

## Respiratory viruses in children younger than five years old with acute respiratory disease from 2001 to 2004 in Uberlândia, MG, Brazil

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*The main viruses involved in acute respiratory diseases among children are: respiratory syncytial virus (RSV), influenza virus (FLU), parainfluenza virus (PIV), adenovirus (AdV), human rhinovirus (HRV), and the human metapneumovirus (hMPV). The purpose of the present study was to identify respiratory viruses that affected children younger than five years old in Uberlândia, Midwestern Brazil. Nasopharyngeal aspirates from 379 children attended at Hospital de Clínicas (HC/UFU), from 2001 to 2004, with acute respiratory disease, were collected and tested by immunofluorescence assay (IFA) to detect RSV, FLU A and B, PIV 1, 2, and 3 and AdV, and RT-PCR to detect HRV. RSV was detected in 26.4% (100/379) of samples, FLU A and B in 9.5% (36/379), PIV 1, 2 and 3 in 6.3% (24/379) and AdV in 3.7% (14/379). HRV were detected in 29.6% (112/379) of the negative and indeterminate samples tested by IFI. RSV, particularly among children less than six months of life, and HRV cases showed highest incidence. Negative samples by both IFA and RT-PCR might reflect the presence of other pathogens, such as hMPV, coronavirus, and bacteria. Laboratorial diagnosis constituted an essential instrument to determine the incidence of the most common viruses in respiratory infections among children in this region.*

Key words: respiratory viruses - respiratory disease - immunofluorescence assay - reverse transcription of polymerase chain reaction - children

Viruses are the most frequent agents that cause acute respiratory infections (ARIs) and are responsible for a considerable percentage of childhood mortality (Williams et al. 2002). In Brazil, some reports from different geographical areas has revealed the viruses as the main cause of respiratory infections, as related in the cities of Fortaleza (Arruda et al. 1991), Rio de Janeiro (Nascimento et al. 1991), São Paulo (Miyao et al. 1999, Vieira et al. 2001), and Curitiba (Tsuchiya et al. 2005).

The most important viruses involved in ARI are: respiratory syncytial virus (RSV), influenza viruses types A and B (FLU A/B), parainfluenza virus (PIV), adenovirus (AdV), human rhinovirus (HRV), and the human metapneumovirus (hMPV) (Miyao et al. 1999, Kuiken et al. 2003, Tsuchiya et al. 2005). The last one was recently identified by Hoogen et al. (2001).

RSV is the main cause of viral lower respiratory tract illness in children (Miyao et al. 1999), particularly in those younger than six months old (mo.) (Queiróz et al. 2002, Moura et al. 2003). In addition, RSV infections are responsible for most cases of severe symptoms such as bronchiolitis with recurrent wheezing and pneumonia (Calegari et

al. 2005) and for leading to a high number of hospitalizations (Tsuchiya et al. 2005).

FLU is a serious public health problem worldwide, were children constitute the age group most affected (Neuzil et al. 2002). Although many infections caused by FLU could be prevented by effective vaccination program, it has been predicted that a pandemic is likely to emerge in a near future (Cox et al. 2003), caused by a virus variant not covered by the current vaccine, requiring, thus, a constant epidemiological surveillance.

PIV seems to have pattern of seasonal occurrence and is considered an important cause of respiratory illnesses, particularly among young children (Monto 2002).

AdV infections are common in all age groups, causing both hospital- and community-acquired epidemics. Moreover, AdV has been associated with hospitalizations of near-fatal asthma patients (Tan et al. 2003) and with cases of acute otitis media in children younger than two years old (Monobe et al. 2003).

HRV is responsible for the majority of common colds during winter, causing upper respiratory infections (Arruda et al. 1991, Savolainen et al. 2003) and is considered a risk factor for acute otitis media (Monobe et al. 2003). In addition, HRV accounts for serious lower respiratory illness in infants and children with bronchopulmonary dysplasia (Kotaniemi-Syrjänen et al. 2003) and contributes for asthma exacerbations (Monto et al. 2001).

Some host factors, such as age, have been shown relevant for increasing the incidence and/or severity of respiratory infections caused by viruses in both children and elderly people (Zamorano et al. 2003). In the present

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study, our main objective was to determine what respiratory viruses are the causative agents of acute respiratory disease in young children in Uberlândia city, Midwestern Brazil, located in a region characterized by dry winters, rainy summers, and mild variations of temperatures.

