MAJOR ARTICLE

A 1-Year Experience with Human Metapneumovirus in Children Aged <5 Years

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Human metapneumovirus (hMPV) is a recently discovered respiratory pathogen. We tested respiratory specimens for the presence of hMPV by reverse-transcription polymerase chain reaction. These specimens were obtained over a 1-year period from children aged <5 years and had negative results by the direct fluorescent antibody test for respiratory syncytial virus, influenza A and B, parainfluenza viruses 1–3, and adenovirus. Overall, 54 (8.1%) of 668 individuals tested positive for hMPV. During March and April of the study period, hMPV was detected in 17.6% and 25.0% of specimens tested, respectively. At least 2 distinct genotypes of hMPV circulated during the study period. Fever, tachypnea, cough, rhinorrhea, retractions of the chest wall, and wheezing were common findings. Of hMPV-positive children, 60.4% were aged <12 months. hMPV accounted for a small but significant proportion of respiratory-tract disease in infants and children.

Respiratory infections are a leading cause of morbidity and mortality in children worldwide. The World Health Organization ranks respiratory-tract infections as the second leading cause of death in children aged <5 years [1]. In June 2001, researchers in The Netherlands reported the discovery of a new respiratory virus, human metapneumovirus (hMPV) [2], in the respiratory secretions of children with respiratory-tract disease. Electron microscopy and genomic sequence analysis of hMPV revealed that this new pathogen was a paramyxovirus and was the first nonavian pathogen of the *Metapneumovirus* genus [2, 3]. The results of phylogenetic studies have indicated that respiratory syncytial virus (RSV), a member of the *Pneumovirus* genus, is the most closely related human pathogen [3].

Preliminary serological evidence from The Netherlands suggested that infection with hMPV is ubiquitous; by age 5 years, nearly every individual tested had evidence of hMPV-specific antibody [2]. Subsequently, hMPV has been reported in Australia [4, 5], Canada [6, 7], the United Kingdom [8, 9], Finland [10], France [11], Spain [12], and the United States [13, 14], which suggests that the virus has a worldwide distribution.

Although there have been only a limited number of reports about hMPV, a picture of the epidemiology of and infection with this virus is beginning to emerge. hMPV may have a seasonal distribution [2, 7–15], although full-year testing for the virus has only been done in The Netherlands [15]. Most infections with hMPV have been detected during winter and spring. Infection in all age groups has been documented [2, 4, 7, 9, 12–15], although young children and elderly persons may be particularly prone [7, 13, 14]. The clinical features associated with hMPV infection in children are consistent with both upper and lower respiratory-tract infections [4, 7, 13]. Jartti et al. [10] have detected hMPV in children who presented with acute wheezing. We have previously reported [13] the clinical features associated with hMPV in infants and young children. To

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Table 1. Respiratory specimens tested for human metapneumovirus (hMPV) from November 2001 to October 2002.

Month	No. tested		hMPV-positive individuals, no. (%)
	Specimens	Individuals	
November	31	28	0 (0)
December	41	35	0 (0)
January	153	122	11 (9.0)
February	132	111	8 (7.2)
March	109	102	18 (17.6)
April	54	48	12 (25.0)
May	47	41	1 (2.4)
June	22	19	2 (10.5)
July	47	34	2 (5.9)
August	31	22	0 (0)
September	37	32	0 (0)
October	90	74	0 (0)
Total	794	668	54 (8.1)[a]

[a] Two individuals had 2 hMPV-positive specimens obtained <7 days apart; therefore, the total no. of hMPV-positive specimens was 56. One individual had 2 hMPV-positive specimens obtained 54 days apart. These were considered to be 2 separate infections.

investigate the epidemiology and to describe further the clinical syndrome associated with this newly emerging respiratory pathogen, we tested respiratory specimens from children that were obtained over a 1-year period. Here we describe the seasonal distribution, phylogenetic analysis, and clinical characteristics of infection with hMPV in Connecticut.

MATERIALS AND METHODS

Specimen collection. From 1 November 2001 to 31 October 2002, respiratory specimens were collected from the Clinical Virology Laboratory at Yale–New Haven Hospital. Specimens were submitted to the Clinical Virology Laboratory at the discretion of the medical teams. Specimens originated from the emergency department, inpatient wards, intensive care units, and the hospital-affiliated primary care outpatient clinic. We obtained all clinical specimens from individuals aged <5 years who had negative results for RSV, parainfluenza viruses (types 1–3), influenza A and B, and adenovirus, according to the results of direct immunofluorescence assay (DFA). For DFA, specimens were processed, applied to slides by cytocentrifugation, stained with SimulFluor Respiratory Screen reagent (Chemicon International), and examined as described elsewhere [16]. An adequate specimen required a minimum of 25 columnar epithelial cells. The collection of specimens and clinical data was approved by the Yale University Human Investigation Committee.

