Journal of Medical Virology 80:1452–1460 (2008)

Prevalence of Human Metapneumovirus Among Hospitalized Children Younger than 1 Year in Catalonia, Spain

Marta Camps,[1]* Sílvia Ricart,[2] Veselka Dimova,[1] Núria Rovira,[2] Carmen Muñoz-Almagro,[2] Juan Jose Garcia,[2] Martí Pons-Odena,[2] Mª Angeles Marcos,[1] and Tomàs Pumarola[1]

[1]*Department of Microbiology, Hospital Clínic i Provincial de Barcelona, Barcelona, Spain*
[2]*Department of Microbiology, Pediatrics Infectious Diseases Unit, Hospital Sant Joan de Déu, Barcelona, Spain*

Human metapneumovirus was discovered recently respiratory virus implicated in both upper and lower respiratory tract infection. In children, the clinical symptoms of human metapneumovirus are similar to those produced by respiratory syncytial virus, ranging from mild to severe diseases such as bronchiolitis and pneumonia. The aim of the present study was to describe the prevalence of human metapneumovirus and other common respiratory viruses among admitted to hospital infants. From January 2006 to June 2006, 99 nasopharyngeal aspirates were collected from hospitalized children younger than 12 months in order to study respiratory viruses. Human metapneumovirus detection was performed by cell culture and two RT-PCR targeting on polymerase and fusion genes. The latter gene was used for phylogenetic analysis. In 67/99 children (67%) at least one viral pathogen was identified, the viruses detected most frequently were respiratory syncytial virus (35%), human metapneumovirus (25%) and rhinovirus (19%). The results obtained in this study, show that: (1) human metapneumovirus is one of the most important viruses among children less than 12 months; (2) children infected with human metapneumovirus were significantly older than those infected by respiratory syncytial virus; (3) human metapneumovirus was associated more frequently with pneumonia whereas respiratory syncytial virus was only detected in patients with bronchiolitis; (4) there was a clear epidemiological succession pattern with only a small overlap among the viruses detected most frequently; (5) all human metapneumovirus samples were clustered within sublineage A2. ***J. Med. Virol. 80:1452–1460, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: human metapneumovirus; hospitalized infants; lower respiratory infection

INTRODUCTION

Human metapneumovirus was discovered recently as a respiratory virus implicated in both upper and lower respiratory tract infection ranging from mild to severe disease in all age groups [van den Hoogen et al., 2001]. Since then, it has been reported in Europe, Asia, Australia, South Africa and America [Peret et al., 2002; Bastien et al., 2003; Jpma et al., 2004; Rao et al., 2004; Sloots et al., 2006; Brooks et al., 2007], suggesting worldwide distribution. Seroprevalence studies have shown that this virus has been present among humans for over five decades [van den Hoogen et al., 2001; Hamelin et al., 2004]. Human metapneumovirus was not discovered previously due to its difficulty to grow in traditional cell cultures, although it has slow growth in the *Rhesus monkey kidney* (LLC-MK2) cell line [Deffrasnes et al., 2005]. Although monoclonal antibodies (MAbs) are being developed [Landry et al., 2005; Percivalle et al., 2005], these are not available commercially for direct antigen detection in nasopharyngeal aspirate (NPA), and molecular assays remain the main approach available currently for human metapneumovirus identification. Human metapneumovirus has a negative-strand RNA genome of approximately 13 kb encapsidated by a helicoidal nucleocapsid and covered with a lipid bilayer [van den Hoogen et al., 2002; Biacchesi et al., 2003]. It has been classified in the *Pneumovirus* subfamily of the *Paramyxovirus* family, which also contains the respiratory syncytial virus and *Metapneumovirus* genus. Currently, two main genotypes A and B with the subtypes A1, A2, B1, and B2

Grant sponsor: Spanish Ministry of Health; Grant number: FIS PI050063.

*Correspondence to: Marta Camps, Laboratory of Microbiology, Hospital Clínic i Provincial de Barcelona, Villarroel 170, 08036 Barcelona, Spain. E-mail: martacs@hotmail.com

Accepted 18 March 2008

DOI 10.1002/jmv.21209

Published online in Wiley InterScience (www.interscience.wiley.com)

are identified by molecular methods [Biacchesi et al., 2003].

The clinical symptoms of human metapneumovirus are similar to those caused by respiratory syncytial virus, ranging from wheeze, cough, fever, bronchiolitis, and even pneumonia [Wolf et al., 2006]. Although some studies have detected human metapneumovirus in all age groups, human metapneumovirus infections could be more severe in children, elderly, and immunocompromised patients [Pelletier et al., 2002; Bastien et al., 2003; Cane et al., 2003; Falsey et al., 2003]. However, several epidemiological and clinical features have yet to be established firmly.

The aim of the present study was to describe the role of human metapneumovirus and other common respiratory viruses including: influenza virus A, B, and C, parainfluenza 1–4 viruses, adenoviruses, respiratory syncytial virus A and B, rhinovirus, coronavirus 229E and OC43 and enterovirus as bronchiolitis, and bronchopneumonia pathogens among hospitalized children younger than 1 year, taking into account that in this age group respiratory viruses are the main etiological agents of lower respiratory tract infections [Shay et al., 1999; Smyth and Openshaw, 2006; Bush and Thomson, 2007]. Additionally, in human metapneumovirus positive samples, a comparative phylogenetic analysis of fusion (F) gene sequences was carried out to study the potential circulation of distinct human metapneumovirus genotypes among patients included.

PATIENTS AND METHODS

Study Population

The present study falls within the framework of the "Study of prevalence, clinical and epidemiological features associated to human metapneumovirus infection among infants and adult population" awarded by the Fondo de Investigaciones Sanitarias (FIS), Spain.