#### MATERIALS AND METHODS

**Samples** - Three hundred and seventy nine samples of children younger than five years old who participated in this study were collected from 2001 to 2004. Children with acute respiratory infection were attended at Hospital de Clínicas of the Universidade Federal de Uberlândia (HC/UFU) and the samples were collected within five days of the onset of the symptoms in the following hospital units: (i) first aid (FA), (ii) pediatric first aid (PFA), (iii) pediatric ward (PW), (vi) pediatric intensive care unit (PICU), and v) neonatal intensive care unit (NICU). Demographic data, such as age, gender, and clinical symptoms were also obtained at the moment of the sample collection. Nasopharyngeal aspirates (NPAs) were collected according to Calegari et al. (2005), by instillation of 0.5 ml of buffered solution (0.9%) into the nostrils of a patient followed by aspiration by a vacuum system using a catheter. Samples were transported on ice to the Laboratório de Virologia and processed as previously described (Queiroz et al. 2002). This study was approved by the Ethics Committee of UFU according to resolution no. 196/1996/CNS. Written consent was obtained from the children's parents.

**Indirect immunofluorescence assay (IFA)** - The assay was carried out by using the Respiratory Panel I Viral Screening and Identification Kit (Chemicon International, Inc., Temecula, CA), following the manufacturer's instructions, for detection of the following respiratory viruses: RSV, FLU A/B, PIV 1, 2, and 3), and AdV.

**Reverse transcription-polymerase chain reaction (RT-PCR) for detection of HRV** - The assay was applied to detect HRV RNA sequence in samples that were negative or indeterminate by IFA. Total RNA was extracted with Trizol (Invitrogen Corporation, Carlsbad, CA), following the manufacturer's instructions. Complementary DNA (cDNA) synthesis and PCR amplification conditions were described by Arruda and Hayden (1993). The only modification we made was that primer 1, which was used for cDNA synthesis, was also added in the first-round PCR mixture, however maintaining the final primer concentration described by these authors. HRV grown in cell culture was a gift from Dr E Arruda (Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo) and was used as positive control.

**Statistical analysis** - Statistical analysis were carried out by using BioEstat version 3.0 (Ayres et al. 2003). Differences between proportions were evaluated by Qui-square test. Incidence of the referred respiratory viruses and the respective confidence intervals (CI) of 95% were calculated;  $p$  values lower than 0.05 were considered statistically significant.

#### RESULTS

From 2001 to 2004, 379 nasopharyngeal specimens of children younger than five years old with acute respira-

tory disease were tested by IFA for RSV, FLU A/B, PIV 1, 2 and 3, and AdV. Samples that were negative or indeterminate by IFA were tested by RT-PCR for detection of HRV.

Approximately 75% (286/379) of all samples were positive for the presence of any of these viruses (Table I). HRV cases responded for 29.6% (112/379), followed by RSV cases (26.4% - 100/379), FLU (9.5% - 36/379), PIV (6.3% - 24/379), and AdV (3.7% - 14/379), whereas samples which showed results that were either negative or indeterminate for those viruses tested responded for 24.6% (Fig. 1).

TABLE I  
Age distribution of the positive cases by indirect immunofluorescence and reverse transcription-polymerase chain reaction assays from 2001 to 2004, in Uberlândia, MG

Age in months	RSV	FLU	PIV	AdV	HRV	Total
< 3	37	2	1	1	19	60
3-6	15	5	2	1	18	41
6-9	12	3	2	2	13	32
9-12	5	2	3	3	13	26
12-24	20	15	7	6	25	73
≥ 24	11	9	9	1	24	54
Total	100	36	24	14	112	286

RSV: respiratory syncytial virus; FLU: influenza virus; PIV: parainfluenza virus; AdV: adenovirus; HRV: human rhinovirus.

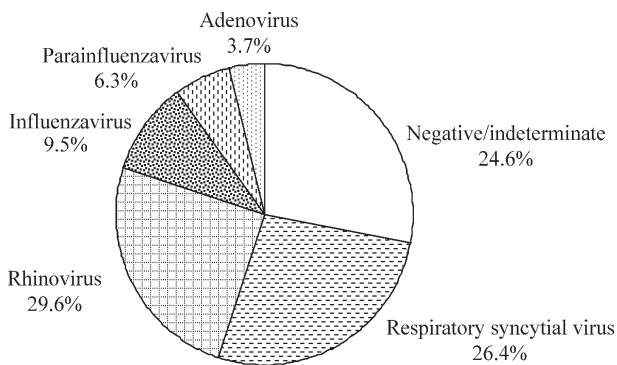


Fig. 1: percentage distribution of the results evaluated by indirect immunofluorescence assay and reverse transcription-polymerase chain reaction.

