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

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Human metapneumovirus (HMPV) was the unique viral pathogen detected by a real-time reverse transcriptase PCR (RT-PCR) assay in 6 (6.4%) of 94 consecutive French children hospitalized for acute bronchiolitis from September 2001 to June 2002. This virus was identified as the third etiological cause of bronchiolitis, after respiratory syncytial virus and rhinovirus (35 [37%] and 21 [22%] of 94 cases, respectively). Phylogenetic analysis of F-gene sequences demonstrated the cocirculation of distinct HMPV genotypes during this study. These findings highlight the need to implement a rapid HMPV RT-PCR detection assay for the clinical diagnosis of respiratory infections in pediatric patients with bronchiolitis.

Human metapneumovirus (HMPV) is a recently discovered pathogen identified as a cause of human respiratory tract infections (14). This new virus (family *Paramyxoviridae*) is responsible for several diseases, from a mild influenza-like syndrome to more severe bronchopneumopathy (bronchitis and pneumonia) in child, adult, elderly, and immunocompromised patients (3, 12). Several retrospective studies have reported the presence of HMPV sequences in 4 to 21% of nasopharyngeal samples of infants with acute bronchiolitis (6, 8, 10, 15). In these previous reports, HMPV was identified by RT-PCR assay either as a unique viral pathogen or as being associated with respiratory syncytial virus (RSV) or other common respiratory viruses, suggesting that this paramyxovirus could play a role as an etiological agent or as a cofactor of childhood bronchiolitis. At the present time, the significance of HMPV infection as the cause of bronchiolitis in French infants hospitalized for acute wheezing remains to be determined in well-defined respiratory disease patients (6).

In the present report, we used a real-time reverse transcription PCR (RT-PCR) assay to detect HMPV RNA genomic sequences in nasopharyngeal aspirates of 94 consecutive infants hospitalized for acute bronchiolitis. To determine the causal role of HPMV in bronchiolitis, all of these respiratory samples were also tested for the presence of other common respiratory viruses by classical immunofluorescence antigens, cell culture detection, or molecular detection assays. Moreover, a comparative phylogenetic analysis of F-gene sequences was carried out to study the potential circulation of distinct HMPV genotypes during the present study.

One-hundred twenty-four consecutive children admitted to

the pediatric emergency department of the University Medical Hospital of Reims (Champagne-Ardenne, France) for acute expiratory wheezing were prospectively enrolled from September 2001 to June 2002. We selected a group of 94 children (55 males and 39 females), because (i) they had demonstrated specific clinical signs of acute bronchiolitis (respiratory effort, expiratory wheezing, and/or crackles) (2), (ii) they were aged 24 months or less (mean age, 6.6 months; range, 12 days to 24 months), and (iii) they were hospitalized in the pediatric unit for the first time of the epidemic season since the beginning of the study and with a maximal time delay of 3 days after the onset of symptoms. Thirty of the 124 children with acute wheezing were excluded from the present study, because they were suffering from cystic fibrosis, chronic asthma, bronchopulmonary dysplasia, congenital heart disease, rhinitis, chronic allergic rhinitis, otitis, or a chronic genetic or acquired immunodepression. Briefly, nasopharyngeal secretions were collected using sterile physiological saline fluid with a disposable mucus extractor at the time of hospital admission according to the recent European Respiratory Society guidelines (4). Nasopharyngeal aspirate samples were then rapidly transported to the virology laboratory, where they were divided into two sterile tubes. Tube 1 was used to perform the immunofluorescence detection assay of viral antigens and classical virus cell culture detection assays, and tube 2 was immediately frozen and stored at -80°C prior to RT-PCR assays. The hospital's ethics committee approved the study, and informed consent was obtained from parents.

Immunofluorescence detection assays for the detection of respiratory syncytial viruses A and B, influenza virus, parainfluenza virus, and adenovirus antigens were performed on tube 1. This aliquot was diluted in 3 ml of a classical viral transport medium as described previously (1). Moreover, 200 μl of this diluted nasopharyngeal secretion specimen was inoculated in

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FIG. 1. Monthly distribution of bronchiolitis cases with an acute viral respiratory infection during a 10-month period (from September 2001 to June 2002). RSV, respiratory syncytial virus; IVA, influenza virus A; PIV3, parainfluenza virus 3; HMPV, human metapneumovirus; RV, rhinovirus; EV, enterovirus.

duplicate onto 24-well plates covered with monolayers of continuous human diploid fibroblasts (MRC-5) or Rhesus monkey kidney (MA-104) cells as previously described (5).