From January 2006 to June 2006, children younger than 12 months hospitalized in a 345-bed children's hospital (Hospital Sant Joan de Déu, Barcelona, Spain) with bronchiolitis or bronchopneumonia, were studied prospectively. Within the first 24 hr of admission, a nasopharyngeal aspirate was collected and delivered to the Laboratory of Microbiology at the Hospital Clínic of Barcelona, where it was processed to study respiratory viruses. A questionnaire about clinical and epidemiological features was also completed during the initial consultation. Routine bacteriological testing (of blood and urine) was only performed in all febrile infants who have *toxic* manifestations, defined as a clinical appearance consistent with the sepsis syndrome [Baraff et al., 1993]. Other respiratory samples were obtained according to clinical indication or the decision of the attending physician.

Bronchiolitis was defined as an acute infection of the lower airway, characterized by increased respiratory effort (tachypnea) and expiratory wheezing and/or crackles associated with common viral infection symptoms (rhinorrhea, cough) in infants younger than 1 year. The presence of lower respiratory infection (wheezing and/or crackles) with a focal infiltrate in the chest X-ray was considered as a bronchopneumonia. Exclusion criteria were underlying medical conditions (cystic fibrosis, metabolic diseases, neurological diseases) and patients with two or more previous episodes of wheeze without the presence of radiological infiltrates.

Criteria for Pediatric Intensive Care Unit (PICU) admission were: acute respiratory failure with hemoglobin saturation $<90\%$ and $>40\%$ fraction of inspired oxygen (FiO_2) supplementation, common episodes of apnea and sepsis according to the International Consensus Conference on Pediatric Sepsis [Goldstein et al., 2005].

Informed consent was obtained from the patients' parents. The Ethical Committees of the Hospital Clínic and Hospital Sant Joan de Déu approved the study protocol.

Detection of Common Respiratory Viruses by Conventional Methods

Specimens for immunofluorescence assay (IFA) were suspended in PBS (phosphate buffered saline), when necessary, and spotted onto a slide that was air-dried and fixed with cold acetone and then stained with a pool of fluorescein-conjugated antibody to influenza virus A, influenza virus B, human parainfluenza virus 1–3, adenovirus, and respiratory syncytial virus (Respiratory Panel 1, Viral Screening and Identification Kit; Light Diagnostics, CHEMICON International Temecula, USA).

Simultaneously, specimens were inoculated into MDCK (Madin Darby Canine Kidney), Hep-2 (human caucasian larynx carcinoma squamous cell) and LLC-MK2 (Rhesus monkey kidney epithelial cell) cell lines (Vircell, Granada, Spain) for isolation of the viruses mentioned above and human metapneumovirus. MDCK and LLC-MK2 tube cultures were incubated at 33 and 37°C, respectively, and maintained with growth essential medium [EMEM (EBSS) + 2 mM glutamine + 1% non-essential amino acids (NEAA)] adding 12 μl (500 μg/ml) of Trypsin/EDTA for optimal growth of influenza viruses and human metapneumovirus. Hep-2 cell line was maintained with the same medium plus 2% fetal bovine serum and incubated at 37°C. Cell cultures were examined twice weekly for the development of a cytopathic effect; positive cultures were harvested and stained for conventional virus identification (IFA). In IFA negative cases, a RT-PCR (reverse transcription-polymerase chain reaction) for common respiratory viruses and human metapneumovirus was undertaken.

Extraction and Amplification of Viral Nucleic Acids

Upon sample collection, an aliquot of each fresh specimen was collected to be used for RT-PCR analysis.

Nucleic acids from either DNA/RNA viruses present in the nasopharyngeal secretion or in infected cell cultures were extracted from 200 μl of specimen using *NucliSense easyMAG* (BioMérieux, NL-5281 RM Boxtel, The Netherlands) according to the manufacturer's instructions. The lysis buffer included 500 molecules of the cloned-amplified product used as an internal control in each reaction tube in order to exclude false negative results due to non-specific inhibitors or extraction failure. Two independent multiplex nested RT-PCR assays able to detect from 1 to 10 copies of viral genomes were carried out using techniques described previously [Coiras et al., 2003; Coiras et al., 2004]. One RT-PCR assay detected influenza virus A, influenza virus B, influenza virus C, respiratory syncytial virus A, respiratory syncytial virus B, and adenovirus. Another RT-PCR assay examined human parainfluenza virus 1–4A and 4B, human coronavirus 229E, human coronavirus OC43, and the generic detection of enterovirus and rhinovirus.

In order to detect human metapneumovirus, extracted RNA was used as a template for cDNA synthesis by random primers according to the manufacturer's instructions (First Strand cDNA Synthesis Kit, Roche Diagnostics, Mannheim, Germany). Amplification reaction was carried out using specific primers amplifying a conserved fragment of 170 bp in the polymerase gene (L): Lf, 5′CAT GCC CAC TAT AAA AGG TCA G 3′; Lr, 5′CAC CCC AGT CTT TCT TGA AA 3′, as described elsewhere [van den Hoogen et al., 2003]. The PCR conditions comprised 45 cycles at 94°C for 1 min (denaturalizaton), 50°C for 50 sec (annealing), 72°C for 1 min (extension) and a final extension at 72°C for 10 min. To detect human metapneumovirus types A and B, in addition to the widely used primers of L gene that do not hybridize to type B [Sarasini et al., 2006], a second nested PCR test using fusion gene (F) primers was performed. First round PCR used the following primers: Ff1, 5′TTC GTT CTA GGA GCA A 3′ and Fr, 5′ GTC TTC CTG TCG TAA CTT TG 3′. The second round used an inner forward primer Ff 5′ ATG CCA ACA TCT GCA GGA C 3′ and the same reverse primer as in the first step, obtaining a final product of 450 pb. Amplified products were analyzed by electrophoresis on 2% agarose gel, stained with ethidium bromide. Fusion gene primers were either selected from published protocols [van den Hoogen et al., 2004] or designed originally based on the sequence data of a Canadian strain available from GenBank, National Center for Biotechnology Information (GenBank) (Accession No. AY297749) [Biacchesi et al., 2003]. As an human metapneumovirus positive control, RNA of human metapneumovirus (subtype A2, according to strain Canada 97/83) was used in the PCR test. Each set of RT-PCR reaction included a positive control for extraction and amplification handling obtained from our viral lysates, when available, and a negative control (viral transport medium containing no nucleic acid). All positive results were confirmed by two sequential assays.

