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Surveillance of eight respiratory viruses in clinical samples of pediatric patients in Southeast Brazil

Luciano M. Thomazelli,[1] Sandra Vieira,[2] Andrea L. Leal,[3] Thereza S. Sousa,[4] Daniele B. L. Oliveira,[3] Miguel A. Golono,[3] Alfredo E. Gillio,[5] Klaus E. Stwien,[6] Dean D. Erdman,[7] Edison L. Durigon[8]

Abstract

Objective: Detection of the eight most common respiratory viruses: Human respiratory syncytial virus (HRSV), influenza virus A and B (IA and IB), parainfluenza viruses 1, 2 and 3 (HPIV1, 2 and 3), adenovirus (Ad) and human metapneumovirus (HMPV), in order to establish the etiology of acute respiratory infections (ARIs) and the epidemiology of these viruses in young children seen at Hospital Universitário, Universidade de São Paulo, in São Paulo, Brazil, during 2003.

Methods: The epidemiological surveillance was conducted in all children younger than 5 years hospitalized at the Hospital for lower respiratory tract infections (LRTI) from January 1, 2003 to December 30, 2003. Nasal and throat samples were scanned for respiratory viruses by polymerase chain reaction and detected by the GeneScan assay.

Results: Of 336 samples collected from 336 patients, 187 (55.6%) were positive for at least one of the respiratory viruses studied. Of all the children, HRSV was identified in 24.1%, HMPV in 17.8%, HPIV3 in 8.3%, Ad in 6.8%, IA in 5%, HPIV1 in 0.6%, but no virus could be detected in 44.1%. Dual virus infections were detected in 7.1% of all samples (12.8% of positive samples). HPIV2 and IB were not detected in the present study.

Conclusions: This study confirms that children younger than 5 years and particularly younger than 1 year have a high hospitalization rate due to HRSV, HMPV, HPIV, influenza and adenovirus. We were able to determine the etiology and epidemiology of most ARIs and trace the seasonal profile of the commonest respiratory viruses among young children.

J Pediatr (Rio J). 2007;83(5):422-428: Influenza, HRSV, parainfluenza, metapneumovirus, respiratory infections.

Introduction

Acute respiratory infections (ARIs) are the most common causes of childhood morbidity and mortality worldwide, accounting for about 30% of all childhood deaths in the developing world.[1] Although they rarely cause death in industrialized countries, ARIs translate into enormous direct and indirect health care costs.[2,3] Viruses account for 50 to 90% of lower respiratory tract infections (LRTI) in young children[4] with most of identified infections being attributed to viruses: human respiratory syncytial virus (HRSV), human parainfluenza viruses (HPIVs) I, II, and III, influenza A and B, adenovirus, and more recently, human metapneumovirus (HMPV).[5-8]

1. Mestre, Universidade de São Paulo (USP), São Paulo, SP, Brazil. Instituto de Ciências Biomédicas, USP, São Paulo, SP, Brazil.
2. Doutora. Professora, Departamento de Pediatria, Faculdade de Medicina, USP, São Paulo, SP, Brazil. Hospital Universitário, USP, São Paulo, SP, Brazil.
3. Mestre, USP, São Paulo, SP, Brazil. Instituto de Ciências Biomédicas, USP, São Paulo, SP, Brazil.
4. Fisioterapeuta, Universidade de São Camilo, São Camilo, SP, Brazil. Instituto de Ciências Biomédicas, USP, São Paulo, SP, Brazil.
5. Doutor, Faculdade de Medicina, USP, São Paulo, SP, Brazil. Hospital Universitário, Universidade de São Paulo, SP, Brazil.
6. Doutor, Faculdade de Medicina, USP, São Paulo, SP, Brazil. Instituto de Ciências Biomédicas, USP, SP, Brazil.
7. PhD, University of Georgia, Atlanta, GA, USA. Centers for Disease Control and Prevention, Atlanta, GA, USA.
8. Professor titular, USP, São Paulo, SP, Brazil. Instituto de Ciências Biomédicas, USP, São Paulo, SP, Brazil.

