JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2006, p. 2739–2742 Vol. 44, No. 8
0095-1137/06/$08.00+0 doi:10.1128/JCM.00164-06

Role of Metapneumovirus in Viral Respiratory Infections in Young Children

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Received 25 January 2006/Returned for modification 12 April 2006/Accepted 8 June 2006

The contribution of human metapneumovirus (hMPV) relative to that of other respiratory viruses as a cause of respiratory infections in children less than 1 year old has been evaluated. From October 2003 to April 2004, nasopharyngeal samples from 211 children less than 1 year old were analyzed to detect respiratory viruses. Respiratory syncytial virus (RSV) was the predominant virus isolated (96 children [45.5%]), followed by influenza A virus, parainfluenza virus, adenovirus, cytomegalovirus, and herpes simplex virus type 1, which were only occasionally detected. From January 2004 to April 2004, a nested retrotranscription-PCR, using in-house primers directed to the matrix protein gene of hMPV, was carried out on samples in which no other viruses were detected. hMPV was detected in 18 (16.2%) children, indicating that this virus was the second-most-frequent cause of viral respiratory infections in children less than 1 year old. The rate of hospitalization for RSV- and hMPV-infected children was higher than 75%. While RSV had a peak from December to February, hMPV was increasingly detected from January to April. The mean age of hMPV-infected children (6.44 ± 3.64 [mean ± standard deviation] months) was significantly higher than that of RSV-infected children (3.99 ± 2.96 [mean ± standard deviation] months). On the other hand, 64.3% of the RSV-infected children and 12.5% of the hMPV-infected children showed high levels of C-reactive protein. Although several authors have reported that clinical symptoms of hMPV-positive patients mirrored those of RSV-positive patients, differences between the two viruses can be found.

Human metapneumovirus (hMPV) has recently been described as a causal agent of acute respiratory disease mainly in children. This virus was first discovered in 2001 in The Netherlands in patients with respiratory infections and classified in the *Pneumovirinae* subfamily of the *Paramyxoviridae* family (24). hMPV has been found in many countries, including Australia (17), Canada (21), Finland (12), the United States (18), France (8), the United Kingdom (22), Spain (10, 27), Ireland (2), and Japan (23).

The clinical manifestations of hMPV-infected children range from mild upper-airway disease to severe pneumonia and include rhinorrhea, nasal congestion, pharyngeal erythema, myalgia, cough, and fever and, in more severe cases, wheeze, dysphonia, stridor, respiratory difficulty, bronchiolitis, pneumonia, and respiratory failure (5, 10, 25, 29). The epidemiology and seasonal distribution of hMPV have been described as similar to those of traditional respiratory viruses. In fact, it has been reported that the epidemiological characteristics and clinical manifestations of hMPV closely resemble those of respiratory syncytial virus (RSV) (1, 24, 25).

Although specific hMPV antigens can be detected from samples or viruses can be isolated from LCC-MK-2 cells, molecular techniques based on genomic amplification are most widely used (2, 8, 10, 12, 17, 18, 21, 22, 23).

The aim of this study was to detect hMPV using an in-house, nested retrotranscription (RT)-PCR with samples obtained from children less than 1 year old and to evaluate the contribution of hMPV relative to that of other respiratory viruses as a cause of respiratory infections as well as to define clinical features and seasonal patterns of hMPV infection.

MATERIALS AND METHODS

Samples. From October 2003 to April 2004, 211 nasopharyngeal samples from 211 children were collected. Among these children, 195 (92.4%) showed symptoms of lower respiratory tract infections, such as bronchitis or pneumonia, which were considered severe. The remaining 16 (7.6%) came to hospital with mild symptoms, such as cough, mucosity, fever, or general discomfort. All the patients were less than 1 year old (mean age, 4.23 ± 3.25 months; range, 0 to 12). The samples were diluted in virus transport medium (ViralPack; Biomedics SL, Madrid, Spain) and sent to the Clinical Virology Laboratory within 24 h after collection. Information about hospitalization for 86 patients was obtained by a review of their medical charts. For 38 of them, biochemical and hematological parameters were analyzed.

Laboratory testing. All the nasopharyngeal samples were decontaminated and stored at 4°C until they were processed for conventional and rapid cultures following standard protocols.

