

Frequent respiratory pathogens of respiratory tract infections in children attending daycare centers

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Abstract

Objectives: To identify and characterize respiratory viruses that infect children from daycare centers with symptoms of respiratory infection and to evaluate the association of clinical and epidemiological disease data with the identified virus.

Methods: We conducted a study between 2003 and 2005 in 176 children with respiratory infection symptoms attending a municipal daycare center. Samples from nasopharyngeal secretion were tested by reverse transcriptase polymerase chain reaction and positive samples for picornavirus were sequenced.

Results: All 782 collected samples were analyzed and 31.8% were positive for at least one of the studied respiratory viruses. Respiratory infections were characterized by the presence of mild symptoms of the upper respiratory tract, the most common of which were runny nose and cough. In the 2 years of study, most cases of infection occurred in autumn and winter, but respiratory viruses were detected throughout all the study period.

Conclusions: Respiratory viruses and respiratory infections caused by them are part of the daily life of children attending daycare centers. Our results show the great impact that respiratory infections have on these children and suggest that more attention must be paid to viral pathogens.

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Introduction

Acute respiratory infections (ARIs) are the major cause of morbidity in young children,^{1,2} particularly in developing countries.³ Children are more susceptible to respiratory infections due to anatomical, physiological and immunological characteristics.⁴ These respiratory infections have a significant impact on health worldwide and are predominantly of viral origin.⁵

Viral pathogens account for 30-40% of ARI cases.⁶ The viruses most frequently involved are human respiratory syncytial virus (HRSV), human metapneumovirus (hMPV), influenza A and B (FluA and FluB), parainfluenza virus (PIV) 1, 2, and 3, and rhinovirus.

Currently, daycare centers are increasingly present in the lives of children aged 0-72 months, and several studies

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suggest that attending a daycare center is an important risk factor for respiratory infections.⁷ Children in preschool and daycare centers have a high frequency of respiratory infections due to contact with other children every day.⁸

Furthermore, infant morbidity associated with daycare centers has economic implications, since diseases result in the increased use of health care services and in increased family expenses on medical care and medications.⁹ However, studies on the viral etiology of ARIs in children attending daycare centers are relatively few in the literature.¹⁰

The purpose of this paper was to make a descriptive study reporting results on daycare-based ARI, carried out over a 24-month period to identify the frequency of viral agents in children with symptoms of respiratory infection.

Materials and methods

Study design and population

We conducted a longitudinal study of ARI cases in children from a daycare center in the city of São José do Rio Preto, SP, Brazil, from July 2003 to September 2005. All children attending the daycare aged 4-72 months and diagnosed by a pediatrician with typical symptoms of respiratory infection, such as sneezing, runny nose, fever, cough, and shortness of breath, were selected to participate in the study. Our study population consisted of 176 children, 100 boys and 76 girls. It is important to note that the daycare center was closed in the holiday period, which corresponded to the months of December, January, and February. This study was approved by the Research Ethics Committee of Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Instituto de Biociências, Letras e Ciências Exatas (IBILCE), by opinion no. 062/2001 on June 11, 2001 in São José do Rio Preto, SP, Brazil. In addition, a consent form for collection of samples was obtained from parents or guardians of all children enrolled.

Sample collection

At the time of sample collection, to ensure that the genetic material found was characteristic of an ongoing respiratory infection and not residual genetic material from previous respiratory infections, a new episode of respiratory infection was considered only if there was an interval of 7 days or more between the end and restart of symptoms.

The collection of material from nasopharyngeal secretion was performed by a single nurse, using 0.5 mL of sterile 1X phosphate buffered saline (PBS) for streamlining the secretion and swabs and neonatal suction catheter to harvest nasal material. The material was stored in a vial containing 3 mL of 1X PBS and informing name and protocol number of the child. Once collected, the nasal aspirates were placed in an insulated cooler with ice and sent to the laboratory (Laboratory for Genomic Studies, Laboratório de Estudos

Genômicos, UNESP, São José do Rio Preto) for processing. The nasal aspirates were diluted in PBS pH 7.2. Samples were divided into aliquots of 250 µL and treated with 25 µL of streptomycin and 2.5 µL of antifungal agents to prevent contamination. After an incubation period of 1 hour, samples were added to 750 µL of Trizol-LS (Gibco) and the material was stored in a freezer at -80 °C for subsequent RNA extraction.

