CONCISE COMMUNICATION

Viroleological Features and Clinical Manifestations Associated with Human Metapneumovirus: A New Paramyxovirus Responsible for Acute Respiratory-tract Infections in All Age Groups

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The human metapneumovirus (HMPV) recently has been identified in respiratory specimens from young children in the Netherlands [1]. This virus has been assigned preliminarily to the Paramyxoviridae family, subfamily Pneumovirinae, and genus Metapneumovirus, on the basis of electron microscopy and molecular studies [1, 2].

The virological features and clinical findings associated with the new human metapneumovirus (HMPV) were examined retrospectively in Canadian patients hospitalized for various respiratory conditions since 1993. Thirty-eight previously unidentified respiratory viruses isolated from rhesus monkey kidney (LLC-MK2) cells were found to be positive for HMPV by reverse-transcription polymerase chain reaction, and those strains clustered in 2 phylogenetic groups. Children aged <5 years and elderly subjects aged >65 years represented 35.1% and 45.9% of the HMPV-infected cases, respectively. In hospitalized children, the most frequent diagnoses were pneumonitis (66.7%) and bronchiolitis (58.3%), whereas bronchitis and/or bronchospasm (60%) and pneumonitis (40%) were most commonly seen in elderly subjects. Of the 15 patients with pneumonitis, 4 (26.7%) had immunosuppressive conditions and 6 (40%) were infants aged <15 months. These findings suggest that HMPV can be associated with severe lower-respiratory-tract infections in very young children, the elderly, and immunocompromised patients.

Materials and Methods

Specimens, cell lines, and nonmolecular detection assays. Respiratory specimens were inoculated throughout the year on the following continuous cell lines in 96-well plates and were incubated for 3 weeks: human laryngeal carcinoma (HEp-2), human foreskin fibroblast, Vero (African green monkey kidney), Mink lung, human lung adenocarcinoma (A-549), human rhabdomyosarcoma (RD), transformed human kidney (293), and human colon adenocarcinoma (HT-29). In addition, samples were inoculated on Madin Darby canine kidney (MDCK) cells during the winter season and on rhesus monkey kidney (LLC-MK2) cells throughout the year in vials containing trypsin (Sigma). The presence of a cytopathic effect (CPE) or a positive hemadsorption test was confirmed by immunofluorescent assays for influenza A and B, parainfluenza viruses 1–4, adenoviruses (Bartels), measles, mumps (Chemicon), and respiratory syncytial virus (RSV; Meridian Diagnostics). Nasopharyngeal samples from children (but not those from adults) were tested for RSV antigens using the Abbott TestPack RSV kit (Abbott Laboratories). Upon request, viral antigens were directly sought in respiratory samples by immunofluorescence testing, by use of the monoclonal an-
tibodies (MAbs) described above, or by ELISA, using the Directigen Flu A test (Becton Dickinson). Serological testing for HMPV was performed with paired serum (diluted 1:10) of a few patients by use of an indirect immunofluorescent assay using LLC-MK2 cells infected with the patients’ corresponding HMPV isolates.

Reverse-transcription polymerase chain reaction (RT-PCR) assays for HMPV and phylogenetic analysis. RNA was extracted from cell culture supernatants by use of the QIAamp Viral RNA Mini kit (QIAGEN) and then was reverse transcribed using the Superscript II RT enzyme (Invitrogen Life Technologies) and random hexamer primers (Amersham Pharmacia Biotech). The cDNA was amplified by use of the Pfu turbo polymerase (Stratagene) and 2 separate HMPV primer pairs: HMPV-F1 (5′-ATGTCTTGGAAAGTGTTGCTTGGTGTTATC-3′) and HMPV-F2 (5′-TCTTCTTACCATGATGGCACCTAC-3′), as well as HMPV-M1 (5′-ATGGAGTCCTACCTAGTACCCAGCCTAACCTGCACA-3′) and HMPV-M2 (5′-AAGTTGCTTGGTGTGTTATCATC-3′). This resulted in amplified regions of 759 and 780 bp in the F (fusion) and M (matrix) genes, respectively. Nucleotide sequences of the amplified F gene products were aligned by use of CLUSTAL W, version 1.74 for Unix. Phylogenetic trees were computed by maximum parsimony, distance and maximum likelihood-based criteria analysis using PAUP* version 4.0.d8.