Sequence of Human Metapneumovirus F Gene

In addition, a second nested PCR targeting a fragment of the F gene was also used for further phylogenetic analysis and to confirm the previous L gene positive results and identify probable human metapneumovirus type B [Bouscambert-Duchamp et al., 2005; Banerjee et al., 2007].

cDNA synthesized previously was used as a template for the first F gene PCR amplification. The reaction mix contained Ff1/Fr primers and termocycler conditions were 35 cycles at 94°C for 1 min (denaturation), 45°C for 2 min (annealing), 72°C for 2 min (extension) and a final extension step of 72°C for 10 min. The second round PCR used an inner primer, Ff, and the same reverse primer used before. In this PCR, annealing reaction was performed at 50°C instead of 48°C, and other conditions and reaction volumes were identical to those applied in the first round. PCR reactions that produced proper bands were purified on QIAquick Gel Extraction Kit (Qiagen, IZASA, Spain) and sequenced with BigDye Terminator v3.1 (Applied Biosystems, Foster City, USA) in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, USA).

Phylogenetic Analysis of Human Metapneumovirus F Gene

Sequences were aligned with different human metapneumovirus subtype strains from Canada, Netherlands, and Japan (Accession Nos. AY145295, AY145298, AY145289, AY145292, AY145287, AY145294, AY145299, AY145297, AY145301, AY145296, DQ362940.1, and AY312232) [Bastien et al., 2003; Galiano et al., 2006; Huck et al., 2006] and avian metapneumovirus C sequence (Accession No. AY590688) was used as an outgroup [Govindarajan et al., 2004]. Nucleotide sequence alignments were generated using the ClustalW algorithm of the MEGA software [Kumar et al., 2004] and the total length of each sequence was 302 nucleotides. Phylogenetic and molecular analyses were conducted using MEGA version 3.0. Phylogenetic trees were constructed by the Phylip program package, version 3.66 (Felsenstein, Department of Genetics, University of Washington, Seattle, WA), using the Neighbor-Joining method supplied by the TreeView 32 program.

Statistical Methods

Prevalence of human metapneumovirus was calculated as the number of children positive by PCR for human metapneumovirus divided by the number of children in the same age group admitted during the same period and hospitalized with lower respiratory tract infection. Quantitative variables were described with means ± standard deviation and significant differences between respiratory syncytial virus and human metapneumovirus groups were analyzed by using Mann–Whitney *U*-test. Qualitative variables were reported as frequencies and percentages. Comparison of proportions was determined by χ^2 or Fisher's exact

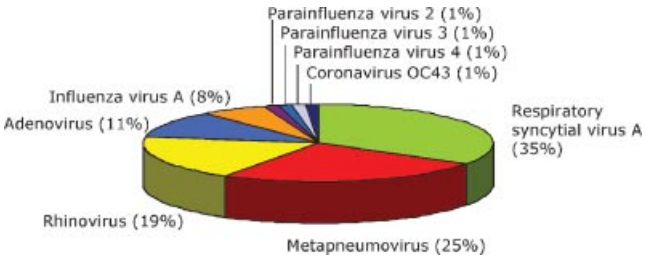

Fig. 1. Distribution of respiratory viruses detected in 99 patients.

test. Probability values of $P < 0.05$ were considered significant. Analysis was performed using the GraphPad Prism3 statistical program (GraphPad Software, Inc., San Diego, CA).

RESULTS

From January to June 2006, of the 120 children younger than 12 months who were hospitalized for lower respiratory tract infection, 99 with nasopharyngeal aspirate for respiratory viruses detection were included prospectively. The median age of the patients was 3.9 ± 4.1 months (range: 14 days to 12 months) and included 59 males (59%) and 40 females (41%).

Clinical diagnoses at the time of admission were bronchiolitis (74%) and (26%) bronchopneumonia, of which 67% and 72%, respectively, had an etiological diagnosis.

In 67/99 children (67%), at least one viral pathogen was identified; 55/99 (55%) had an infection with one of the viruses investigated, whereas 12/99 (12%) had a co-infection of at least two viruses. A total of 80 viruses were identified, while 32/99 (33%) patients remained without an etiological diagnosis. The most frequent virus detected was respiratory syncytial virus (35%) followed by human metapneumovirus (25%) and rhinovirus (19%) (Fig. 1). Viral co-infections were observed in 12 (12%) cases, with adenovirus and rhinovirus the most common viruses identified. In one patient with bronchopneumonia, a triple infection was detected (respiratory syncytial virus + adenovirus + rhinovirus).

Blood and urine cultures were obtained from 32 patients with criteria of *toxic* manifestations. None of the specimens of blood or urine grew a pathogen. Three bronchial aspirates and one bronchoalveolar lavage were collected. An *Haemophilus influenzae* and a *Streptococcus pneumoniae* were recovered from bronchial aspirate and bronchoalveolar lavage, respectively.

Diagnosis of bronchiolitis was significantly more frequent in respiratory syncytial virus single-infected children ($P < 0.001$), whereas human metapneumovirus infection was significantly more common among infants with pneumonia ($P < 0.001$) (Table I). Human metapneumovirus infected children were significantly older (mean age: 6.2 ± 5.14 months) than those infected by respiratory syncytial virus (mean age: 2.5 ± 3.37 months) ($P = 0.009$) (Tables II, III).