Molecular viral detection

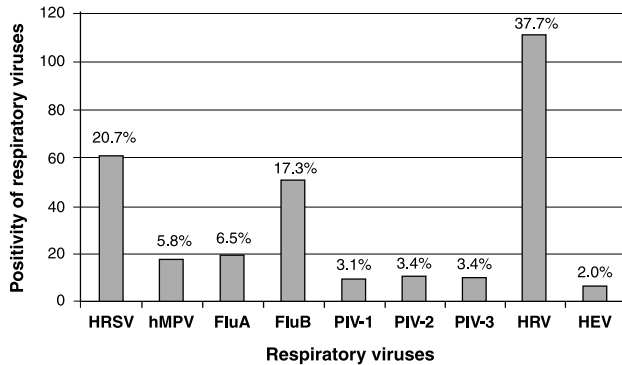
Aliquots (250 µL) of each sample were frozen at -70 °C, each with 750 µL of Trizol-LS (Gibco). The extraction of total RNA / DNA followed the instructions of the manufacturer of Trizol. The nucleic acid extracts were diluted with 50 µL of MilliQ water treated with diethylpyrocarbonate (Sigma) and containing 0.5 µL of RNase OUT (Invitrogen) at a final concentration of ~ 1 unit/µL. Extracts were immediately tested by reverse transcriptase polymerase chain reaction (RT-PCR). Reverse transcription was performed with High Capacity cDNA Archive kit (Applied Biosystems), according to manufacturer's instructions. For amplification of the samples, we used the method of polymerase chain reaction (PCR) with specific primers for detection of HRSV, hMPV, FluA, FluB, PIV-1, PIV-2, PIV-3, and picornavirus.¹¹⁻¹⁴ Typing of picornaviruses to detect rhinovirus or enterovirus was performed by direct sequencing with the specific primers OL26 (5'- GCA CTT CTG TTT CCC C -3') and OL27 (5'- CGG ACA CCC AAA GTA -3').¹⁵ The PCR products were purified by column with the QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions, and the BigDye® Terminator v 3.1 Cycle Sequencing Kit was used for fluorescent reaction. Samples were precipitated and then sequenced on a 3130xl Genetic Analyzer sequencer (Applied Biosystems). All sequences of rhinovirus and enterovirus samples were analyzed for homology with the sequence corresponding to the 5' untranslated region of the genomes of the respective virus using the Basic Local Alignment Search Tool (BLAST) program (Table 1).

Results

A total of 782 samples were collected throughout the 2 years of study. Of the 782 samples tested, 37.6% (294/782) were positive for at least one of the respiratory viruses analyzed. Among the positive samples, we found 20.7% (61/294) of HRSV, 5.8% (17/294) of hMPV, 6.5% (19/294) of FluA, 17.3% (51/294) of FluB, 3.1% (9/294) of PIV-1, 3.4% (10/294) of PIV-2 and PIV-3, 37.7% (111/294) of human rhinovirus (HRV), and 2% (6/294) of human enterovirus (HEV), as illustrated in Figure 1.

We analyzed the age distribution of the children from the daycare center according to each of the nine viral types found. As can be seen in Table 2, children above 24 months were the most affected by respiratory viruses, except FluA

and HEV, which affected more children under 24 months. Not all children who had symptoms of respiratory infection were positive for a virus, and 124 of the 176 children studied (70.4%) were really affected by some viral respiratory infection. Boys were infected in 56.4% (70/124) of the cases and girls in 43.5% (54/124). There were cases in which the same child was affected by a virus more than once in different collections. The most prevalent virus in both genders was HRV (Table 2).



FluA and FluB = influenza type A and B; HEV = human enterovirus; hMPV = human metapneumovirus; HRSV = human respiratory syncytial virus; HRV = human rhinovirus; PIV-1, PIV-2, and PIV-3 = parainfluenza virus type 1, 2, and 3.

Figure 1 - Prevalence of respiratory viruses in samples of nasopharyngeal aspirates collected from children attending a daycare center

All episodes of respiratory infection were characterized by the absence of severe symptoms and were generally limited to upper airways, with no need of hospitalization during the respiratory infection. The most frequent symptom in the study population was runny nose (93.2%), followed by cough (58.2%), nasal obstruction (14.6%), wheezing (3.7%), and fever (2.4%). Some symptoms considered common in respiratory infections, such as shortness of breath and sibilance, were not observed in our study population.