Briefly, samples were inoculated in different cell lines from human fetal lung fibroblasts (MRC-5), monkey kidney (LLC-MK2), and canine kidney (MDCK) with MEM medium supplemented with 50 IU of penicillin, 50 μg of streptomycin, and 0.25 μg/ml of trypsin and incubated at 37°C in a CO_2 incubator and checked periodically for 15 to 20 days. When a cytopathic effect was observed or before the culture was discarded as negative, the cell monolayer was scraped, washed with phosphate-buffered saline, mounted on a slide, fixed with cold acetone, and stained by indirect immunofluorescence assay with a mix of specific monoclonal antibodies against influenza A and B viruses, RSV, adenovirus, and parainfluenza virus types 1, 2, and 3.

Samples were also inoculated in two shell vials of MRC-5 cells and incubated in a CO_2 incubator for 48 h at 37°C. After this time, the cell monolayer of one shell vial was fixed with cold acetone and stained with a mixture of monoclonal antibodies against the above-mentioned respiratory viruses. When the first shell vial was positive, the cell monolayer of the second shell vial was separated using

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TABLE 1. Viruses identified in nasopharyngeal samples from children with respiratory infections

Virus detected	No. of positive children/no. of children tested (%)		
	Total	With respiratory symptoms	
		Severe	Mild
RSV	96/211 (45.5)	90/191 (47.1)	6/20 (30.0)
hMPV	18/111 (15.3)	16/103 (15.5)	2/8 (25.0)
Influenza A virus	3/211 (1.4)	3/191 (1.6)	0/20
Parainfluenza virus	3/211 (1.4)	2/191 (1.0)	1/20 (5.0)
Adenovirus	1/211 (0.5)	1/191 (0.5)	0/20
CMV	1/211 (0.5)	1/191 (0.5)	0/20
HSV-1	1/211 (0.5)	1/191 (0.5)	0/20
Total	123/211 (58.3)	114/191 (59.7)	9/20 (45.0)

glass balls in a shaker and put into six wells of a slide, fixed with cold acetone, and stained with each antibody used in the pool.

The immunostaining of nasopharyngeal cells was also performed. The swab was introduced in phosphate-buffered saline and vortexed. This solution (or 500 μl of sample solution if the swab was not present) was centrifuged at 6,000 rpm to pellet the cells. These were put into six wells of a slide, fixed with formaldehyde for 20 min, and stained by monoclonal antibodies against the above-mentioned respiratory viruses. All monoclonal antibodies were purchased from Dako Ltd. (Ely, United Kingdom).

hMPV detection. Viral RNA was purified from nasopharyngeal samples diluted in virus transport medium by using the guanidine isothiocyanate method. Briefly, 200 μl of guanidine isothiocyanate was added to 50 μl of the sample suspension and the mixture incubated at 60°C for 10 min. Nucleic acids were precipitated by addition of 250 μl of isopropanol and further centrifugation at 14,000 rpm for 15 min. The pellet was washed with 1 ml of 75% ethanol, dried, resuspended in 50 μl of diethyl pyrocarbonate-treated water, and stored at −20°C. The presence of hMPV was tested by nested RT-PCRs. The first round of amplification was carried out using the Titan one-tube RT-PCR system (Roche Corporation, Indianapolis, IN) and primers Met1N (5′-CAAGGTGCA GCAATGTCTGT-3′, positions 2408 to 2427 in the hMPV genome NC 004148) and Met2N (5′-TGCAATTTTGGCCTGTGTTA-3′, positions 2764 to 2745), which were deduced from the matrix protein gene. Five microliters of extracted RNA was added to 20 μl of a RT-PCR mixture according to the manufacturer's instructions. Amplification was performed using a GeneAmp PCR system 9600 thermal cycler (Applied Byosistems, Foster City, CA) using the following conditions: retrotranscription at 48°C for 45 min; denaturation at 94°C for 2 min; 40 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 30 seconds; and 1 final cycle at 68°C for 10 min.

To increase sensibility, a second round of amplification was carried out. Three microliters of the previous amplification reaction mixture was added to 22 μl of a PCR mixture containing 12.5 pmol each of primers Met3N (5′-AACCATAC GGGATGGTATCAA-3′, positions 2541 to 2562) and Met4N (5′-GCTTGGTC TGCTTCACTGCT-3′, positions 2742 to 2723), 50 μM of each deoxynucleoside triphosphate (Gibco BRL, Carlsbad, CA), and 1 U of *Taq* DNA polymerase (Gibco BRL) in PCR buffer (1×) supplied by the manufacturer. The amplification protocol was as follows: denaturation at 95°C for 5 min; 40 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and 1 final cycle at 72°C for 10 min.

PCR products were analyzed using 2% agarose-TBE (Tris-borate-EDTA) gel electrophoresis, stained with ethidium bromide, and examined with UV light. The results were regarded as positive when a band of 200 bp was present.