It was observed a significant difference among the total proportions of the cases along the age groups ( $p < 0.05$ ). This difference was also observed to RSV cases according to age distribution ( $p = 0.00$ ), in which the age group corresponding to children younger than three mo. was more affected (37/100) than the other age groups: 3-6 mo. ( $p = 0.00$ ), 6-9 mo. ( $p = 0.00$ ), 9-12 mo. ( $p = 0.00$ ), 12-24 ( $p = 0.03$ ), and  $\geq 24$  mo. ( $p = 0.00$ ). For HRV, no significant difference was found in the distribution of cases according to age group ( $p > 0.05$ ).

Regarding nosology, the most common symptoms related to HRV, FLU, PIV, and AdV infections were those involving the upper respiratory tract, mostly in children older than one year old (Table II). On the other hand, RSV infections were related mostly to bronchiolitis and pneumonia/bronchopneumonia cases (lower respiratory tract), especially in children younger than six mo. ( $p < 0.05$ ), responding for 60 and 46% of the cases, respectively. HRV were detected in 25% of bronchiolitis cases in children less than six mo. and in 15.4% of pneumonia/bronchopneumonia cases at the same age group. For influenzaviruses, almost 2/3 (26/36) of the FLU infections, involved the upper respiratory tract, whereas the lower respiratory tract disease responded for 11.1% (4/36).

Regarding seasonality, the outbreaks occurred mainly during the months with low temperatures of Uberlândia (Fig. 2). RSV cases were detected from February to June – late summer to late autumn in this region, with highest incidence from April to May, revealing a clear seasonality.

Peak of FLU cases occurred shortly after, in mid-winter, and extended to the end of the season. PIV and AdV cases did not show a clear seasonality, although these viruses were detected during the coldest rather than the hottest months. Similar data were observed for HRV, having, however, a peak of incidence during the coldest months, mostly in June with 28% (31/112) of the cases.

**DISCUSSION**

The application of IFA and RT-PCR allowed us to detect a viral agent in 75.5% (286/379) of the nasopharyngeal specimens of children under five years of age with acute respiratory disease. In Brazil, Souza et al. (2003), in order to determine the incidence of viral respiratory infectious episodes in young children detected a viral agent in 42.8% of the samples by using IFA. Yet, Tsuchiya et al. (2005) identified a viral agent in 30% of the nasopharyngeal aspirate or bronchoalveolar lavages samples tested using shell vial culture and IFA.

TABLE II  
Main nosological diagnostic of the respiratory infections according to age and to etiological agent

Symptoms Age in months	Bronchiolitis					Pneumonia/BCP					URTI				
	HRV	RSV	FLU	PIV	AdV	HRV	RSV	FLU	PIV	AdV	HRV	RSV	FLU	PIV	AdV
<3	6	22	-	1	-	2	6	-	-	1	3	7	1	-	-
3-6	12	7	1	-	-	2	3	2	-	-	6	3	2	2	1
6-9	2	7	-	-	-	-	-	-	-	1	8	2	2	2	1
9-12	2	4	-	-	2	2	-	-	-	-	4	1	2	1	1
12-24	1	2	1	-	1	3	2	-	1	-	15	10	12	5	4
≥ 24	1	2	1	-	1	3	2	-	1	-	15	10	12	5	4
Total	23	43	2	1	3	9	12	2	1	2	49	29	26	14	8

URTI: include flu, cold, and acute otitis media; HRV: human rhinovirus; RSV: respiratory syncytial virus; FLU: influenzavirus; PIV: parainfluenzavirus; AdV: adenovirus