Regarding the seasonal distribution of viral respiratory infections among the children from the daycare center, it was observed that rhinovirus was detected more frequently in autumn, but the virus occurred throughout all the study period, with low frequency in the summer months (December, January, and February) (1%, n = 1). In all years, we observed that, in the summer, the number of respiratory infections was reduced. When we analyzed outbreaks per year, we noted that, in 2003, the most prevalent virus was HRSV, in 2004 there was a decrease in infections by HRSV and an increase in HRV outbreaks, and in 2005 HRSV was the predominant virus once again (Figure 2).

Discussion

This study shows that respiratory viruses are common in upper respiratory infections and are frequently found in children attending a daycare center in São José do Rio Preto, since, from the 782 samples of nasopharyngeal aspirates,

Table 1 - Specific primers used for virus detection

Virus	Primer	Sequence	BP	Reference
HRSV	sense (+)	5'- AAC AGT TTA ACA TTA CCA AGT GA -3'	379	Mazzulli et al., 1999 ¹¹
	antisense (-)	5'- TCA TTG ACT TGA GAT ATT GAT CG -3'		
hMPV	sense (+)	5'- GAG CCA ATT GAA AAT CCC AGA CA -3'	343	Falsey et al., 2003 ¹²
	antisense (-)	5'- GAA AAC TGC CGC ACA ACA TTT AG -3'		
FluA	sense (+)	5'- CTA AGG GCT TTC ACC GAA GA -3'	191	Claas et al., 1992 ¹³
	antisense (-)	5'- CCC ATT CTC ATT ACT GCT TC -3'		
FluB	sense (+)	5'- ATG GCC ATC GGA TCC TCA AC -3'	240	Claas et al., 1992 ¹³
	antisense (-)	5'- TGT CAG CTA TTA TGG AGC TC -3'		
PIV-1	sense (+)	5'- CCG GTA ATT TCT CAT ACC TAT G -3'	317	Echevarria et al., 1998 ¹⁴
	antisense (-)	5'- CCT TGC AGC GGA GTT GTT AAG -3'		
PIV-2	sense (+)	5'- CCA TTT ACC TAA GTG ATG GAA T -3'	204	Echevarria et al., 1998 ¹⁴
	antisense (-)	5'- GCC CTG TTG TAT TTG GAA GAG A -3'		
PIV-3	sense (+)	5'- ACT CCC AAA GTT GAT GAA AGA T -3'	102	Echevarria et al., 1998 ¹⁴
	antisense (-)	5'- TAA ATC TTG TTG TTA AGA TTG A -3'		
HRV	sense (+)	5'- GGC CCC TGA ATG YGG CTA A -3'	114	Arruda & Hayden, 1993 ¹⁵
	antisense (-)	5'- GAA ACA CGG ACA CCC AAA GTA -3'		

BP = base pair; FluA and FluB = influenza type A and B; hMPV = human metapneumovirus; HRSV = human respiratory syncytial virus; HRV = human rhinovirus; PIV-1, PIV-2, and PIV-3 = parainfluenza virus type 1, 2, and 3.

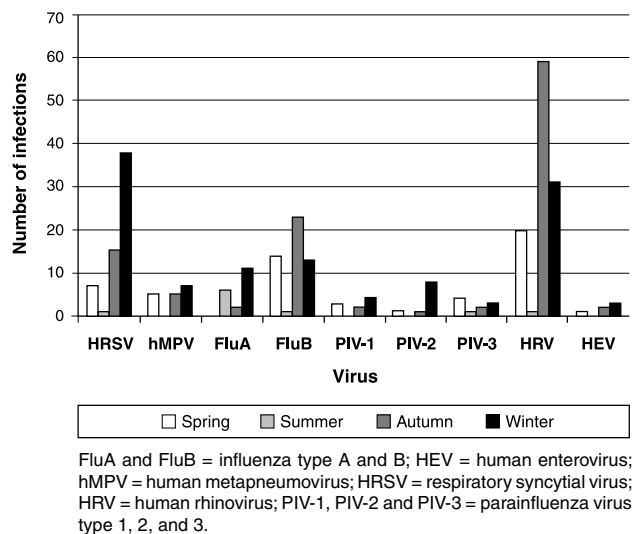
Table 2 - Distribution of each respiratory virus according to gender and age of the children