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In order to provide surveillance of the major responsible agents of ARIs and trace their etiological and epidemiological profile, we used a reverse transcription polymerase chain reaction (RT-PCR) assay based on GeneScan software, which offers the possibility of improved sensitivity and more timely diagnoses of respiratory viral infections, and has been shown to provide rapid results with equivalent or greater sensitivity than direct antigen detection or virus isolation for detection of these viruses.[9,10]

Methods

Target population

This is a prospective trial of children aged less than 5 years with LRTI within 7 days of onset, admitted to the emergency room (ER), intensive care unit (ICU) or pediatric wards of the Hospital Universitário (HU) of Universidade de São Paulo (USP), São Paulo,Brazil. The HU provides care to students and low-income individuals (around 500,000 people) who live close to the University, with admission of nearly 400 infants with LRTI to the pediatric department every year. The study period started on January 1 and ended on December 30, 2003. The patients were enrolled in the study by one of the authors (SV) after written consent was obtained from the child's parents. On examination, patient history, socioeconomic background, clinical signs and diagnosis at admission were recorded in a standard form.

The inclusion criteria were: all children under 5 years with LRTI presenting with one or more of the following physical symptoms: dyspnea (respiratory rate > 50), chest retractions, wheezing, rales, stridor, and cyanosis; and radiological pulmonary changes (hyperinflation, condensation). The exclusion criteria were: chronic respiratory disease (> 7 days of LRTI onset); and patients seen at the HU from Friday 6 p.m to Monday 8 a.m., for operational reasons.

Virological studies

A nasal wash was obtained after washing the nostrils with saline and collecting the suctioned specimen into a cup within a maximum of 24 h after admission. Specimen collection protocols and procedures were approved by the Research Ethics Committee of ICB-USP. All specimens were transported to the laboratory at 4 ºC within 2 h of collection, with immediate extraction thereafter.

Prevention of carryover contamination

To reduce the opportunity for amplicon contamination, we separated the pre- and post-assay into three different rooms, changed gloves regularly, pre-measured reagents into aliquots and used multiple negative specimen controls in each test. Tips equipped with sealing filters were used for pipetting the reagents, and all areas and equipment were decontaminated with sodium hypochlorite prior to and after pipetting. In addition, the GeneScan analysis further reduced the risk of contamination by permitting sensitive amplicon detection without the risks inherent to nested PCR[11].

Nucleic acid extraction

Aliquots (250 µl) of each specimen were dispensed into a tube, each containing 750 µl of Trizol-LS (Invitrogen) and frozen at -70 ºC until their extraction. The total RNA/DNA extraction followed the Trizol manufacturer's instructions. Nucleic acid extracts were diluted with 50 µl of UltraPure nuclease-free water (Gibco-BRL) containing RNAse inhibitor (RNA guard-Applied Biosystems) at a final concentration of ~1 unit/µl. Extracts were tested by RT-PCR immediately or stored at -70 ºC.

Primers

Previously described oligonucleotide primers for HRSV, HPIV I, II, and III, influenza A and B, adenovirus and HMPV, were used[9,12,13] (Table 1). For GeneScan analysis, the positive strand primer of each primer set was 5'-end-labeled with fluorescent 6-carboxyfluorescein (6-FAM).

RT-PCR

RT-PCR assays were developed with the High Capacity cDNA Archive kit (Applied Biosystems). For each specimen, 100 µl reactions were prepared by adding 50 µl of RNA extract to 50 µl of nuclease-free water containing 1 x RT buffer, 1 x dNTP mixture, 2.5 U/µl Multscribe reverse transcriptase and 1 x random primers, and distributed into 0.2 mL tubes. Thermocycling was performed on a MicroAmp 9600 thermocycler (Applied Biosystems) programmed for 25 ºC for 10 min and 37 ºC for 120 min.

