Detection of human bocavirus and human metapneumovirus by real-time PCR from patients with respiratory symptoms in Southern Brazil

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The introduction of newer molecular methods has led to the discovery of new respiratory viruses, such as human metapneumovirus (hMPV) and human bocavirus (hBoV), in respiratory tract specimens. We have studied the occurrence of hMPV and hBoV in the Porto Alegre (PA) metropolitan area, one of the southernmost cities of Brazil, evaluating children with suspected lower respiratory tract infection from May 2007-June 2008. A real-time polymerase chain reaction method was used for amplification and detection of hMPV and hBoV and to evaluate coinfections with respiratory syncytial virus (RSV), influenza A and B, parainfluenza 1, 2 and 3, human rhinovirus and human adenovirus. Of the 455 nasopharyngeal aspirates tested, hMPV was detected in 14.5% of samples and hBoV in 13.2%. A unique causative viral agent was identified in 46.2% samples and the coinfection rate was 43.7%. For hBoV, 98.3% of all positive samples were from patients with mixed infections. Similarly, 84.8% of all hMPV-positive results were also observed in mixed infections. Both hBoV and hMPV usually appeared with RSV. In summary, this is the first confirmation that hMPV and hBoV circulate in PA; this provides evidence of frequent involvement of both viruses in children with clinical signs of acute viral respiratory tract infection, although they mainly appeared as coinfection agents.

Key words: human metapneumovirus - human bocavirus - real-time PCR - respiratory tract infection

Respiratory tract infections are caused mostly by viruses, including respiratory syncytial virus (RSV), influenza viruses A (FLU A) and B (FLU B), parainfluenza viruses (PIV), human adenovirus (ADV) and human rhinoviruses (RNV). Recently, with the advent of molecular techniques and the introduction of these methods into the clinical laboratory, new respiratory viruses, such as the human metapneumovirus (hMPV) and human bocavirus (hBoV), were identified in respiratory tract specimens collected from patients with clinical symptoms of respiratory diseases (Neske et al. 2007). hBoV is the latest pathological parvovirus discovered in Sweden, isolated from pooled nasopharyngeal aspirate specimens (Allander et al. 2007). The incidence rates are between 3% and 19% in respiratory samples from children with acute respiratory disease. However, the pathogenic role of hBoV is not clear because other viruses have been frequently detected in hBoV-positive samples from children with acute respiratory tract infection (ARTI) (Longtin et al. 2008). Similarly, hMPV is a new member of the Paramyxoviridae family, Pneumovirus subfamily (Chan et al. 2007), showing a incidence rate between 3.9-9% in young children with ARTI (Manoha et al. 2007). Molecular techniques are powerful tools to search for respiratory pathogens that may be present in clinical samples, especially from patients suffering from respiratory infections (Manning et al. 2006). We have studied the occurrence of hBoV and hMPV in the Porto Alegre (PA) metropolitan area, one of the southernmost cities of Brazil, evaluating children suspected to have a lower respiratory tract infection during a 13-month period.

PATIENTS, MATERIALS AND METHODS

Studied population - This study included infants (< 24 months) admitted to the paediatric emergency room of Santo Antônio Children's Hospital, PA, who presented clinical signs of viral respiratory tract infection with lower airway obstruction, together with widespread crackles and/or wheezing. Nasopharyngeal aspirates were prospectively collected from May 2007-June 2008. This routine has been applied to all patients when a viral respiratory tract infection was suspected. For all eligible cases, a specific questionnaire was completed. The study was approved by the Ethical Committee of Clinical Hospital of Porto Alegre (06-549).

Nucleic acid extraction and cDNA synthesis - A total of 455 nasopharyngeal aspirates (NPA) were kept frozen at -70ºC and were used for total nucleic acid extraction with the QIAmp UltraSens Virus Kit (Qiagen).

Real-time polymerase chain reaction (PCR) - A two-step reverse-transcriptase PCR method was used for amplification and detection of RNA viruses (RSV, FLU A and B, PIV 1, 2 and 3, RNV and hMPV). The reverse transcription step was performed using random hexamers and SuperScript III First-Strand (Invitrogen), according to the manufacturer's recommended protocol. Multiplex real-time PCR assays were developed in two panels: RSV, FLU A and B (panel 1) and PIV 1, 2 and 3 (panel 2). The other viruses (RNV, ADV, hBoV and hMPV)

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were detected by a singleplex virus-specific PCR. All amplifications and detections were performed using the LightCycler instrument (Roche Diagnostics) and identification of individual viruses was assessed by melting curve analysis. Primers were specifically designed from consensus genome regions obtained from the DNA Data Bank of Japan; they have closely matched annealing temperatures so that virtually identical conditions could be applied for the PCR cycle (Table I).