RNA extraction, reverse-transcription polymerase chain reaction (RT-PCR), and sequencing. RNA from each respiratory specimen was extracted with the QiaAmp Viral RNA Mini Kit (Qiagen), according to the manufacturer's protocol.

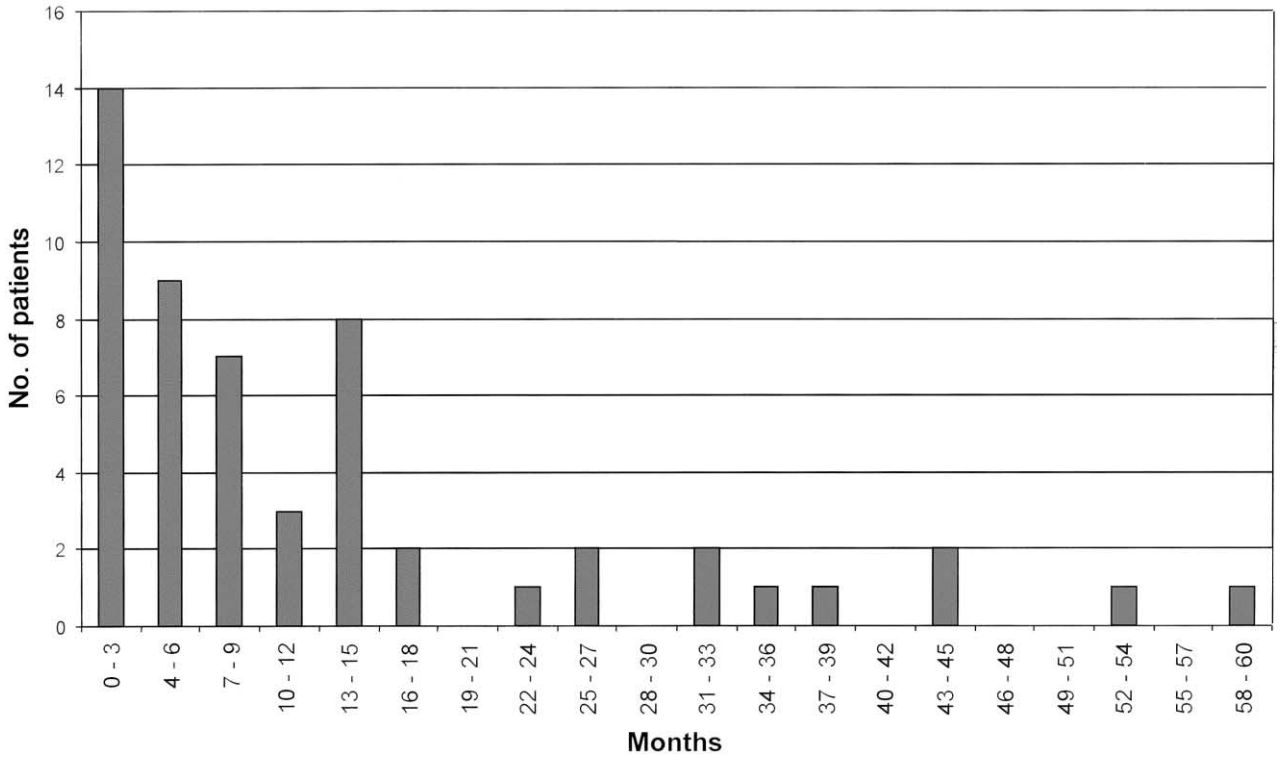


Figure 1. Age distribution of human metapneumovirus–positive children

Table 2. Clinical features associated with human metapneumovirus (hMPV).

Clinical feature	Patients, no. (%)[a]
Age <12 months	32 (60.4)
Male	36 (67.9)
Comorbidity[b]	18 (34.0)
Fever	41 (77.4)
Tachypnea	41 (77.4)
Cough	36 (67.9)
Rhinorrhea	34 (64.2)
Retractions	28 (52.8)
Wheeze	27 (51.0)
Hypoxia[c]	20 (37.7)
Abnormal chest radiograph results (n = 36)	20 (55.6)
Infiltrates	10 (27.8)
Hyperinflation	5 (13.9)
Peribronchial cuffing	4 (11.1)

[a] One of 54 hMPV-positive individuals tested positive for respiratory syncytial virus 1 day after the hMPV-positive specimen was obtained. This individual's clinical features were not included in the data. Therefore, the total no. of patients was 53, unless otherwise noted.