Screening PCR

The amplification assay was performed separately. For each specimen, 3 µl of cDNA were distributed in a column of the 96-well plate containing 1 x PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 25 pM of each primer set and Ultra pure water for 25 µl. Each row of the plate comprised primers for a different virus, and the last one comprised primers for adenovirus; therefore, extracted DNA instead of cDNA was applied to this row. Thermocycling was programmed for 94 ºC for 2 min, 40 cycles of 94 ºC for 1 min, 54 ºC for 1 min, and 72 ºC for 1 min for cDNA/DNA amplification; and 5 min at 72 ºC for final amplicon extension. A clinical isolate of HRSV (Dean Erdman, CDC, Atlanta) was used as positive control of extraction and PCR reaction, and sterile PBS was used as a negative control in each reaction plate.

GeneScan (GS) analysis

Following RT-PCR, amplified products of each specimen (plate column) were pooled and purified by precipitation with ethanol to remove the excess salt. 1 µl of the purified amplification product was transferred to sequencing tubes (Applied Biosystems) containing 12 µl of deionized formamide (Applied

Table 1 - Reverse transcription polymerase chain reaction assay panel showing virus-specific primers

Virus	Primer ID	Gene	Polarity	Sequences (5' > 3')	Amplicon size (bp)
HRSV	RSVAB-F1-FAM	F	+	AACAGTTTAACATTACCAAGTGA	380
	RSVAB-R1		-	TCATTGACTTGAGATATTGATGC	
HPIV1	HPIV1-F1-FAM	HN	+	CCGGTAATTTCTCATACCTATG	317
	HPIV1-R1		-	CCTTGGAGCGGAGTTGTTAAG	
HPIV2	HPIV2-F1-FAM	HN	+	CCATTTACCTAAGTGATGGAAT	203
	HPIV2-R1		-	GCCCTGTTGTATTTGGAAGAGA	
HPIV3	HPIV3-F1-FAM	HN	+	ACTCCCAAAGTTGATGAAAGAT	102
	HPIV3-R1		-	TAAATCTTGTTGTTGAGATTGA	
Influenza A	FLUA-F1-FAM	NS1	+	CTAAGGGCTTTCACCGAAGA	192
	FLUA-R1		-	CCCATTCTCATTACTGCTTC	
Influenza B	FLUB-F1-FAM	NS1	+	ATGGCCATCGGATCCTCAAC	241
	FLUB-R1		-	TGTCAGCTATTATGGAGCTG	
Adenovirus	ADENO-F1-FAM	Hexon	+	CCC(AC)TT(CT)AACCACCACCG	167
	ADENO-R1		-	ACATCCTT(GCT)C(GT)GAAGTTCCA	
HMPV	MPVF-F1-FAM	F	+	GAGCAAATTGAAAATCCCAGACA	347
	MPVF-R1		-	GAAAACTGCCGCACAACATTTAG	

Biosystems) and 0.5 µl of ROX 50-500 DNA Ladder (Applied Biosystems) as internal size standard, denatured at 90 ºC for 5 min and cooled on ice. Amplicon analysis was then performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with GeneScan software (version 3.1.2), using a 47-cm capillary with POP-4™ polymer. Electropherograms were examined and specimens showing discrete amplicon peaks within ≤ 1 nt of the respective positive control peak were considered positive.

Statistical analysis

The results were compared using either the chi-square test or Fisher's exact test. The Mann-Whitney test was applied to compare continuous or ordinal measures. The significance level adopted was 0.05.

Results

Patients

Three hundred thirty-six patients were included in the study. The distribution of patients according to the unit of admission was as follows: 32% for ICU, 61.2% for ER and 6.8% for the pediatric ward. The children's ages ranged from 18 days to 4.6 years, with a median age of 5 months. The proportion of males and females was 56.5% and 43.5%, respectively. The most common respiratory symptoms were cough (86%), fever (69%), rhinitis (42.1%) and wheezing (38.9%). Clinical diagnoses at admission were pneumonia (64%), bronchiolitis (30.9%), and acute otitis media (4.3%).