Thirteen patients required hospitalization to the Pediatric Intensive Care Unit (PICU); 11 were first diagnosed as bronchiolitis and two as bronchopneumonia. Eleven out of 13 patients admitted to the PICU had an etiological diagnosis: 2 respiratory syncytial virus, 3 rhinovirus, 1 human metapneumovirus, 1 human metapneumovirus + rhinovirus, 2 respiratory syncytial virus + adenovirus, 1 *H. influenzae* and 1 *S. pneumoniae* + respiratory syncytial virus. The duration of hospitalization was 2–14 days (mean 6.2 ± 3.8). Although the differences were not statistically significant, patients with a dual viral infection required more days to recover (8.3 ± 4.9 days) than those with a single infection (5.28 ± 3.25 days).

Overall, 20 human metapneumovirus cases were detected by RT-PCR, of which 5 could also be recovered from the LLC-MK2 cell line. All samples that were

TABLE I. Etiological Pathogens by Clinical Diagnosis

	Bronchiolitis (n = 74)	Pneumonia (n = 25)
Respiratory syncytial virus A	21 (45%)	0
Human metapneumovirus	7	8 (44%)
Rhinovirus	6	3
Influenza virus A	2	1
Adenovirus	2	1
Coronavirus OC43	1	0
Parainfluenza virus 3	0	1
Parainfluenza virus 4	0	1
Human metapneumovirus + rhinovirus	2	0
Human metapneumovirus + influenza virus A	2	0
Human metapneumovirus + adenovirus	1	0
Rhinovirus + adenovirus	0	2
Respiratory syncytial virus A + adenovirus	2	0
Respiratory syncytial virus A + rhinovirus	1	0
Respiratory syncytial virus A + Parainfluenza virus 2	1	0
Respiratory syncytial virus a + adenovirus + rhinovirus	0	1
Respiratory syncytial virus A + *Streptococcus pneumoniae*	1	0
Haemophilus influenzae	1	0
Total	50/74 (67%)	18/25 (72%)

TABLE II. Baseline and Clinical Characteristics of Respiratory Syncytial Virus and Human Metapneumovirus Infected Patients

	RSV (n = 27)	hMPV (n = 20)
Median age (months)	2.5 ± 3.37	6.2 ± 5.14
Sex		
Male	13	12
Female	14	8
Premature infants (<36 weeks of gestational age)	7	8
Clinical diagnosis		
Bronchiolitis	26	12
Bronchopneumonia	1	8
Median length of stay (days)	5.85 ± 4.09	5.6 ± 5.98
PICU admission	5	2

Data are numbers unless otherwise specified.

human metapneumovirus positive by RT-PCR targeting L gene were also confirmed by RT-PCR based on the F gene. Sequencing the F amplicons of human metapneumovirus strains identified from NPA and their subsequent phylogenetic analysis showed that only type A2 of human metapneumovirus circulated among patients included during the study period (Fig. 2). All human metapneumovirus samples tested were found in the same cluster sharing a nucleotide identity of 88.4% with a slightly higher amino acid similarity of 89%. Notably, when the same sequences were examined leaving out P15 and P19 patients, the similarity increased to 91%. In the phylogenetic tree, both sequences seemed to form a different cluster within the subtype A2 (Fig. 2).

The present study is based on the first half of the year, thus it is difficult to establish a distribution of viruses detected during the follow-up. Nonetheless, during these 6 months, there were different distribution patterns among the viruses detected most frequently (Fig. 3).

DISCUSSION

Acute respiratory tract infections are an important cause of morbidity and mortality in children, and it is well known that a high percentage of these infections are caused by respiratory viruses [Williams et al., 2002; Jennings et al., 2004; Meissner, 2005; Pierangeli et al., 2007]. To date, respiratory syncytial virus has been an important trigger of acute respiratory tract infection, although the role of other respiratory viruses such as rhinovirus or human metapneumovirus [van den Hoogen et al., 2001], human coronavirus-NL63 [van der Hoek et al., 2005], human coronavirus-HKU1 [Woo et al., 2005] and human bocavirus [Allander et al., 2007] are gaining attention increasingly.

In the present report, respiratory viruses were involved in 67% of either bronchiolitis or bronchopneumonia patients admitted to hospital. Despite the fact that both human metapneumovirus and respiratory syncytial virus are common pathogens in children, patients infected with human metapneumovirus were significantly older than respiratory syncytial virus-infected patients, a finding that has been reported elsewhere [Williams et al., 2006; Wolf et al., 2006; Chung et al., 2007].

Despite the fact that a limited range of age population has been selected, similar results to previous studies were found [Wright et al., 1989; Chung et al., 2007; Weigl et al., 2007], suggesting that during the first years of life respiratory viruses are the most important pathogens causing severe respiratory tract infections resulting in hospital admission. Several studies have demonstrated that human metapneumovirus is more likely to be associated with bronchiolitis in early childhood [Xepapadaki et al., 2004; Garcia-Garcia et al., 2007; do Carmo Debur et al., 2007]. However, other investigations including the present study found that human metapneumovirus was associated more frequently with bronchopneumonia compared to human metapneumovirus–bronchiolitis cases [Choi et al., 2006; Brooks et al., 2007]. These variable results may reflect that human metapneumovirus is not exclusively a bronchiolitis pathogen and can, also cause bronchopneumonia among infants. It probably indicates that human metapneumovirus causes a spectrum of lower respiratory tract illness, with a tendency toward the more severe end, namely pneumonia. In the study sample, human metapneumovirus was present in 25% of patients causing either bronchiolitis or broncopneumonia, a percentage second only to respiratory syncytial virus (35%) and surpassing rhinovirus (19%). Compared to other studies, a higher rate of human metapneumovirus infection was obtained, considering that it is

TABLE III. Baseline and Clinical Characteristics of Patients Included in the Study

	Total (n = 99)
Median age (months)	3.9 ± 4.146
Sex	
Male	59
Female	40
Premature infants (<36 weeks of gestational age)	31
Clinical diagnosis	
Bronchiolitis	74
Bronchopneumonia	25
Median length of stay (days)	6.182 ± 5.36
PICU admission	13

Data are numbers unless otherwise specified.

