in approximately 29% of the samples confirms the importance of this agent as frequent cause of ARI in children, particularly those older than 24 months 65.82%, ($p=0.029$), test significance. Although most studies conducted in temperate climates show RSV to be a frequent cause of ARI in children younger than 5, predominantly in infants, (Moraes et al. 1997, Patwari et al. 1996, Robertson et al. 2004), the present study has revealed somewhat surprisingly that in this region of Brazil RSV was predominantly detected in children between 2 and 5 years of age. Selection bias was discarded in order that both the children's pediatric ICU with ARI as the children's ward was also included in the study.

As expected, RSV was significantly associated with bronchiolitis 68.2% ($p\leq 0.001$), a finding that confirms most previously published studies (Peret et al. 1998, Pierangeli et al. 2007, Vicente et al. 2003, Jartti et al. 2004).

Detection of HRV in 23% of the ARI episodes reiterates that this virus is not only a common agent of respiratory infections in adults, but also a frequent pathogen in hospitalized children with ARI, in a way similar to that previously reported for children with ARI from a slum in another region of the country (Arruda et al. 1991, Savolainen et al. 2003). Interestingly, 71,4% of the HRV related episodes occurred in children younger than 24 months. Most of the HRV related episodes occurred in patients with upper respiratory tract illness, in agreement with a report by Heikkinen & Järvinen (2003). However, the virus was also detected in 22.7% of the cases of bronchiolitis and in 28.8% of the cases of pneumonia, as well as in association with 31.7% of the cases with

bronchospasm, suggesting an association of this agent with more severe symptoms of lower respiratory tract. Similar results have been reported by Papadopoulos et al. (2002) and by Hayden (2004). Although bronchospasm and pneumonia were the most frequent diagnoses in children infected with HRV, such association was not statically significant when comparison was done with the other clinical diagnoses grouped together. Importantly, co-infections with RSV may have resulted in overestimation of the severity of HRV related ARI episodes.

HMPV was detected in 3.3% of the ARI episodes tested. This frequency is not dissimilar to those found by Jartti et al. (2004), who found 4%, and Boivin et al. (2002), who found 6% among hospitalized children. For HMPV related ARI episodes, the most frequent diagnoses were pneumonia and bronchospasm, in keeping with previous studies (Estrada et al. 2007, Boivin et al. 2002).

For FLUA, FLUB, PIV1, PIV2 and PIV3, lower rates of detection were found, similar to the report by Bourgeois et al. (2006). While additional tests would be needed to confirm the etiologies of the episodes that tested negative in the present study, a significant portion of them would probably be attributable to other viral agents, such as adenoviruses, coronaviruses and human bocaviruses (Kuiken et al. 2003).

In conclusion, this study has confirmed that not only is RSV the most important virus in association with bronchilitis in infants, but also can be frequently detected in children between 2 and 5 years of age hospitalized for ARI in this region. In addition, HRV was frequently detected in association with ARI requiring hospitalization.

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