Age/gender	Positive samples (n = 294)	HRSV (n = 61)	hMPV (n = 17)	FluA (n = 19)	FluB (n = 51)	PIV-1 (n = 9)	PIV-2 (n = 10)	PIV-3 (n = 10)	HRV (n = 111)	HEV (n = 6)
≤ 24 months	104 (35.4%)	21 (7.1%)	4 (1.4%)	10 (3.4%)	14 (4.8%)	3 (1.0%)	4 (1.4%)	4 (1.4%)	40 (13.6%)	4 (1.4%)
> 24 months	190 (64.6%)	40 (13.6%)	13 (4.4%)	9 (3.0%)	37 (12.6%)	6 (2.0%)	6 (2.0%)	6 (2.0%)	71 (24.1%)	2 (0.7%)
Boys	70 (23.8%)	28 (9.5%)	9 (3.0%)	11 (3.7%)	28 (9.5%)	6 (2.0%)	7 (2.4%)	6 (2.0%)	47 (16.0%)	5 (1.7%)
Girls	54 (18.4%)	20 (6.8%)	8 (2.7%)	8 (2.7%)	15 (5.1%)	3 (1.0%)	3 (1.0%)	3 (1.0%)	38 (12.9%)	0 (0.0%)

FluA and FluB = influenza type A and B; HEV = human enterovirus; hMPV = human metapneumovirus; HRSV = human respiratory syncytial virus; HRV = human rhinovirus; PIV-1, PIV-2 and PIV-3 = parainfluenza virus type 1, 2 and 3.

37.6% (n = 294) were positive for a respiratory virus. These results are similar to those from the study conducted by Tsuchiya et al. in Curitiba, state of Paraná, Brazil,¹⁶ which identified a viral agent in 30% of the nasopharyngeal aspirate or bronchoalveolar lavage samples tested using shell vial culture and immunofluorescence assay (IFA). Other study analyzed nasopharyngeal specimens of children under 5 years of age with acute respiratory disease, and the application of IFA and RT-PCR allowed to detect a viral agent in 75.5% of the samples.¹⁷ However, these studies were performed with hospitalized children. In Bahia, Brazil, one of the few studies conducted with daycare children found 43% positivity for any of the respiratory viruses.¹⁸ However, in this case, only children under 24 months were selected, and the literature reports a high percentage of viral infection in children from this age group. Therefore, the lower positivity rate of our work is probably because most children from the daycare center were older than 24 months.

In our study, the most frequent respiratory pathogen found was HRV (37.7%), followed by HRSV (20.7%) and FluB (17.3%). In a study conducted by Souza et al.¹⁸ with children from daycare centers, of the 264 samples available for testing by RT-PCR and hybridization, 78% were rhinovirus positive. A study by van der Zalm et al.¹⁹ indicates that the occurrence of HRV infection is 13 times higher than that of HRSV infection, and also that the number of medical visits made during an episode of HRV infection is seven times higher than during HRSV infection. Overall, their study confirms the high occurrence of HRVs in respiratory tract infections. Despite the fact that rhinovirus presents high positivity, there are few data available in Brazil about the characteristics and

**Figure 2** - Seasonal distribution of viral respiratory infections among daycare children between July 2003 and September 2005

functions of this viral type in respiratory infections, which complicates the diagnosis and demonstrates the need for further studies to obtain new information.

HRSV was the second most prevalent virus, an unexpected high frequency, because our samples were from daycare children, and HRSV is mostly found in samples from children attending hospitals, who present more severe cases of infection.^{20,21}

FluB was the third virus most commonly found in our samples, followed by FluA, which presented 6.5% positivity.

The study by van der Zalm et al.¹⁹ found higher rates of influenza infection, with 83% of type A and 17% of type B. This difference can be explained by the fact that this study was performed with hospitalized children, who had more severe infections characteristic of FluA. The children in our study had mild symptoms of respiratory infection, which is a characteristic of FluB infection, justifying thus the higher incidence of this less aggressive virus.