Demographic and clinical data. A retrospective review of the virological and bacteriological laboratory findings was performed for all patients with positive HMPV culture results. In addition, an extensive review of the clinical findings was possible for the vast majority (76%) of patients positive for HMPV.

Results

Laboratory findings. Unidentified viruses collected on an ongoing basis in a regional virology laboratory were included in this study on the basis of the following criteria: (1) positive CPE in LLC-MK2 cells; (2) negative hemadsorption testing with human red blood cells and immunofluorescence staining with MAbs against the common respiratory viruses; (3) positive RT-PCR assays for the HMPV F and M genes; and (4) negative RT-PCR or PCR assays for common respiratory viruses using cell culture supernatants [2]. On this basis, 38 HMPV isolates (including 11 previously reported in [2]) from 37 patients were retrospectively identified in the Quebec City’s virology laboratory during 1993–2001. Viruses were recovered from nasopharyngeal aspirates (47.4%), nasal and/or pharyngeal swabs (28.9%), endotracheal aspirates or bronchoalveolar lavages (10.5%), and other unspecified respiratory specimens (13.2%). In most cases (86.7%), HMPV isolates were recovered during a 5-month period, from December to May.

The HMPV isolates only initially grew on LLC-MK2 cells. The characteristic CPE consisted of small, round, granular, and refringent cells, without large syncytium in most cases. The CPE was apparent after a mean incubation time of 17.3 days (range, 3–23 days). A seroconversion for HMPV was observed in the 2 patients (aged 49 and 83 years) for whom acute and convalescent serum were available (from <1:10 to 1:80 over 2 weeks and from <1:10 to 1:160 over 4 weeks, respectively). ELISA or immunofluorescence assay (IFA) tests for influenza A and B, RSV, parainfluenza 1–3, and adenovirus antigens were performed for 23 (60.5%), 11 (28.9%), 2 (5.3%), and 1 (2.6%) of the original samples, respectively. Nine (23.7%) of the 38 samples were found to be positive for other viral (3 influenza A and 2 RSV by ELISA only; 1 measles by culture) or bacterial (1 Staphylococcus aureus, 1 Streptococcus pneumoniae, and 1 Stenotrophomonas maltophilia) pathogens.

One of the children with ALL died of Streptococcus pneumoniae.

Clinical findings. Twenty-six HMPV-infected patients were hospitalized in acute care hospitals for a febrile respiratory condition, 7 patients were living in long-term care facilities at the time they developed a respiratory illness, and 4 patients consulted at a private clinic for a flu-like illness. The study population included 17 men and 20 women with a mean age of 45.5 years (median, 49.0 years; range, 2 months to 99 years), including 13 (35.1%) aged <5 years and 17 (45.9%) aged ≥65 years. Detailed clinical findings were obtained for 28 (75.7%) of the 37 patients.

Twelve patients were aged <5 years (median, 15 months), including 11 (91.7%) who were hospitalized for their respiratory condition during a median of 4 days, and 3 (25%) who required a stay in the intensive care unit (ICU). Four (33%) of these children had an underlying disease consisting of acute lymphoblastic leukemia (ALL; n = 2, both receiving chemotherapy, with 1 being neutropenic), prematurity (n = 1), and epilepsy (n = 1). The most frequently reported symptoms were fever (91.7%), dyspnea (83.3%), cough (75%), and wheezing/stridor (50%). All infected children had a diagnosis of either pneumonitis (66.7%) and/or bronchiolitis (58.3%). One-fourth of them also had concomitant otitis media. Among the 8 patients with pneumonitis, a copathogen was found in respiratory secretions of 3 patients: RSV by antigen detection in 1 patient and a bacteria in 2 patients (Staphylococcus aureus and Streptococcus pneumoniae). One of the children with ALL died of ARDS a few weeks after her last culture positive for HMPV. Although no autopsy was performed, all premortem cultures and antigenic tests from nasopharyngeal aspirates and bronchoalveolar lavage were otherwise negative.