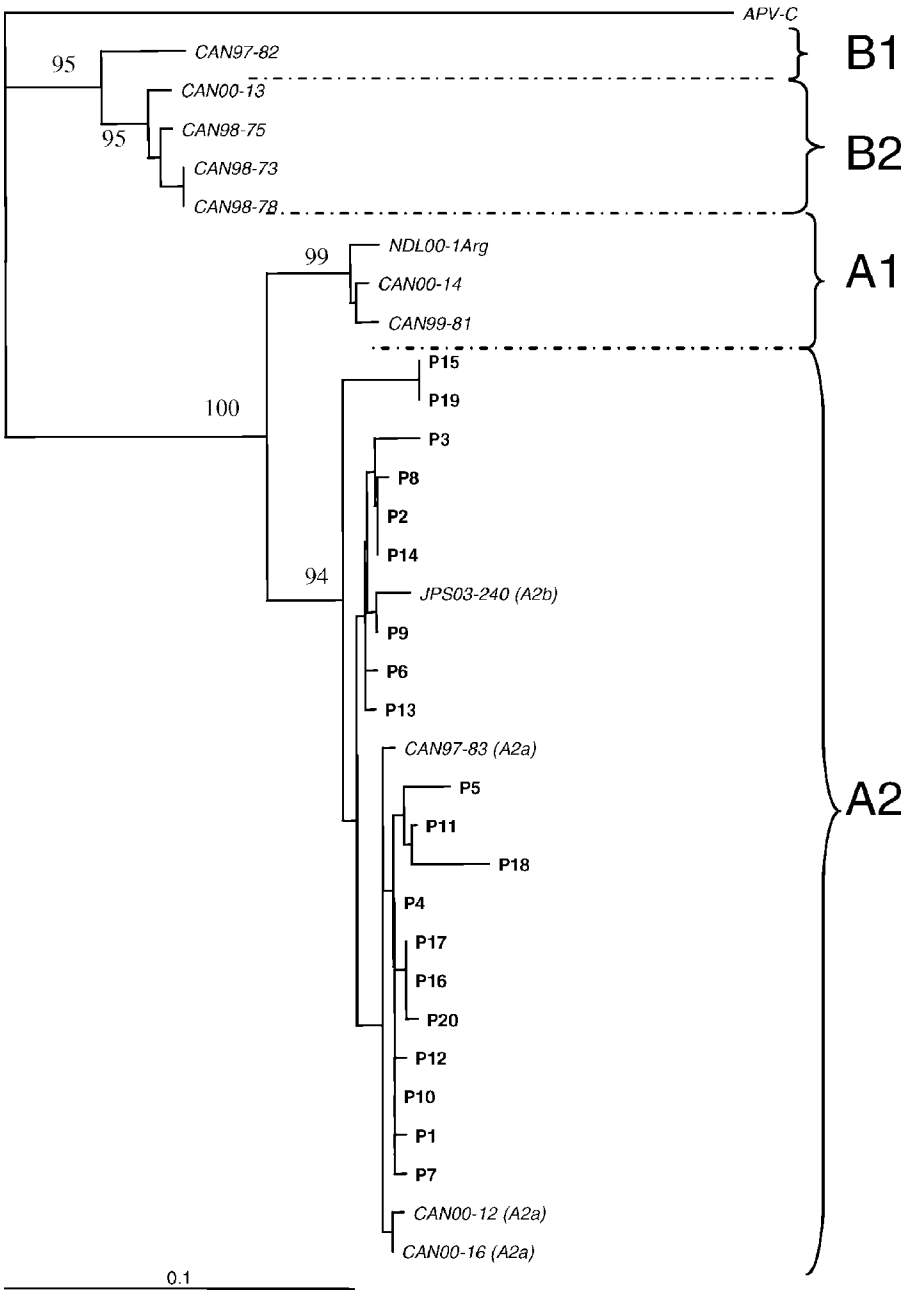

Fig. 2. Neighbor-Joining phylogenetic tree of partial F gene (302 bp) of human metapneumovirus. *Note*: Reference strains are in italics and patient samples in bold. Bootstrap values based on the consensus tree are plotted at the main internal branches to show support values.

responsible normally for 5–10% of hospitalizations of children suffering from acute respiratory tract infections [Jpma et al., 2004; Deffrasnes et al., 2007]. However, these rates reflect data from only the first half of the year, corresponding to the peak infection period and thus, increasing the proportion of human metapneumovirus cases. Probably, it has led to an overestimate of the real prevalence.

Dual viral infections are frequent in childhood, mainly in infants, but to date they were not associated with an increased severity of illness [Williams et al., 2004; Wolf et al., 2006; van Woensel et al., 2006]. In contrast, other studies have found an increase of 5- to 10-fold in the severity of disease in human metapneumovirus and respiratory syncytial virus dual infections [Greensill et al., 2003; Semple et al., 2005]. The present study showed 12% of viral coinfections, and although no human metapneumovirus plus respiratory syncytial virus cases were detected, coinfected patients required longer recovery.