RESULTS

In total, 455 NPAs collected from 433 patients spanning a one-year period were tested. The average age of the study population was 5.46 (4.90-6.02) months and 57.6% were male. Only hBoV was influenced by age. The average age of hBoV-positive children was 7.5 months and the average age of hBoV-negative children was 5.1 months ($p < 0.05$). The average age of hMPV-positive was 5.9 months, whereas hMPV-negative was 5.4. hMPV and the other viruses included in this study showed no significant difference between positive and negative samples. When we evaluated the influence of gender, no significance differences for any of the viruses were seen, even considering the fact that male patients were more frequently infected with all viruses. Of the 455 samples tested, 409 (89.9%) were positive for at least one of the 10 respiratory viruses tested in this protocol. RSV was detected in 222 samples (49.3%), FLU A in 94 (20.9%), FLU B in 30 (6.7%), PIV 1 in seen (1.6%), PIV 2 in 2 (0.4%), PIV 3 in 26 (5.8%), ADV in nine (2%), RNV in 112 (24.9%), hMPV in 66 (14.5%) and hBoV in 60 (13.2%). A unique causative viral agent was identified in 201 (44.2%) samples. Of these 409 virus-positive samples, 199 were also positive for additional viruses,

TABLE I

Primer sequences designed for real-time polymerase chain reaction and melting temperatures of each virus (*de novo*)

Virus	Sequence (5'→ 3')	Target gene	Melting temperature (ºC)
RSV	RSV-LC-F1: TGGGAGAGGTAGCTCCAGAA RSV-LC-R: CAGATCTRTCCCCTGCTGCT	Nucleo-capsid protein	78.4
FLU A	INFA-LC-F: TCACCATTGCCTTCYCTTCC INFA-LC-R: CTGCTTCTCCAAGCGAATCT	Nuclear export protein	80.8
FLU B	INFB-LC-F: CTGATGTCCATCAAGCTCCA INFB-LC-R: CCTTTGACATCTGCATCACG	Nucleoprotein	84.9
PIV 1	PIV1-LC-F: GGAAAACAAWTAGTTCATATTGGTC PIV1-LC-R: TGCATTGTTGTTGCAATCAGT	Hemagglutinin-neuraminidase	79.9
PIV 2	PIV2-LC-F: TGATGGAATCAATCGCAAAA PIV2-LC-R: GAAAGCAAGTCTCAGTTCAGCTA	Hemagglutinin-neuraminidase	76.9
PIV 3	PIV3-LC-F: AATCGAGAGTRAACCCAGTCATAA PIV3-LC-R1: TGTTATAGTGTGTAATGCAGCTYGT	Hemagglutinin-neuraminidase	82
ADV	ADNU-LC-F: TTCCCCATGGCNCACAAYAC ADNU-LC-R: TGCCKRCTCATRGGCTGRAAGTT	Hexon protein	84.7
RNV	RNV-A-LC-F1: TAGTAGACCTGGCAGATGAGG RNV-A-LC-R1: ACACGGGGCTCTTCRCAC RNV-A-LC-F2: TACTAAACCTGGCAGATGAGG RNV-A-LC-R2: ACAATAGGCTCTTCACAC	5' UTR	86
hMPV	HMPV-LC-F11: ATGGCAAAGCATTAGGCTCA HMPV-LC-R11: TGTTGGATGACCTGGCAAT HMPV-LC-R12: TGTTAGATGACCTGGCAAT HMPV-LC-R13: TGTTAGATGACCTGGCGAT	Nonfunctional nucleoprotein	81.3
hBoV	HBOV-LC-F1: GTCCAGAAAGAGGGGAGAGG HBOV-LC-R1: GCTGATTGGGTGTTCCTGAT	Nucleoprotein 1	86.1

ADV: human adenovirus; FLU: influenza virus; hBoV: human bocavirus; hMPV: human metapneumovirus; PIV: parainfluenzaviruses; RNV: human rhinoviruses; RSV: respiratory syncytial virus.

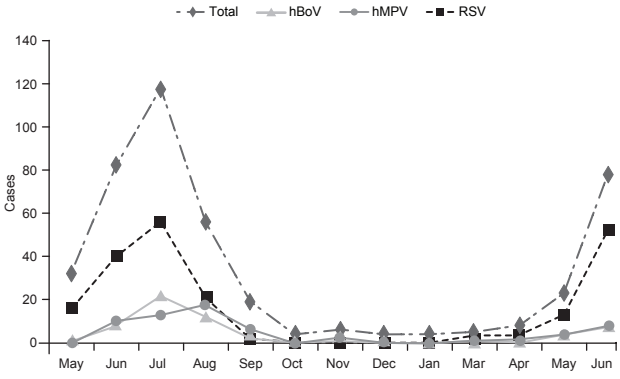
resulting in a coinfection rate of 43.7%. Double infections were detected in 144 (31.6%) samples, triple infections were identified in 47 samples (10.3%) and eight (1.8%) samples showed four different viruses.