Clinical specimens

All the 336 samples were tested by GeneScan RT-PCR; of these 149 (54.4%) specimens were negative and 187 (55.6%) were positive for at least one of the respiratory viruses included in the test. The HRSV was detected in 81 (24.1%) of all samples, HMPV was detected in 60 (17.8%), HPIV3 in 28 (8.3%), adenovirus in 23 (6.8%), influenza A virus in 17 (5%), and HPIV1 was detected in just 2 (0.6%) samples. HPIV2 and influenza B virus were not detected in the present study. The total results are summarized in Table 2.

Simultaneous detection of two viruses

Dual virus infection was detected in 24 samples (7.1% of the total or 12.8% of the positive samples). Detection of the HRSV amplified product occurred with HPIV1 (once), HPIV3 (twice), adenovirus (twice), influenza A virus (three times), and HMPV (five times). Dual infections involving HMPV were detected with HPIV3 (once), with influenza A virus (once), and with adenovirus (four times). Furthermore, HPIV3 was detected with influenza A virus (once) and with adenovirus (four times).

Epidemiological data x virus type

When physical symptoms and clinical diagnosis were compared with virus type no significant association was found; both simple infections and co-infections often consisted of pneumonia, followed by bronchiolitis, wheezing, and otitis. The highest rate of pneumonia was found in patients with adenovirus (88.9%), whereas bronchiolitis was more frequent in those with HRSV (54%), wheezing in those with HMPV (16.7%), and otitis in patients with the influenza virus (18.2%). When patient sex and etiology were compared, no significant association was found; with 55% of males and 45% of females showing positive results. The analysis of etiology

Table 2 - Total results of simple infections and co-infections

	HRSV	HMPV	HPIV3	Adenovirus	Flu A	HPIV1	TOTAL
HRSV	**68**	05	02	02	03	01	81
HMPV	05	**49**	01	04	01	-	60
HPIV3	02	01	**20**	04	01	-	28
Adenovirus	02	04	04	**13**	-	-	23
Flu A	03	01	01	-	**12**	-	17
HPIV1	01	-	-	-	-	**01**	02

* Number of samples containing each set of viruses (row x column). In boldface, simple infections.
Flu A = influenza A virus; HMPV = human metapneumovirus; HPIV1 = human parinfluenza I; HPIV3 = human parinfluenza III; HRSV = human respiratory syncytial virus.

by age distribution shows that the largest number of positive cases occurs in infants aged less than 1 year (75%); the median age of patients by respective results were: 4 months for HRSV, 4.5 months for HMPV, 5 months for HPIV, 7 months for adenovirus, 8 months for the influenza virus and 6.5 months for co-infections.

Seasonality of respiratory viruses

Considering the sampling date, we were able to obtain a graph for the number of positive samples to each studied virus according to the month of the year, showing the seasonality of the major respiratory viruses during 2003 (Figure 1).