To avoid cross-contamination in RT-PCR, procedures for extraction of viral RNA were carried out in a room physically separated from that used for performing RT-PCRs. Furthermore, positive and negative controls were included in all PCR assays.

Statistical analysis. Chi-square, Fisher exact, and Student's *t* tests were performed using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA).

RESULTS

Among the 211 nasopharyngeal samples collected, 105 (49.8%) contained one identifiable virus. Among them, RSV was detected in 96 (45.5%) children, followed by influenza A virus in 3 (1.4%), parainfluenza virus in 3 (1.4%), adenovirus in 1 (0.5%), cytomegalovirus (CMV) in 1 (0.5%), and herpes simplex virus type 1 (HSV-1) in 1 (0.5%) (Table 1). To study the role of hMPV as a cause of respiratory infections in our population, nested RT-PCR assays were carried out on the 111 samples received since January. hMPV was found in 18 (16.2%) children, indicating that hMPV was the second-most-frequently detected virus. The main clinical manifestations reported were bronchitis and pneumonia (90.5%). RSV and hMPV caused 47.1% and 15.5% of these severe respiratory infections, respectively (Table 1).

The analysis of the temporal distribution of the respiratory viral infections showed that the episodes were clearly most frequent from December to February (69.7% of all cases) (Table 2). A further analysis of the distribution of the most frequent viruses (RSV and hMPV) showed that while RSV had a peak from December to February, hMPV was increasingly detected from January to April (Fig. 1). It is worth noting that the highest detection rates (>60%) were observed in those months coinciding with the RSV-associated peak and with high detection of hMPV (Table 2).

The ages of the children were also analyzed (Table 3). Thus, while the mean age of hMPV-infected children was 6.44 ± 3.65 (mean ± standard deviation) months, the mean ages of RSV-infected and negative children were significantly lower (3.99 ± 2.96 and 3.94 ± 3.29 [means ± standard deviations], respectively) ($P = 0.015$).

A review of the medical charts for 86 children showed that while 46.5% of the negative patients needed to be hospitalized, 88.5% and 77.8% of RSV- and hMPV-infected children, respectively, were hospitalized ($P = 0.009$ and $P = 0.04$, respectively). No significant difference between RSV- and hMPV-infected children was observed (Table 3).

TABLE 2. Monthly distribution of respiratory infection cases

Virus	No. of cases during[a]:						
	October (12)	November (18)	December (70)	January (56)	February (21)	March (19)	April (15)
RSV	0	5	44	32	13	1	1
hMPV	ND	ND	ND	1	2	7	8
Other	1 (P)	3 (2 IA, 1 P)	2 (1 IA,1 P)	2 (1 C, 1 H)	0	0	1 (A)
Total	1	8	46	35	15	8	10

[a] The value in parentheses following each month is the number of children tested during that month. ND, not done; A, adenovirus; C, CMV; IA, influenza A virus; H, HSV-1; P, parainfluenza virus.

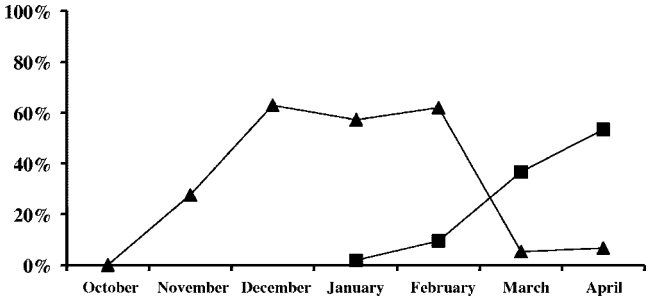

FIG. 1. Monthly distribution of RSV (triangles) and hMPV (squares) given as percentages of RSV- and hMPV-positive samples.

Hematological and biochemistry studies were carried out with 38 children (Table 3). Thus, 5 of 8 (62.5%) hMPV-positive children showed leukocytosis, compared with 10 of 14 (71.4%) RSV-positive patients and 8 of 16 (50%) negative patients ($P = 0.48$). On the other hand, 9 of 14 (64.3%) RSV-positive children and only 1 of 8 (12.5%) hMPV-infected children showed high levels of C-reactive protein (CRP) ($P = 0.019$).

DISCUSSION

Viral infections, which represent more than 90% of acute diseases in children, are considered the most important cause of lower respiratory tract illness commonly observed during wintertime (26).

Identification rates for virus in these infectious processes range from 35 to 87% (11, 13), despite significant advances made in diagnostic technology, supporting the existence of undiagnosed pathogens causing respiratory infections. A first finding of the present report is that only 58.3% of the respiratory infections could be related to the presence of known viruses.