Thirteen patients required hospitalization to the Pediatric Intensive Care Unit, and respiratory syncytial virus and rhinovirus were the main respiratory viruses involved. Either as a single pathogen or in co-infection,

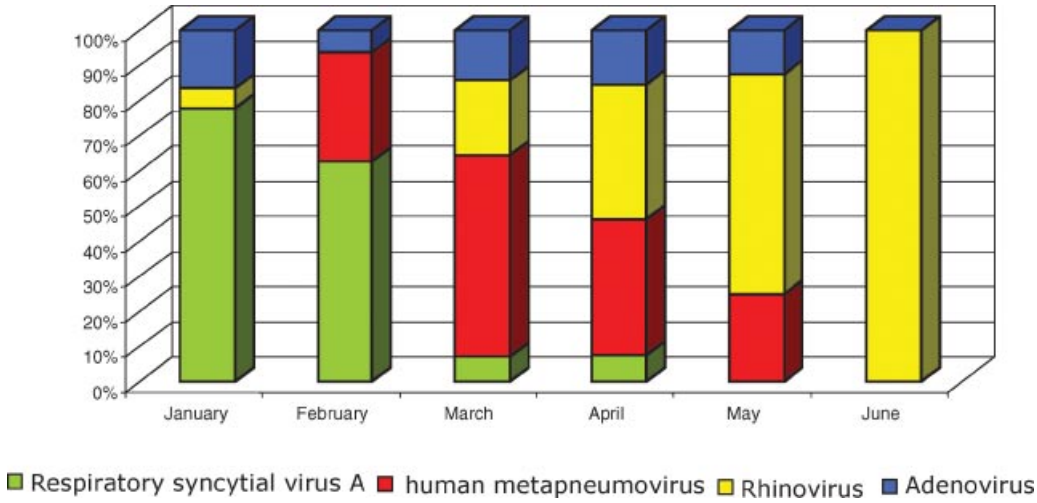

Fig. 3. Seasonal distribution of the most frequently detected viruses: respiratory syncytial virus, human metapneumovirus, rhinovirus and adenovirus during the study period.

they required more days in the Pediatric Intensive Care Unit. Interestingly, one rhinovirus-infected patient spent 11 days at the Pediatric Intensive Care Unit, although further studies are needed to evaluate its role in the severity of illness. Respiratory syncytial virus has been studied widely and in most cases involved in severe acute respiratory infections, however, it has been shown that rhinovirus can also be a life-threatening virus among children with lower respiratory infection [Guittet et al., 2003; Calvo et al., 2007].

Currently, four distinct major human metapneumovirus phylogenetic lineages, A1, A2, B1, and B2 have been described [van den Hoogen et al., 2004]. During the study period, 20 human metapneumovirus cases were detected, all belonging to type A2. Maertzdorf et al. [2004] highlighted that some strains, particularly B1 and B2 sublineages, could not be detected by the widely used L6-L7 pair primers published by van den Hoogen et al. [2001]. Nonetheless, all samples were tested by either L and F genes RT-PCR, so it was considered that during the follow-up only human metapneumovirus subtype A2 circulated among patients included in this study. Interestingly, in a study undertaken during the same year based on 171 children with upper respiratory infection attended in different primary care centers of Catalonia, it was observed a cocirculation of both lineages among 10 human metapneumovirus identified (unpublished data). Recently, Huck et al. [2006] described a novel human metapneumovirus sublineage within A2 group, which they divided into A2a and A2b. In the present report, the sequences within the group A2 shared a nucleotide identity of 88.4%. When patients P15 and P19 were excluded, the identity of sequences reached 91%, suggesting that these patients may belong to another cluster.

One limitation of this study is that follow-up was done during a short period of time, even though respiratory syncytial virus, human metapneumovirus and rhinovirus cases occurred in a defined succession with only small overlap between the viruses, as has been reported in other studies [Choi et al., 2006; Pierangeli et al., 2007]. Further studies would be valuable to investigate how these seasonal patterns change, taking into account either the entire year and a wider age group, thus establishing a true pattern of respiratory viruses.

Overall, the results obtained in this study, show that: (1) human metapneumovirus is one of the most important viruses among hospitalized children less than 12 months, and therefore an important cause of severe lower respiratory tract infection in this age group; (2) children infected with human metapneumovirus were significantly older (mean 6.2 months) than those infected by respiratory syncytial virus (mean 2.5 months); (3) human metapneumovirus was associated more frequently to pneumonia whereas respiratory syncytial virus was only detected in bronchiolitis cases; (4) human metapneumovirus is associated with a spectrum of lower respiratory tract illness, with a greater predominace, in this population, with pneumonia; (5) although respiratory syncytial virus and human metapneumovirus circulate mainly during the winter season, there was a clear succession with a small overlap between viruses; (6) all human metapneumovirus sequences were clustered within sublineage A2.

ACKNOWLEDGMENTS

This study has been funded by the Fondo de Investigaciones Sanitarias from the Spanish Ministry of Health (FIS PI050063). We would like to thank Montse Bernat and Joan Claria from the sequence service of Hospital Clínic who performed the sequencing of the samples. Human metapneumovirus control (subtype A2, according to strain Canada 97/83) was kindly provided by Dra. Vilma Savy, WHO National Influenza Center, Respiratory Virus Service, Virology Department, National Institute of Infectious Diseases, ANLIS "C.G. Malbran", Buenos Aires, Argentina.

REFERENCES

Allander T, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, Vuorinen T, Waris M, Bjerkner A, Tiveljung-Lindell A, van den Hoogen BG, Hyypia T, Ruuskanen O. 2007. Human bocavirus and acute wheezing in children. Clin Infect Dis 44:904–910.

Banerjee S, Bharaj P, Sullender W, Kabra SK, Broor S. 2007. Human metapneumovirus infections among children with acute respiratory infections seen in a large referral hospital in India. J Clin Virol 38:70–72.

Baraff LJ, Bass JW, Fleisher GR, Klein JO, McCracken GH Jr, Powell KR, Schriger DL. 1993. Practice guideline for the management of infants and children 0 to 36 months of age with fever without source. Agency for Health Care Policy and Research. Ann Emerg Med 22: 1198–1210.

Bastien N, Ward D, Van Caeseele P, Brandt K, Lee SH, McNabb G, Klisko B, Chan E, Li Y. 2003. Human metapneumovirus infection in the Canadian population. J Clin Microbiol 41:4642–4646.

Biacchesi S, Skiadopoulos MH, Boivin G, Hanson CT, Murphy BR, Collins PL, Buchholz UJ. 2003. Genetic diversity between human metapneumovirus subgroups. Virology 315:1–9.