Retrospectively, total RNA was extracted from 200 μ l of nasal aspirate sample (tube 2) using a rapid extraction protocol according to the manufacturer's recommendations (high pure viral nucleic acid kit; Roche Diagnostics, Mannheim, Germany). Nucleic acids were eluted in a final volume of 50 μ l of DEPC (diethyl-pyrocabonate) sterile water, as described by the manufacturer's recommendations, and was stored at -80°C until used. Detection of human rhinovirus and enterovirus RNA sequences was carried out using a picornavirus RT-PCR detection assay followed by a differential southern blotting procedure as previously described (1). HMPV RNA genomic sequences were detected by real-time RT-PCR amplification of the nucleoprotein (N) gene. Briefly, a 199-bp product was simultaneously amplified and detected using 300 nmol of each primer (forward primer, 5' GTGATGCACTCAAGAGATACCC 3'; reverse primer, 5' CATTGTTTGACCGGCCCATAA 3'), 200 nmol of the detection probe (5' FAM-CTTTGCCATACTCAATGAACAAC-TAMRA 3'), and the Taq-Man One-Step RT-PCR Master Mix Reagents kit according to the manufacturer's recommendations (Applied Biosystems, Tib-Molbiol, Berlin, Germany). The reverse primer and detection probe were identical to those previously developed by MacKay et al. (9), whereas the forward primer was newly designed. Real-time RT-PCR cycling conditions were 30 min at 45°C and 10 min at 95°C , followed by 45 cycles of 95°C for 15 s and 60°C for 1 min in an ABI PRISM 7700 (Applied Biosystems, Tib-Molbiol). The primers and the probe were tested for their specificity against respiratory viruses, such as respiratory syncytial viruses A and B, influenza viruses A and B, adenovirus, enterovirus, rhinovirus, and coronavirus as described previously (9). The assay detected as few as 0.01 50% tissue culture infectious doses of HPMV isolates cultured on LLC-MK2 cells per reaction tube (12). In order to check the quality of each RNA sample, simultaneous codetection of GAPDH was carried out using the TaqMan GAPDH control

reagents according to the manufacturer's recommendations (Applied Biosystems, Tib-Molbiol).

For each respiratory sample positive by HMPV real-time RT-PCR detection assay, a 450-bp sequence of the HMPV fusion protein (F) gene was amplified in a second RT-PCR assay using previously published primers and RT-PCR procedures (13). The PCR products were purified using a Quiaquick gel extraction kit (QIAGEN, Courtaboeuf, France). Both strands were sequenced on a CEQ 2000 XL DNA Analysis System sequencer using a fluorescent dye terminator kit (Beckman Coulter, Villepinte, France). The nucleotide sequences were edited using Sequence Navigator software (Applied Biosystems) and then aligned using the CLUSTAL X 1.8 multiple alignment software (EMBL, Heidelberg, Germany). Phylogenetic trees were generated by the neighbor-joining method using Njplot 1.8 software (Infobiogen, Paris, France). For bootstrap analysis, sequences were added randomly, and one tree was determined at each step (100 bootstrap replicates).

We identified a potential unique causative viral agent in 68 (72%) of 94 children with acute bronchiolitis: 35 (51%) were RSV, 21 (31%) were rhinovirus, 6 (9%) were HMPV, 3 (4.5%) were parainfluenza virus type 3, 2 (3%) were nontyped enterovirus strains, and 1 (1.5%) was influenza virus A strain. Of the 94 patients, 31 (33%) demonstrated a mixed infection, including a rhinovirus in 29 (94%) of these cases. An associated HMPV-rhinovirus was identified in 2 (6.5%) of these 31 respiratory dual infections. No coinfection of HMPV-RSV was observed in the present study. HMPV was detected in children from November 2001 to March 2002, with a peak in January. These HMPV infections were mainly detected during the RSV epidemic period. Moreover, a smaller number of HMPV infections appeared in March 2002, concomitant with RVH respiratory infection cases (Fig. 1). The median age of children with HMPV was 4.5 months (range, 2 to 6 months). All HMPV-positive patients developed classical clinical signs of bronchiolitis, and only one of them required oxygenotherapy on admission. HMPV-infected and HMPV-rhinovirus-coinfected patients were hospitalized for an average of 2.4 days

FIG. 2. Phylogenetic relationships observed in maximum likelihood analysis of the human metapneumovirus fusion nucleotide sequences. Bootstrap proportions were plotted at the main internal branches of the phylogram to show the support values. The scale refers to the distance between sequences. CAN, Canada; NLD, The Netherlands; FR, France. Canadian strains are under GenBank accession numbers AY145242 to AY145301; the Dutch HPMV strain is under GenBank accession number AF371367; the French strains are under GenBank accession numbers AY626963 to AY626969.