Among the 201 samples positive for a single virus, 122 (60.7%) were positive only for RSV, 40 (19.9%) only for FLU A, 18 (8.9%) only for RNV, 10 (4.9%) only for hMPV, four (2%) only for FLU B, four (2%) only for PIV 3, two (1%) only for PIV 1 and one (0.5%) only for hBoV. The other pathogens were not identified alone. Interestingly, both hBoV and hMPV were far more frequently found in mixed viral infections. For hBoV, 98.3% of all positive samples (59 cases) were from patients with mixed infections. Similarly, 84.8% of all hMPV-positive (56 cases) results were also observed in mixed infection. Both hBoV and hMPV normally appeared with RSV.

The temporal distribution for all viruses during the 2007-2008 period showed that the majority of the viral episodes were more frequent in June-July 2007 and June 2008 (61.1% of all cases), which represents the winter months in the Southern Hemisphere. In this period, hMPV showed a peak of infection (n = 18) that overlapped with the RSV-associated peak (n = 56). In the hBoV analyses, we observe that the peak of infection (n = 22) overlapped with the RSV distribution in both years, again correlating with the winter season. The monthly distributions of the viruses detected over a 13-month period in this study are shown in Table II and Figure.

DISCUSSION

In this study, hMPV was found in 14.5% of all children with suspected lower respiratory tract infection. The prevalence found in our study is similar to the 4-18% prevalence reported elsewhere using real-time PCR methods (Boivin et al. 2003, Mullins et al. 2004, Xepapadaki et al. 2004, Kuypers et al. 2005). Do Carmo Debur et al. (2007) studied hospitalized children with ARTI in Curitiba, Brazil, and found a prevalence of 6.4% for hMPV (do Carmo Debur et al. 2007). Da Silva et al. (2008) assessed the presence of hMPV in NPAs from children with ARTI and reported that this virus caused 5.6% of cases in the region of Campinas, Brazil (da Silva et al. 2008). It is interesting to note that Curitiba and Campinas are located north of PA and the lower infection rates found by these authors could be related to other variables, such as population density, socioeconomic factors and climate changes.



Monthly distributions of respiratory syncytial virus (RSV), human bocavirus (hBoV) and human metapneumovirus (hMPV) infection. Total: all samples analyzed in the month. hBoV: hBoV frequency in the month; hMPV: hMPV frequency in the month; RSV: RSV frequency in the month.

TABLE II

Monthly distributions for all viruses over 13-month study period

		Total	hBoV	hMPV	RNV	FLU A	FLU B	RSV	PIV 1	PIV 2	PIV 3	ADV
2007	May	32	1	0	10	3	1	16	1	1	0	0
	Jun	82	8	9	12	21	1	40	2	0	1	1
	Jul	118	22	13	18	32	8	56	2	1	12	1
	Aug	56	12	18	22	13	7	21	1	0	10	2
	Sep	19	2	6	10	5	2	2	0	0	3	1
	Out	4	1	0	1	0	1	0	0	0	0	0
	Nov	6	0	2	3	0	1	0	0	0	0	0
	Dec	4	0	0	1	2	1	0	0	0	0	0
2008	Jan	4	0	0	1	2	1	0	0	0	0	0
	Mar	5	0	1	4	0	0	3	0	0	0	0
	Apr	8	0	2	8	1	0	4	0	0	0	0
	May	23	4	4	9	3	3	13	0	0	0	1
	Jun	78	7	8	10	10	4	53	1	0	0	2

there were no samples collected for February 2008. ADV: human adenovirus; FLU: influenza virus; hBoV: human bocavirus; hMPV: human metapneumovirus; PIV: parainfluenzaviruses; RNV: human rhinoviruses; RSV: respiratory syncytial virus.