The second group of patients included 6 HMPV-infected patients between the age of 15 and 65 years (median, 44.5 years) with 2 (33.3%) specifically hospitalized for their respiratory condition, and 1 (16.7%) who required a stay in the ICU. Four
(66.6%) of these patients had an underlying medical condition (lung tumor, active lymphoma, atherosclerotic coronary heart disease, and epilepsy). The most frequently noted symptoms were fever (83.3%), cough (83.3%), dyspnea (50.0%), and sore throat (50.0%). Three (50%) of the HMPV-infected patients developed a pneumonitis, including the 2 patients with immunosuppressive conditions, whereas the other 3 only presented a flu-like syndrome. Among patients with pneumonitis, a copathogen was identified in respiratory samples of 1 patient that consisted of influenza A antigens. None of these patients died as a result of their respiratory-tract infection.

Clinical findings of 10 patients with HMPV infections aged >65 years (median, 74.5 years) were reviewed. Six (60%) patients were hospitalized for their respiratory condition a median of 10.5 days, with 2 being (20%) hospitalized in the ICU. All 10 patients had at least 1 underlying disease, including chemotherapy-treated leukemia or lymphoma, with resulting neutropenia (n = 2), chronic pulmonary condition (n = 3), chronic cardiovascular condition (n = 4), and neurologic disease (n = 3; Parkinson disease, myotonia, and Alzheimer disease). The most frequently reported symptoms were cough (100%), fever (80%), and dyspnea (70%). Four (40%) of the patients developed a pneumonitis, and 6 (60%) had a diagnosis of bronchitis and/or bronchospasm. Of the patients with pneumonitis, only 1 had a copathogen (Stenotrophomonas maltophilia) identified in sputum. Two patients died of pneumonitis, including 1 with leukemia (HMPV in bronchoalveolar lavage and S. maltophilia in sputum) and 1 with Alzheimer disease (no copathogens). Neither of these 2 patients had an autopsy performed.

**Phylogenetic analysis.** Nucleotide comparison of 22 HMPV F gene sequences revealed 2 major phylogenetic groups with 80.2%–83.3% similarity among groups versus 94.2%–100% (group 1) and 92.6%–100% (group 2) similarity within groups (figure 1). At the amino acid level, similarity varied from 94%–96.5% between groups and 96.8%–100% (group 1) and 97.2%–100% (group 2) among isolates within the same group. Strains from both groups cocirculated during certain years (2000 and 2001), and HMPV sequences from different years were found in the same subcluster (strains 3-1997, 33-2001, and 35-2001). Interestingly, 2 strains (5-1998 and 6-1998) isolated on the same day from 2 patients with flu-like illnesses in the same long-term care facility had F gene sequences that differed by 2 nonsilent mutations. When the convalescent serum sample from one of the subject who seroconverted for HMPV was used to isolate respiratory viral pathogens [1]. The LLC-MK2 cells did not display CPE until later (mean time before appearance, 17.3 days), with no hemadsorption activity. Our phylogenetic analysis of the F gene revealed 2 major groups of sequences. These 2 groups have been noted elsewhere in a smaller number of isolates from Europe [1] and in a subset of isolates reported in the present study from North America [2]. It is noteworthy that the similarity between members of the 2 groups was greater at the amino acid level than at the nucleotide level, which suggests a functional and/or immunological conservation of the F protein. Interestingly, we observed cocirculation of the 2 HMPV groups during a single year, which was similar to circulation patterns described for RSV during community outbreaks [12, 13]. In addition, isolates from 2 patients who resided at the same nursing home that were recovered during the same outbreak had slightly different F gene sequences, suggesting cocirculation of >1 strain in the same institution.