(standard deviation, 2.5), meaning the duration of hospital stay was not statistically different from those observed for bronchiolitis infants not infected by HMPV ($P > 0.5$, Mann-Whitney test; Epi-Info 6.1).

Nucleotide sequences of a 450-bp amplified fragment in the F gene were obtained from seven of the patients infected by HMPV. Phylogenetic analyses of the F-gene sequences indicated the presence of two distinct subclusters of strains (subgroups A1 and A2). All of these strains clustered along with isolates belonging to the HMPV genogroup A, including the initial Dutch reference strain (13) (Fig. 2). During our study cohort, we observed that FR02-3 and FR02-4 strains, which belong to two different subgroups, were cocirculating during January, whereas FR01-1 and FR01-2 as well as FR02-4 and FR02-5, which belong to the same subgroup, were cocirculating during December 2001 and January 2002, respectively (Fig. 2).

Our findings provided evidence that HMPV was one of the leading etiological causes of bronchiolitis with RSV and human rhinovirus, these latter two being the predominant respiratory pathogens in our study. Using a reliable real-time RT-PCR assay, we identified HMPV as the unique viral pathogen in 6 (6.4%) of the 94 children presenting with acute bronchiolitis. Only one French retrospective study reported the presence of 16 cases of HPMV bronchiolitis in a cohort of 337 infants hospitalized for various respiratory illnesses (6). Our prospective study is the first to assess the prevalence rate of HMPV infection in a clinically well-defined cohort of French

pediatric patients with bronchiolitis. Our prevalence of HMPV infection is close to those reported by Xepapadaki et al. (5 out of 56 [9%]) (15). Similarly, a recent study detected HMPV in 4% of a large clinically well-defined cohort of 292 children hospitalized for acute expiratory wheezing, including bronchiolitis and asthma cases (8). Our findings indicate that HMPV is the third etiological agent of acute bronchiolitis, after RSV and human rhinovirus, during the study period. Moreover, our data confirm the major role of human picornaviruses (enteroviruses and rhinoviruses) as etiological causes of bronchiolitis in young children (8, 11).

In the present report, HMPV was detected as a unique respiratory pathogen in 6.4% (6 out of 94) of the cases and was associated with rhinovirus in only 2.1% (2 out of 94) of the children. Despite the overlapping distribution of HMPV and RSV, we did not detect any dual infections with these two viruses as previously described (8, 15). The small number of mixed viral infections with HMPV could be explained by the use of classical rather than molecular assays for the detection of RSV and other common respiratory viruses, which are known to be more sensitive techniques (5). Some recent studies indicated that HMPV could play a role as a cofactor, especially in association with RSV, and that this dual respiratory infection may increase the severity of the respiratory disease (7). In this report, we observed only two mixed infections with HMPV and rhinovirus without an increased severity index of clinical symptoms (data not shown). The role of HMPV as a cofactor increasing the severity of the initial viral infection needs to be assessed in further longitudinal studies comparing the severity of the disease and the frequency of HMPV coinfections (7).

During this study, HMPV seasonal distribution was characterized by two peaks in winter and spring (Fig. 1), and therefore it appeared to be different from those previously observed in Finland, Canada, and Greece (2, 5, 7). Moreover, phylogenetic analysis of fusion gene sequences of our strains demonstrated high nucleotide identity with viruses belonging to genogroup A, which was previously defined as the genetic cluster of European HMPV strains (Fig. 2) (13). On the other hand, we observed the cocirculation of genetically distinct HMPV strains at several periods of the study. This suggests that potential successive HMPV reinfections can occur in young children during the same epidemic season, as has been described for RSV (5).

Finally, our findings suggest that HMPV is a common and frequent causative agent of bronchiolitis in young French children. Moreover, our data indicate that several genetically distinct viral strains could cocirculate during several periods of the same epidemic season, suggesting the possibility of successive reinfection. Taken together, these findings highlight the need to implement a rapid HMPV RT-PCR detection assay for the clinical diagnosis of respiratory infections in pediatric patients with bronchiolitis. The use of rapid HMPV detection RT-PCR assays in clinical virology practice would be of major interest for the development of future therapeutic and preventive strategies to fight the viral causes of childhood bronchiolitis.

Nucleotide sequence accession numbers. All of the French HMPV sequences reported in this publication (FR01-1 to

FR01-2 and FR02-3 to FR02-7) have been submitted to GenBank (accession numbers AY626963 to AY626969).

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