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

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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 commercial 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; Biacchi 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

INTRODUCTION

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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 Clinic 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., 1999]. 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 symp-
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 (denaturation), 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′-TTG TAT CTA GGA GCA A 3′ and Fr, 5′-GTC TTC CGT 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 extracellular, a positive control for cDNA synthesis and 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.

**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 χ² or Fisher’s exact
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%) |

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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 bronchopneumonia, 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.

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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 over-estimate 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,
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 predominance, in this population, with pneumonia; (5) although respiratory syncytial virus and human metapneumovirus circulation 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.

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