Bouscambert-Duchamp M, Lina B, Trompette A, Moret H, Motte J, Andreoletti L. 2005. Detection of human metapneumovirus RNA sequences in nasopharyngeal aspirates of young French children with acute bronchiolitis by real-time reverse transcriptase PCR and phylogenetic analysis. J Clin Microbiol 43:1411–1414.

Brooks WA, Erdman D, Terebuh P, Klimov A, Goswami D, Sharmeen AT, Azim T, Luby S, Bridges C, Breiman R. 2007. Human metapneumovirus infection among children, Bangladesh. Emerg Infect Dis 13:1611–1613.

Bush A, Thomson AH. 2007. Acute bronchiolitis. BMJ 335:1037–1041.

Calvo C, García-García M, Blanco C, Pozo F, Flecha I, Pérez-Breña P. 2007. Role of rhinovirus in hospitalized infants with respiratory tract infections in Spain. Pediatr Infect Dis J 26:904–908.

Cane PA, van den Hoogen BG, Chakrabarti S, Fegan CD, Osterhaus AD. 2003. Human metapneumovirus in a haematopoietic stem cell transplant recipient with fatal lower respiratory tract disease. Bone Marrow Transpl 31:309–310.

Choi EH, Lee HJ, Kim SJ, Eun BW, Kim NH, Lee JA, Lee JH, Song EK, Kim SH, Park JY, Sung JY. 2006. The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000–2005. Clin Infect Dis 43:585–592.

Chung JY, Han TH, Kim SW, Kim CK, Hwang ES. 2007. Detection of viruses identified recently in children with acute wheezing. J Med Virol 79:1238–1243.

Coiras MT, Perez-Brena P, Garcia ML, Casas I. 2003. Simultaneous detection of influenza A, B, and C viruses, respiratory syncytial virus, and adenoviruses in clinical samples by multiplex reverse transcription nested-PCR assay. J Med Virol 69:132–144.

Coiras MT, Aguilar JC, Garcia ML, Casas I, Perez-Brena P. 2004. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. J Med Virol 72:484–495.

Deffrasnes C, Cote S, Boivin G. 2005. Analysis of replication kinetics of the human metapneumovirus in different cell lines by real-time PCR. J Clin Microbiol 43:488–490.

Deffrasnes C, Hamelin ME, Boivin G. 2007. Human metapneumovirus. Semin Respir Crit Care Med 28:213–221.

do Carmo Debur M, Bordignon J, Duarte dos Santos CN, Vidal LR, Nogueira MB, de Almeida SM, Raboni SM. 2007. Acute respiratory infection by human metapneumovirus in children in southern Brazil. J Clin Virol 39:59–62.

Falsey AR, Erdman D, Anderson LJ, Walsh EE. 2003. Human metapneumovirus infections in young and elderly adults. J Infect Dis 187:785–790.

Galiano M, Trento A, Ver L, Carballal G, Videla C. 2006. Genetic heterogeneity of G and F protein genes from Argentinean human metapneumovirus strains. J Med Virol 78:631–637.

Garcia-Garcia ML, Calvo C, Casas I, Bracamonte T, Rellan A, Gozalo F, Tenorio T, Perez-Brena P. 2007. Human metapneumovirus bronchiolitis in infancy is an important risk factor for asthma at age 5. Pediatr Pulmonol 42:458–464.

Goldstein B, Giroir B, Randolph A. 2005. International pediatric sepsis consensus conference: Definitions for sepsis and organ dysfunction in pediatrics. Pediatr Crit Care Med 6:2–8.

Govindarajan D, Yunus A, Samal S. 2004. Complete sequence of the G glycoprotein gene of avian metapneumovirus subgroup C and identification of a divergent domain in the predicted protein. J Genet Virol 85:3671–3675.

Greensill J, McNamara PS, Dove W, Flanagan B, Smyth RL, Hart CA. 2003. Human metapneumovirus in severe respiratory syncytial virus bronchiolitis. Emerg Infect Dis 9:372–375.

Guittet V, Brouard J, Vabret A, Lafay F, Guillois B, Duhamel JF, Freymuth F. 2003. Rhinovirus and acute respiratory infections in hospitalized children. Retrospective study 1998–2000. Arch Pediatr 10:417–423.

Hamelin ME, Abed Y, Boivin G. 2004. Human metapneumovirus: A new player among respiratory viruses. Clin Infect Dis 38:983–990.

Huck B, Scharf G, Neumann-Haefelin D, Puppe W, Weigl J, Falcone V. 2006. Novel human metapneumovirus sublineage. Emerg Infect Dis 12:147–150.

Jennings LC, Anderson TP, Werno AM, Beynon KA, Murdoch DR. 2004. Viral etiology of acute respiratory tract infections in children presenting to hospital: Role of polymerase chain reaction and demonstration of multiple infections. Pediatr Infect Dis J 23:1003–1007.

Jpma FF, Beekhuis D, Cotton MF, Pieper CH, Kimpen JL, van den Hoogen BG, van Doornum GJ, Osterhaus DM. 2004. Human metapneumovirus infection in hospital referred South African children. J Med Virol 73:486–493.

Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis an sequence alignment. Brief Bioinform 5:150–163.

Landry ML, Ferguson D, Cohen S, Peret TC, Erdman DD. 2005. Detection of human metapneumovirus in clinical samples by immunofluorescence staining of shell vial centrifugation cultures prepared from three different cell lines. J Clin Microbiol 43:1950–1952.

Maertzdorf J, Wang CK, Brown JB, Quinto JD, Chu M, de Graaf M, van den Hoogen BG, Spaete R, Osterhaus AD, Fouchier RA. 2004. Real-time reverse transcriptase PCR assay for detection of human metapneumoviruses from all known genetic lineages. J Clin Microbiol 42:981–986.