There is little information about the clinical presentation of HMPV infection. In the original report by van den Hoogen et al. [1], 27 of the 28 infected subjects were aged <5 years and all were diagnosed with a range of ARTI reported as being mild to severe. Our data provide substantial additional information on the clinical manifestations of HMPV infection, although the retrospective nature of our study introduces a bias toward patients who were sicker. We showed that HMPV infection is restricted not only to the very young children but also occurs in elderly subjects, and, similar to findings with other community-acquired respiratory viruses, being very young or elderly or having comorbid and/or immunosuppressive conditions may predispose to serious manifestations of infection [3–8]. Notably, of the 4 patients requiring mechanical ventilation, 2 had neoplasias (ALL and lung tumor) and 2 were aged <1 year. Furthermore, 5 (83.3%) of the 6 infected immunocompromised patients developed a pneumonitis, and 3 (50%) were admitted to the ICU. In both very young children and elderly patients, the most frequent diagnoses were bronchiolitis/bronchitis and/or pneumonitis, whereas middle-age adults presented with a virus, also may contribute to LRTI [7, 8]. However, despite extensive diagnostic testing, a substantial portion of respiratory-tract infections still cannot be attributed to any known pathogens [9, 10]. Our study suggests that HMPV can be added to the list of human respiratory viral pathogens in all age groups and that it could be as frequently isolated as parainfluenza viruses during some winter seasons, accounting for 7.1% (29.3% in subjects aged >65 years) of all positive cultures from respiratory specimens. This adds evidence to preliminary studies from The Netherlands [1] and Australia [11], which reported HMPV detection by RTPCR in 10% and 1.5%, respectively, of samples from children with unexplained respiratory-tract infections.

We were able to report the growth of HMPV in LLC-MK2 cells, an established cell line used to identify parainfluenza viruses, but not in other cell lines (MDCK and Hep-2) that are often used to isolate respiratory viral pathogens [1]. The LLC-MK2 cells did not display CPE until later (mean time before appearance, 17.3 days), with no hemadsorption activity. Our phylogenetic analysis of the F gene revealed 2 major groups of sequences. These 2 groups have been noted elsewhere in a smaller number of isolates from Europe [1] and in a subset of isolates reported in the present study from North America [2]. It is noteworthy that the similarity between members of the 2 groups was greater at the amino acid level than at the nucleotide level, which suggests a functional and/or immunological conservation of the F protein. Interestingly, we observed cocirculation of the 2 HMPV groups during a single year, which was similar to circulation patterns described for RSV during community outbreaks [12, 13]. In addition, isolates from 2 patients who resided at the same nursing home that were recovered during the same outbreak had slightly different F gene sequences, suggesting cocirculation of >1 strain in the same institution.

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flulike syndrome or a common cold complicated by pneumonitis in immunocompromised patients of this age group.

Although further studies are needed to definitively link HMPV to ARTI, our data strongly suggest this is the case. Indeed, HMPV was the only pathogen isolated from the respiratory tract of most hospitalized patients. For instance, of the 15 HMPV-infected patients with pneumonitis, only 5 (33.3%) had positive test results for another pathogen (RSV, n = 1; influenza A, n = 1; and bacteria, n = 3), with the 2 viral antigen tests not confirmed by culture and possibly representing false positive results [14, 15]. It is possible, however, that some coinfecting pathogens were not detected, because either testing was not done (RSV antigen detection assays were only used for pediatric patients) or highly sensitive assays (e.g., PCR) were not used to assess the full range of respiratory pathogens. Nevertheless, the lack of other respiratory pathogens in most patients and seroconversion to HMPV in 2 patients during the course of their illness suggest that HMPV is a true pathogen of both the upper and lower respiratory tracts. Previously reported experimental infection of cynomolgus macaques supports this conclusion [1]. However, a definitive role for HMPV in fatal pneumonitis (which was presumed in 3 of our patients) awaits confirmation from biopsy or autopsy samples, which were not available in our study. It is now important to conduct a variety of prospective case-control studies, including those that will be able to determine the amount of ARTI attributable to HMPV.
infection, the full clinical spectrum of disease caused by HMPV, and risk factors that may predispose to serious HMPV disease.

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References