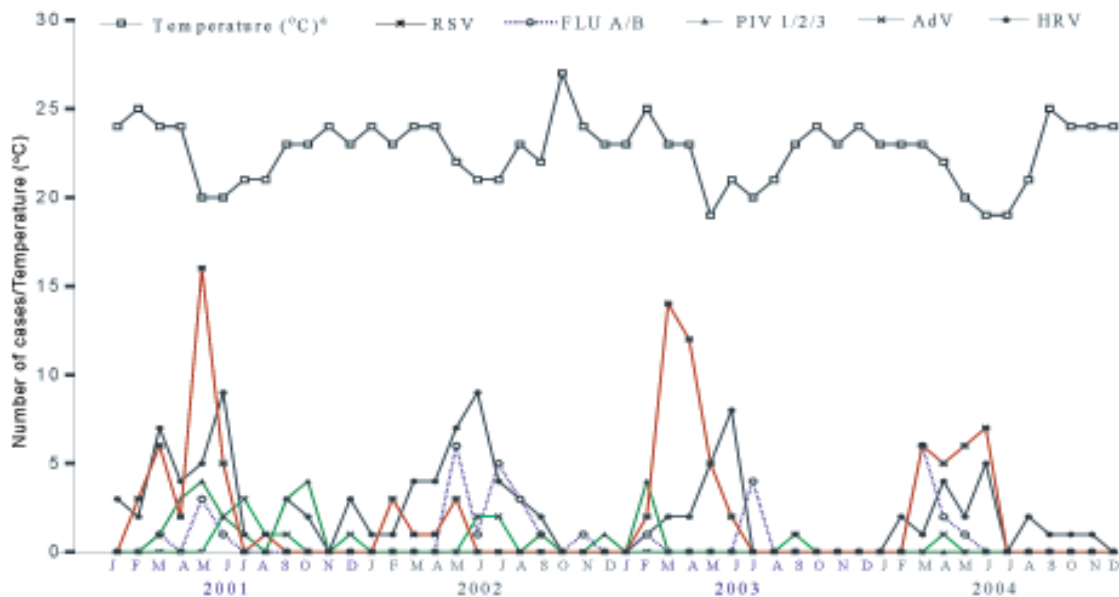


Fig. 2: monthly incidence of the respiratory virus among children up to five years old by immunofluorescence and reverse transcription-polymerase chain reaction methods from January 2001 to December 2004, in Uberlândia, MG; a: data from Laboratório de Climatologia e Recursos Hídricos/Universidade Federal de Uberlândia.

Moreover, Monobe et al. (2003) using multiplex nested RT-PCR from middle ear fluid of children with acute otitis media, identified a virus in 42% of the specimens. Finally, Tan et al. (2003) revealed that a viral agent was found in 52% of patients with near fatal asthma, asthma or chronic pulmonary disease, using PCR. Thus, the present study reveals the importance of applying at least two methods in order to improve the identification of respiratory pathogens.

The detection of RSV by IFA in approximately one fourth of the samples, demonstrated that this virus is a frequent agent of respiratory disease in children, particularly for those in their first six mo. of age. Some reports have shown that the RSV is a frequent cause of acute respiratory infections in children younger than five years old, with incidence reaching 17% (Moura et al. 2003), 18% (Bosso et al. 2003), 22% (Cintra et al. 2001), and 28% (Checon et al. 2002) of the investigated cases.

The finding that RSV infections were mainly associated with bronchiolitis and pneumonia/bronchopneumonia (60 and 46% of the cases, respectively), demonstrated that this agent was an important cause of lower respiratory tract infections, results that were also found by Nascimento et al. (1991), Vieira et al. (2001), Moura et al. (2003), and Tsuchiya et al. (2005). Moreover, the fact that approximately half of RSV infections (52/100) occurred in children under 6 mo., showed that patients in this age group are the most vulnerable to infections caused by RSV, despite the presence of maternal antibodies (Queiróz et al. 2002). Also in the same age group, severity of the infection caused by RSV was observed in those patients that presented bronchiolitis and pneumonia/bronchopneumonia (40.3 and 34.6%, respectively).

The frequency and severity of RSV infections in this age group seem to be a consequence of complex and multifactorial events, involving especially immunological factors (Queiróz et al. 2002). In spite of the fact that infants are relatively immature in their ability to mount immune response to respiratory virus infections, maternal antibodies are an important means of protection (Crowe 2001). Although Glezen et al. (1981) suggested that infants with high levels of maternal RSV-neutralizing antibodies developed mild illness, lactates and young children are the groups most affected by RSV (Queiróz et al. 2002, Checon et al. 2002, Moura et al. 2003, Bosso et al. 2004). Also, it still remains unclear whether the severity of RSV infection could be triggered by cross-reactivity between RSV subtype-specific maternal antibodies and a different RSV variant.

Anatomic restrictions of the neonatal bronchial tree could also explain the severity in neonatal and young infants infected with RSV, mediated by hyper reactivity of substance P and its receptor NK1 on target cells early in infancy than later in life (Piedimonte 2001). Also, according to the hygiene hypothesis (Benten et al. 2005), repeated respiratory infections could induce immune maturation in children, decreasing the incidence and the severity of respiratory diseases.