Meissner HC. 2005. Reducing the impact of viral respiratory infections in children. Pediatr Clin North Am 52:695–710, v.

Pelletier G, Dery P, Abed Y, Boivin G. 2002. Respiratory tract reinfections by the new human Metapneumovirus in an immunocompromised child. Emerg Infect Dis 8:976–978.

Percivalle E, Sarasini A, Visai L, Revello MG, Gerna G. 2005. Rapid detection of human metapneumovirus strains in nasopharyngeal aspirates and shell vial cultures by monoclonal antibodies. J Clin Microbiol 43:3443–3446.

Peret TC, Boivin G, Li Y, Couillard M, Humphrey C, Osterhaus AD, Erdman DD, Anderson LJ. 2002. Characterization of human metapneumoviruses isolated from patients in North America. J Infect Dis 185:1660–1663.

Pierangeli A, Gentile M, Di Marco P, Pagnotti P, Scagnolari C, Trombetti S, Lo Russo L, Tromba V, Moretti C, Midulla F, Antonelli G. 2007. Detection and typing by molecular techniques of respiratory viruses in children hospitalized for acute respiratory infection in Rome, Italy. J Med Virol 79:463–468.

Rao BL, Gandhe SS, Pawar SD, Arankalle VA, Shah SC, Kinikar AA. 2004. First detection of human metapneumovirus in children with acute respiratory infection in India: A preliminary report. J Clin Microbiol 42:5961–5962.

Sarasini A, Percivalle E, Rovida F, Campanini G, Genini E, Torsellini M, Paolucci S, Baldanti F, Marchi A, Grazia Revello M, Gerna G. 2006. Detection and pathogenicity of human metapneumovirus respiratory infection in pediatric Italian patients during a winter–spring season. J Clin Virol 35:59–68.

Semple MG, Cowell A, Dove W, Greensill J, McNamara PS, Halfhide C, Shears P, Smyth RL, Hart CA. 2005. Dual infection of infants by human metapneumovirus and human respiratory syncytial virus is strongly associated with severe bronchiolitis. J Infect Dis 191:382–386.

Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. 1999. Bronchiolitis-associated hospitalizations among US children, 1980–1996. JAMA 282:1440–1446.

Sloots TP, Mackay IM, Bialasiewicz S, Jacob KC, McQueen E, Harnett GB, Siebert DJ, Masters BI, Young PR, Nissen M. 2006. Human metapneumovirus, Australia, 2001–2004. Emerg Infect Dis 12: 1263–1266.

Smyth RL, Openshaw PJ. 2006. Bronchiolitis. Lancet 368:312–322.

van der Hoek L, Sure K, Ihorst G, Stang A, Pyrc K, Jebbink MF, Petersen G, Forster J, Berkhout B, Uberla K. 2005. Croup is associated with the novel coronavirus NL63. PLoS Med 2:e240.

van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD. 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 7:719–724.

van den Hoogen BG, Bestebroer TM, Osterhaus AD, Fouchier RA. 2002. Analysis of the genomic sequence of a human metapneumovirus. Virology 295:119–132.

van den Hoogen BG, van Doornum GJ, Fockens JC, Cornelissen JJ, Beyer WE, de Groot R, Osterhaus AD, Fouchier RA. 2003. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. J Infect Dis 188:1571–1577.

van den Hoogen BG, Herfst S, Sprong L, Cane PA, Forleo-Neto E, de Swart RL, Osterhaus AD, Fouchier RA. 2004. Antigenic and genetic variability of human metapneumoviruses. Emerg Infect Dis 10: 658–666.

van Woensel JB, Bos AP, Lutter R, Rossen JW, Schuurman R. 2006. Absence of human metapneumovirus co-infection in cases of severe respiratory syncytial virus infection. Pediatr Pulmonol 41:872–874.

Weigl JA, Puppe W, Meyer CU, Berner R, Forster J, Schmitt HJ, Zepp F. 2007. Ten years' experience with year-round active surveillance of up to 19 respiratory pathogens in children. Eur J Pediatr.

Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. 2002. Estimates of world-wide distribution of child deaths from acute respiratory infections. Lancet Infect Dis 2:25–32.

Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, Wright PF, Crowe JE Jr. 2004. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N Engl J Med 350:443–450.

Williams JV, Wang CK, Yang CF, Tollefson SJ, House FS, Heck JM, Chu M, Brown JB, Lintao LD, Quinto JD, Chu D, Spaete RR, Edwards KM, Wright PF, Crowe JE Jr. 2006. The role of human metapneumovirus in upper respiratory tract infections in children: A 20-year experience. J Infect Dis 193:387–395.

Wolf DG, Greenberg D, Kalkstein D, Shemer-Avni Y, Givon-Lavi N, Saleh N, Goldberg MD, Dagan R. 2006. Comparison of human metapneumovirus, respiratory syncytial virus and influenza A virus lower respiratory tract infections in hospitalized young children. Pediatr Infect Dis J 25:320–324.

Woo PC, Lau SK, Tsoi HW, Huang Y, Poon RW, Chu CM, Lee RA, Luk WK, Wong GK, Wong BH, Cheng VC, Tang BS, Wu AK, Yung RW, Chen H, Guan Y, Chan KH, Yuen KY. 2005. Clinical and molecular epidemiological features of coronavirus HKU1-associated community-acquired pneumonia. J Infect Dis 192:1898–1907.

Wright AL, Taussig LM, Ray CG, Harrison HR, Holberg CJ. 1989. The Tucson Children's Respiratory Study. II. Lower respiratory tract illness in the first year of life. Am J Epidemiol 129:1232–1246.

Xepapadaki P, Psarras S, Bossios A, Tsolia M, Gourgiotis D, Liapi-Adamidou G, Constantopoulos AG, Kafetzis D, Papadopoulos NG. 2004. Human metapneumovirus as a causative agent of acute bronchiolitis in infants. J Clin Virol 30:267–270.