Discussion

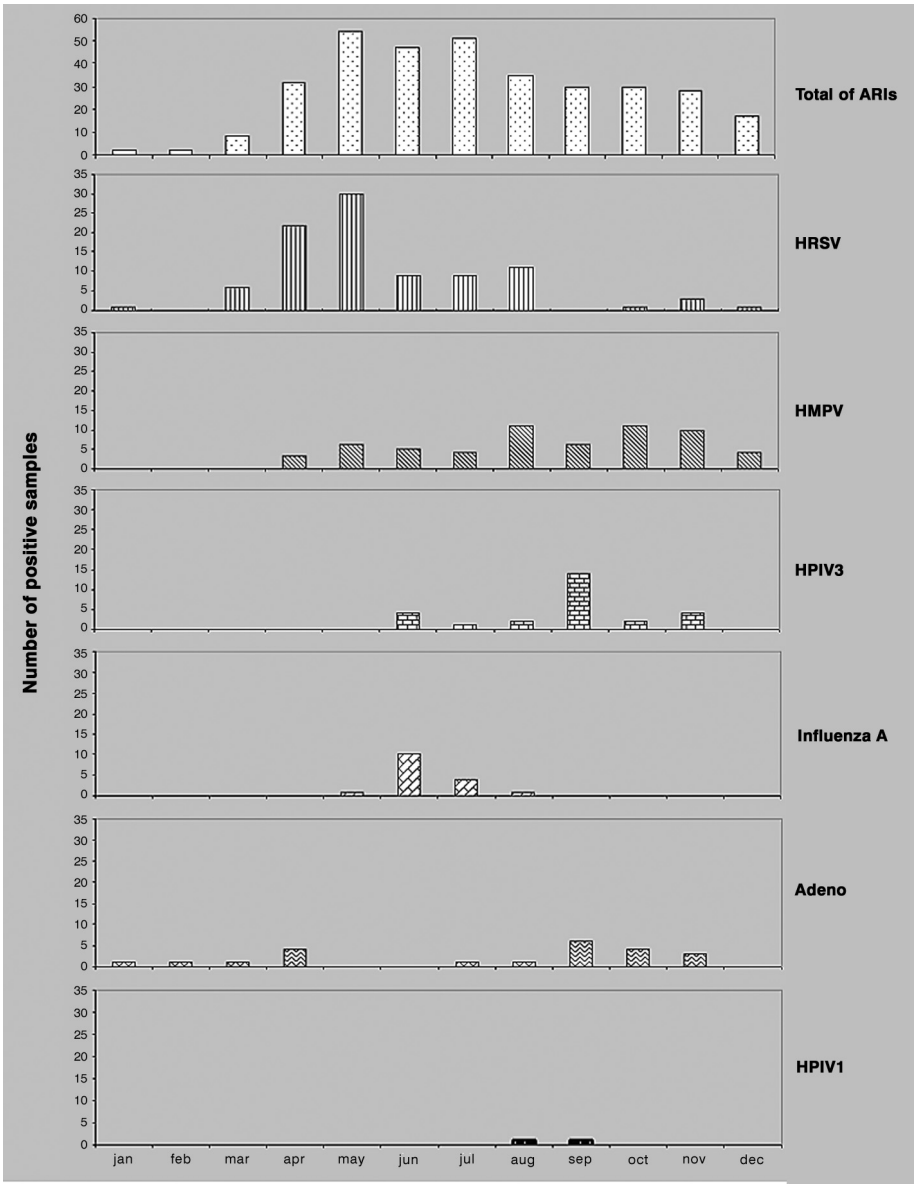
This work provided a first insight into the etiology of ARI in children admitted to the HU. HRSV was the most common cause of ARI detected in 43.3% of positive samples (68 of simple infections and 13 of dual infections), followed by the recently discovered HMPV, detected in 32.1% of positive samples (49 of simple infections and 11 of dual infections). Other studies have found that HMPV incidence can vary from year to year, sometimes rivaling or exceeding HRSV incidence[13,14]. HRSV has a worldwide distribution, with outbreaks occurring yearly and with an unusually predictable and regular pattern. In temperate climates, HRSV causes annual epidemics during the winter months[15], while epidemiological data from tropical regions have shown an association between RSV outbreaks and rainy seasons[16,17]. Brazil is a very large country with five geographic regions, each one with unique climate characteristics. São Paulo is located in southeastern Brazil, with a subtropical climate. Our data show that there, HRSV outbreaks begin in late autumn or early winter, peaking in May and lasting for 5 months. Epidemic outbreaks of HRSV showed a temperature-dependent pattern and no association with rainfall, peaking in the coldest months of the year. This pattern of HRSV outbreaks is similar to that observed by other authors[18,19] and that observed in the southernmost South American countries such as Chile, Uruguay, and Argentina[20-22]. Although the clinical symptoms of HMPV-positive patients are HRSV-like, the distribution of HMPV throughout the year was very different from that of HRSV. Our data show that HMPV outbreaks occurred during autumn, winter, and spring, with most of the positive cases in the spring. These findings are similar to those obtained by Galiano et al.,[23] who detected a larger frequency of HMPV during spring, but our conclusions are biased by the length of the sampling period (1 year). The true timing of HMPV infection needs to be determined in studies performed over longer periods of time.