RSV was the most frequently identified virus (45.5%), supporting its role as the main virus associated with respiratory infections in young children, causing 45 to 90% of these infections (4). Other viruses usually involved in respiratory infections are adenovirus, coronavirus, parainfluenza virus, and influenza virus, with rates between 2 and 15% (9, 11). In our study, only three cases of influenza A virus (1.4%), three of parainfluenza virus (1.4%), and one of adenovirus (0.5%) infection were detected.

As described above, molecular techniques based on PCR technology can be developed to detect new viruses that could be the causes of some respiratory infections without identified etiology agents. In this field, hMPV was recently described as a cause of respiratory infections, with prevalences between 2 and 25% (2, 14, 27, 28, 29). The importance of this virus is supported by seroprevalence studies showing that all children tested had been exposed to hMPV by the age of 10 years (5, 6). The study of the role of hMPV in our population found a prevalence of 15.3%, supporting the assumption that hMPV is the second-most-frequent cause of viral respiratory infections in children less than 1 year old.

Several respiratory viruses, such as influenza virus, parainfluenza virus, and RSV, are clearly more frequently detected in wintertime. Some studies have reported that hMPV is also a seasonal virus. Our study has shown that this virus is more frequently detected in March and April, supporting data found by other authors (3, 10, 23). This seasonality could explain the low prevalence rates reported by other authors, who analyzed samples collected in winter months and probably during the RSV-associated peak (9, 27).

The majority of the hMPV infections was reported in children younger than 5 years old, of whom the most susceptible were less than 2 years old (5, 7, 15). In the present study, the relatively high median age of hMPV-infected children was similar to that reported by other authors (10). Nevertheless, the median age for RSV-infected children, which was consistent with previous studies (19), was significantly lower. The existence of a longer-lasting maternal immunity to hMPV than to RSV and the finding that the pathogenesis of hMPV disease favors older children have been proposed as potential explanations (16, 20). Further research is needed to answer these questions.

It has been reported that clinical features associated with hMPV appear to be similar to those observed with RSV (20, 24). The severities of the infections caused by hMPV were determined by the hospitalization rates for infected children. The hospitalization rates for hMPV- and RSV-infected patients in our study were similar and higher than 75%, similar to rates reported in other studies (30), supporting the fact that both viruses cause severe respiratory infections in children less than 1 year old. Nevertheless, other reports have found that the percentage of hospitalizations caused by hMPV was much smaller than that attributable to RSV (1). A possible explanation for this low rate is that these studies were carried out in winter months and stopped before the end of hMPV transmission in the community. The seasonality of hMPV, which seems to be different from that of other respiratory viruses, could be the cause of an underestimation of the real impact of this virus in several studies.

Leukocytosis and elevation of CRP levels are parameters widely used to identify acute infection. An analysis of these parameters showed that more than 50% of the children infected by both RSV and hMPV presented leukocytosis. On the other hand, while more than 60% of the RSV-infected children showed high levels of CRP, the number of hMPV-infected children with increases in CRP was clearly low (only one case). These findings might be notable for those involved in establishing diagnostic algorithms for the investigation of viral respiratory infections.

In summary, although clinical symptoms in hMPV-positive patients mirrored those in RSV-positive patients, similar to results reported in other hMPV studies, differences in the ages of the infected patients, in the seasonalities of both viruses, or

TABLE 3. Ages, hospitalization rates, and laboratory parameters for RSV- and hMPV-infected children

Group	Avg age (mo) ± SD	No. of children in indicated group/no. of children tested (%)		
		Hospitalized	Leukocytosis	High levels of CRP
RSV+	3.99 ± 2.96	23/26 (88.5)	10/14 (71.4)	9/14 (64.3)
hMPV+	6.44 ± 3.64	14/17 (77.8)	5/8 (62.5)	1/8 (12.5)
Negative	3.94 ± 3.29	21/43 (46.5)	8/16 (50)	8/16 (50)

in viral infection-associated biochemical parameters, such as level of CRP, can be found. Further studies are needed to evaluate these parameters in relation to hMPV infection. In any case, the importance of hMPV as a cause of severe respiratory infections supports their inclusion in routine laboratory tests to diagnose these infections.

ACKNOWLEDGMENTS

J.A.B. is a recipient of a fellowship (FIS 00/3160) from the Fondo de Investigaciones Sanitarias, Madrid, Spain. This work was supported by project grant MCT-03-BIO06008-C0301 from the Ministerio de Ciencia y Tecnología.

We thank Lucía Montoto-Rodríguez for her help in the preparation of the manuscript.

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