Regarding the age of children with respiratory infections, although there were cases of respiratory infection in all age groups, we found that there were more infections in children between the ages of 13 and 48 months. According to Fiterman et al.,²² age is a risk factor for mortality from respiratory diseases. In a study conducted in Taiwan, of the 523 children infected by a virus, 32.5% were younger than 1 year old, 37.7% were aged between 1 and 3 years, and 17.2% between 3 and 6 years old.²³ Children affected by HRV typically have higher ages than those affected by other viruses such as HRSV, for example, which is usually detected in children younger than 1 year.^{24,25} However, in our study, the majority of HRSV-positive children was older than 24 months. This result can be explained by the fact that most children in our study population were from this age group.

In our study, when we analyzed the viral positivity for any of the nine viruses in relation to gender, there was a greater percentage of infections in boys (56.4%) compared to girls (43.5%). However, the number of boys included in our study is higher than the number of girls. Several studies have reported the high susceptibility of boys to respiratory infections; among them, a study in the Amazon also observed that boys were more frequently hospitalized due to respiratory diseases and had a 1.5 times greater risk of hospitalization for respiratory diseases as compared to girls.²⁶ Among other factors, anatomical differences between boys (for example, the smaller airway diameter) may be the cause of this preponderance of boys with regard to respiratory infections.^{27,28}

The predominant clinical symptoms in our study were runny nose (93.2%) and cough (58.2%), which shows the absence of severe symptoms. These results are consistent with studies by Thomazelli et al.,²⁹ in which one of the most common symptoms was cough, found in 86% of the cases of respiratory infection. Pecchini et al.³⁰ also found cough (92.3%) and runny nose (64.7%) as the most common symptoms. However, in their study and in other conducted in India,³¹ different from our results, fever was also a common symptom, found in high percentages. Our study population showed only mild symptoms, and this may have occurred due to the fact that these children live daily in the daycare center and have close contact with other children, and are daily exposed to different viral types, which gives them immunity without presenting severe symptoms of respiratory infection.

Regarding the seasonal distribution of viral respiratory infections among the children in the daycare center, it was found that outbreaks of infection occurred throughout all the study period, especially in autumn and winter. We observed that, in the summer, the number of respiratory infections was greatly reduced. However, infections were reduced in December, January, and February (summer) because it was vacation time in the daycare centers of the city; consequently, there was a little contact between the children and possibly a reduction of transmission of pathogens, which could have influenced the reduction of respiratory viruses in these 3 months. A study by Rosa et al.²⁶ in the Amazon region showed that hospitalizations due to respiratory diseases were lower in the months of December, January and February, which is consistent with our results. Furthermore, it is known that the circulation of respiratory viruses shows different patterns according to each region.³²

We have also observed interference between the annual outbreaks of HRSV and HRV. Previous studies have ascribed specific patterns to the seasonal and annual variations in respiratory virus epidemics and suggested that interference between viruses is the reason for peak fluctuations.^{33,34} However, these studies report the interference between outbreaks of HRSV and influenza infections. We suggest that, in addition to influenza, HRSV may have a possible interference also with HRV. Thus, further research is needed to elucidate these hypotheses.

It is important to note some limitations linked to the results, since, although São José do Rio Preto has 43 daycare centers, only one was authorized by the City Department of Education to implement this study. Hence, we believe that undertaking the study in a single daycare is not enough sample to represent the distribution of respiratory viral infections and to assess how respiratory viruses circulate in the city. Moreover, the percentage of viruses detected may be due to the lack of homogeneity of population in terms of age, because there were variations in the number of children in each age group. As already said, the literature reports a high percentage of viral infection in children under the age of 24 months, and the majority of children in our study population were older than this age group.

Therefore, we highlight the importance of further studies with larger number of daycare centers and with homogenization of age groups, which would allow a comparison between the results and a delineation of the profile of the children affected by respiratory infections, as well as increased knowledge about the behavior of viral agents that circulate in the daycare centers of the city.

Nevertheless, this work is relevant to the investigation of respiratory infections in daycare centers, considering the scarcity, in the literature, of this type of study in the Brazilian population. Our study is one of the few conducted in Brazil with children attending daycare centers and shows

the clinical and epidemiological importance of respiratory tract infections caused by respiratory viruses in children. Furthermore, our results reinforce the importance to be given to rhinovirus.

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