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.
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 μl 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 μl of DEPC (diethyl-pyrocarbonate) 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′ GTGATGCACTCA AGAGATACCC 3′; reverse primer, 5′ CATTGTTGGACCG GCCCCATAA 3′), 200 nmol of the detection probe (5′ FAM- CTTTGCCATATCTCAAGAAACAT-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 co-detection 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. 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.
HMPV infection in a clinically well-defined cohort of French prospective study is the first to assess the prevalence rate of infants hospitalized for various respiratory illnesses (6). Our incidence of 16 cases of HMPV bronchiolitis in a cohort of 337 infants 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 infections (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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