Among the 84.8% of hMPV-positive specimens that were found in association with another respiratory pathogen, RSV was the most prevalent virus, which is similar to the findings reported by others (Boivin et al. 2003, Viazov et al. 2003). Greensill et al. (2003) analyzed bronchoalveolar lavage fluids collected from infants with RSV bronchiolitis and found that 70% of them were co-infected with hMPV (Greensill et al. 2003). It has been reported that simultaneous presence of hMPV and RSV may simply reflect a considerable seasonal distribution overlap of the two viruses (Xepapadaki et al. 2004). However, another recently published study seems to indicate that the association between hMPV and RSV may act in concert, potentiating their pathogenic effects and exacerbating the clinical symptoms of the respiratory disease (Bouscambert-Duchamp et al. 2005). Vicente et al. (2003) did not find hMPV coinfections with other respiratory viruses in a paediatric population with ARTI, a finding that is discordant from the majority of recently published studies (Vicente et al. 2003). In the present report, hMPV was detected as a unique respiratory pathogen in only 2.2% of the cases. Surprisingly, the results obtained by Cuevas et al. (2003) in Aracajú (Northwest Brazil) showed that hMPV alone was responsible for 17% of ARTI. Although the detection of viral nucleic acid in respiratory specimens does not prove that hMPV is responsible for the clinical manifestation, the association between a respiratory tract disease and the presence of the virus suggests a causative role.

In this study, hMPV seasonal distribution was characterized by peaks within the winter period, similar to those previously observed elsewhere (Mullins et al. 2004, van den Hoogen et al. 2004, Gray et al. 2006). In another Brazilian study, do Carmo Debur et al. (2007) observed that hMPV circulated during winter and spring (do Carmo Debur et al. 2007). The seasonal occurrence of hMPV infection largely overlaps with that of RSV infections (Chan et al. 2007).

The average age of 5.9 months for hMPV-positive children in our study is different from the majority of reported data, which exhibit an average of 11.5 months (Mullins et al. 2004, Williams et al. 2004, Kuypers et al. 2005). However, it is similar to another study performed in Brazil, which reported an average age of 4.4 months. In both studies, the distribution of hMPV infection was the same for boys and girls (do Carmo Debur et al. 2007). Similar results were observed by Reina et al. (2008) in Spain.

According to the literature, the prevalence of hBoV infection among hospitalized children with respiratory tract infection ranges between 1.5-18.3% (Bastien et al. 2006, Kaplan et al. 2006, Allander et al. 2007). In our study, we found a prevalence of 13.2%, which is one of the highest reported so far. This result may be explained by the pre-selection of children with severe clinical manifestations. Rather than geographic differences in hBoV circulation, this discrepancy may be explained by differences observed in the cohort studied so far. Several reports included exclusively hospitalized children while others enrolled either hospitalized or outpatient children (Pozo et al. 2007).

Results of our study show a coinfection rate of 98.3%, with only one case of a single hBoV infection. In the majority of previous studies, the coinfection rates ranged from 18-72% (Allander et al. 2005, Kaplan et al. 2006). RSV was the most prevalent pathogen associated with hBoV in all studies, including ours. In a recent report, Esposito et al. (2008) suggested that the association of hBoV with another respiratory pathogen would be clinically important and that the presence of this virus alone does not seems to lead to significant symptoms in children attending an emergency room (Esposito et al. 2008). The high coinfection rates may also suggest that hBoV could increase the pathogenicity potential of other viruses. An alternative hypothesis is that hBoV infection results in long-term production of the virus, even long after withdrawal of symptoms, which could facilitate the infection by other pathogens (Naghipour et al. 2007).

hBoV infections have been reported to occur more frequently during the cold seasons: winter and spring months (Völz et al. 2007). Our study suggests that hBoV also has a seasonal distribution similar to hMPV, occurring in winter months. Human parvovirus B19, the only other parvovirus that is pathogenic to humans, is also seasonal, with peak occurrences in spring and summer (Heegaard & Brown 2002). Bastien et al. (2006) observed no seasonal prevalence, which may be reflected by the low prevalence in their study (Bastien et al. 2006). Nonetheless, as highlighted by Schildgen et al. (2008), it is very difficult to draw any definitive conclusion on the seasonal distribution of a new viral agent based only on analyses of a single season because there is much variation of the peak respiratory season from year-to-year (Schildgen et al. 2008).

Most children infected with hBoV are younger than 24 months, but older children may also be affected (Schildgen et al. 2008). Our work indicates that the average age is 7.5 months for hBoV-positive children.

In line with other recent reports, our study also suggests that hMPV and hBoV are almost exclusively found associated with other viral pathogens (Semple et al. 2005, Ong et al. 2007). When a single sample can contain as many as three or more viruses, it becomes difficult to evaluate the individual significance of each virus. One way to solve this problem would be to quantify the viral nucleic acids. Quantitative analysis of NPAs is methodologically difficult because the quality of the specimens obtained varies from person to person depending on sampling technique and condition of patients' nasal mucosa (Christensen et al. 2008). It is important to note that all of our data were a consequence of the analysis of only one epidemic period.

In conclusion, using a real-time PCR-based technique, this study has confirmed that hMPV and hBoV circulate in the PA metropolitan area and has provided evidence of frequent involvement of both viruses in ARTI, although a causal relationship still needs to be demonstrated by including a control group of healthy individuals.

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