Results obtained for FLU, PIV, and AdV cases in other age groups of this study are in agreement with Erhart et al. (2004). In these cases, maternal antibodies could prob-

ably provide an important mechanism of protection in young children (Crowe 2001).

The detection of influenzaviruses in 9.5% of the cases indicated that this agent is an important cause of respiratory disease among children (Heikkinen et al. 2004), similar to related by Neuzil et al. (2002). However, the number of FLU positive samples might be increased by using PCR based methods (Ellis & Zambon 2002). Regarding the nosology, 2/3 of FLU cases were related to upper respiratory tract infections, whereas the lower respiratory tract disease were observed in 11.1% of the cases. However, it is important to be aware about the emergence of pandemic strains that has been emerging along the years, such as H5N1 (Barrera & Reyes-Terán 2005).

Detection of HRV in 29.6% of the cases demonstrated that this virus is not only a common agent of respiratory infections in adults, but it is also frequently detected in children, as previously reported (Arruda et al. 1991, Savolainen et al. 2003). Although most of HRV cases occurred in patients with upper respiratory tract disease, as reported by Heikkinen and Järvinen (2003), the virus was also detected in 25% of bronchiolitis and in 15.4% of pneumonia/bronchopneumonia cases in children younger than six mo., indicating that HRV was probably involved with more severe symptoms of lower respiratory tract. Similar results were reported by Papadopoulos et al. (2002) and by Hayden (2004). However, co-infection with other pathogens, which might enhance the severity of the HRV-related diseases, can not be discarded (Malcolm et al. 2001). Moreover, atopic diseases (not evaluated here) and young age could also be enhancing the disease severity by HRV (Lemanske et al. 2005).

Regarding gender (results not shown) male children were more frequently affected by respiratory viruses than females, in agreement to studies reported by Monto (2002).

In general, respiratory viruses were detected mostly during the coldest and driest months of Uberlândia, which has a semitropical climate, characterized by an interchange of dry and mildly cold winters and rainy summers. Circulation of the respiratory viruses shows different patterns, according to the region. In tropical regions, such as Salvador, in Northeastern Brazil (Moura et al. 2003), respiratory viruses were detected mostly in rainy seasons, whereas in subtropical regions, as São Paulo, in Southeastern Brazil (Vieira et al. 2001) and Porto Alegre, in Southern Brazil (Stralliotto et al. 2001), they were mainly detected during the coldest months, as similarly found in Uberlândia, which however has a short time period with low temperatures.

In this study, RSV was detected from February to June (late summer to late autumn), with peak in April and May (autumn), as similarly related in Ribeirão Preto (SP) by Cintra et al. (2001) and in Vitória (ES) by Checon et al. (2002), and has not been detected in other months. In regions with lower temperatures during winter, such as São Paulo (Vieira et al. 2001), Botucatu (Bosso et al. 2004), and Porto Alegre (Stralliotto et al. 2001) cities, RSV circulation extended to July or August (mid-winter). Thus, it seems that RSV circulation accompanies low temperatures. Moreover, RSV circulation showed, in our study, an alternate pattern of incidence. In 2001 and 2003, there were

more RSV cases in comparison to 2002 and 2004. This is apparently related to the lower temperatures registered in Uberlândia in 2001 and 2003.

The detection of FLU from March to August, with peak in May, suggests that this virus is also more frequently related to the coldest months of the year, which is in accordance to data reported by Monto (2002). Similarly, HRV was mainly detected during the months with lower temperatures in Uberlândia and is in agreement with results reported by Monto (2002). PIV and AdV showed no apparent regular seasonal pattern. For AdV, similar data was reported by Souza et al. (2003).

Additional studies are needed in order to elucidate the etiology in those cases that showed either negative or indeterminate results, which might indicate the presence of other pathogens, such as human metapneumovirus, coronavirus or bacteria. hMPV is considered an important pathogen that causes respiratory infections in children younger than five years old (Kuiken et al. 2003). Difficulties related to the methodology, such as infection time point of sample collection or the sample collection procedure itself might also have been the cause of these results.

Laboratorial investigation has proved to be necessary for viral identification in acute respiratory disease among children of Uberlândia region. However, continuous efforts are important to consolidate an epidemiological surveillance of the main respiratory viruses affecting children. This study represents the first information of the state of Minas Gerais.

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