Among the parainfluenza viruses, the most detected ones were of type 3, found in 28 samples (8.3% of total samples). The HPIV type 2 was not detected and type 1 was detected in just two samples. In the literature, type 3 is the most frequent among HPIVs and type 1 and 2 are not detected or are detected in just a few samples[24,25]. The two positive samples for HPIV1 were collected in the winter, as well as most of the positive samples for HPIV3, which showed their peak of incidence in late winter, just after the low of the HRSV and HMPV peaks.

The influenza B virus was not detected and the influenza A virus was detected in 17 samples, collected mostly at the end of fall/beginning of winter. These results are consistent with the surveillance studies done by the Center for Disease Control and Prevention, which indicated that 99.8% of the isolated influenza viruses were influenza A virus and that 0.2% were influenza B virus[26].

To conclude the epidemiological profile of the main respiratory viruses in 2003, adenovirus was observed throughout the year in 23 samples (6.8% of total samples), with small peaks at the beginning of fall and spring.

The results presented here indicate the feasibility of using an RT-PCR panel in surveillance work for the detection of RNA containing viral pathogens. By using this technique, we were able to detect the eight commonest pathogens of the respiratory tract within 1 working day, showing that this method is



Adeno = adenovirus; ARIs = acute respiratory infections; LRTI = lower respiratory tract infections; HMPV = human metapneumovirus; HPIV1 = human parainfluenza I; HPIV3 = human parainfluenza III; HRSV = human respiratory syncytial virus; RT-PCR = reverse transcription polymerase chain reaction.

Figure 1 - Total number of patients with LRTI seen at the Hospital during 2003 and the number of GeneScan RT-PCR-positive samples derived from these patients according to the month of the year

well-suited for use in epidemiological studies as well as in rapid microbiological studies in the clinical setting.

One of the goals of our study was to provide baseline data, which health authorities could consider for long-term surveillance plans. Moreover, another potential advantage of using this method is its ability to detect dual infection. Currently, the reported frequency of dual respiratory viral infections varies widely, and the importance of such infection is unclear[27,28]. In this study, multiple infections were seen in 24 patients, that is, dual viral infections were detected in 7.1% of total samples or 12.8% of positive samples. The rate of multiple infections is above that observed in the literature, and can be explained by the greater sensitivity of the fluorescence-based PCR panel when compared to direct antigen detection or virus isolation,[10,29] and by the larger number of respiratory viruses included in this test. The analysis of age distribution according to viral infection shows that the largest number of positive cases, either simple infections or co-infections, occurs in children aged less than 1 year, thus being in line with the international literature[2,5].

When physical symptoms and clinical diagnosis were compared with etiology no association could be found, so it is impossible to identify the virus type based only on clinical signs; however a relatively higher rate of pneumonia was found in patients with adenovirus and HPIV (88.9% and 75% respectively), as well as a higher rate of bronchiolitis in patients with HRSV and HMPV (54% and 27.8%, respectively). Besides, there is no significant association between patient sex and viral etiology. The main objective of this study was not the clinical analysis of infections; so diagnosis at admission was considered in lieu of the definitive one, proving unsuitable for the association between definitive diagnosis and viral etiology.

Our results support data indicating that RSV and HMPV are among the most important agents of ARI in childhood. The epidemic period of respiratory infections observed in São Paulo can be helpful for the planning and implementation of some preventive strategies. Longitudinal studies should be performed to confirm the results obtained here. Efficient strategies such as control of nosocomial infections caused by respiratory viruses, use of antiviral therapy, and more judicious use of antibiotics in viral ARI could be some of several other benefits generated by longitudinal studies of the clinical and epidemiological aspects of these infections.

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Correspondence:
Luciano Matsumiya Thomazelli
Av. Professor Lineu Prestes, 1374, ICB2, Laboratório Virologia
CEP 05508-000 – São Paulo, SP – Brazil
Tel.: +55 (11) 3091.7293
Fax: +55 (11) 3091.7354
E-mail: lucmt@usp.br