[b] History of prematurity, chronic lung disease (including bronchopulmonary dysplasia), complex congenital heart disease, or immunodeficiency.

[c] Defined as an 0_2 saturation <90%.

RNA specimens were then tested by RT-PCR for the presence of hMPV. Random hexamer primers were used to create a cDNA library for each specimen. RT reactions were performed with MuMLV RT (New England Biolabs), according to the manufacturer's specifications. Each cDNA was subsequently tested by PCR with HotStar *Taq* polymerase (Qiagen), according to the manufacturer's specification.

The primers used in the initial testing of the respiratory specimens were based on sequence data of a Dutch strain available from GenBank (accession number AF371367) and targeted the hMPV F gene. After the amplification and sequencing of the F gene of the first strain of hMPV that we identified, primers were synthesized that targeted regions of the hMPV F gene that were conserved among Dutch, Australian (GenBank accession number AF442516, which subsequently became available), and Connecticut strains. The forward primer, 5′-GCGCGTTCTGAGGACAGGTTGG, and reverse primer, 5′-GCGCTCAAGCCGGATGGTTTTGG, produce an amplicon that corresponds to nt 111–392 of the hMPV F gene (GenBank accession number AF371367). G/C clamps for each primer are underlined. All respiratory specimens were tested using these conserved primer sets. Primers were synthesized at the Yale Oligonucleotide Laboratory, Department of Pathology. Each set of RT and PCR reactions contained appropriate negative controls. PCR amplification cycles were as follows: 95°C for 15 min; 35 cycles of 94°C for 1 min, 45°C–55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. Sequencing was performed on Applied Biosystems 377 DNA automated sequencers at the W. M. Keck Biotechnology Resource Lab, Yale University School of Medicine.

Phylogenetic analysis. Sequence alignment and phylogenetic analysis were performed with Lasergene (DNASTAR) software. Maximum-likelihood phylogenetic trees were constructed using the PHYLIP program DNAML, with the default transition to transversion ratio of 2.0 and 1 jumble. Five hundred bootstrap data sets were created using the PHYLIP program SEQBOOT. The phylogenetic analysis included representative sequences of hMPV from Connecticut, The Netherlands, Australia, and Canada and corresponded to a 120-bp region spanning nt 3304–3443 of the hMPV genome (GenBank accession number AF371337).

Clinical data. Medical records of all hMPV-positive individuals were reviewed. Demographic data and clinical characteristics of each individual were recorded on a standard collection form. Clinical data include 19 patients reported elsewhere [13].

RESULTS

Testing respiratory specimens for hMPV. From 1 November 2001 to 31 October 2002, 794 respiratory specimens from 668 individuals were tested by RT-PCR for the presence of hMPV. Overall, 54 individuals (8.1%) tested positive for hMPV (table 1). Three individuals had 2 specimens that tested positive for hMPV. For 2 of these individuals, the specimens were obtained within a 7-day period. These positive specimens were considered to represent the same hMPV infection. The third individual had 2 hMPV-positive specimens obtained 54 days apart. No respiratory specimens were obtained from this patient during the interval between the 2 specimens. Sequence analysis of these 2 positive specimens revealed rare polymorphisms (a 0.5% difference). Because there was a significant interval between the 2 specimens being obtained and because the patient's respiratory symptoms resolved after the initial illness, we considered these to be separate events, although we cannot rule out the possibility of prolonged shedding of hMPV.

Clinical features associated with hMPV. The clinical features associated with hMPV were consistent with our previous findings [13]. The median age of patients was 7.5 months, and a majority (60.4%) of children who tested positive for hMPV were aged <12 months (figure 1 and table 2). Curiously, 67.9% of the hMPV-positive patients were male. One individual, on subsequent testing performed 24 h after the hMPV-positive specimen was obtained, had a respiratory specimen that tested positive for RSV. Because this patient's clinical features may have reflected infection with RSV, hMPV, or both viruses, this patient's data were not included in the analysis. Overall, nearly half of hMPV-positive children (26 [49.1%] of 53) were hospitalized.

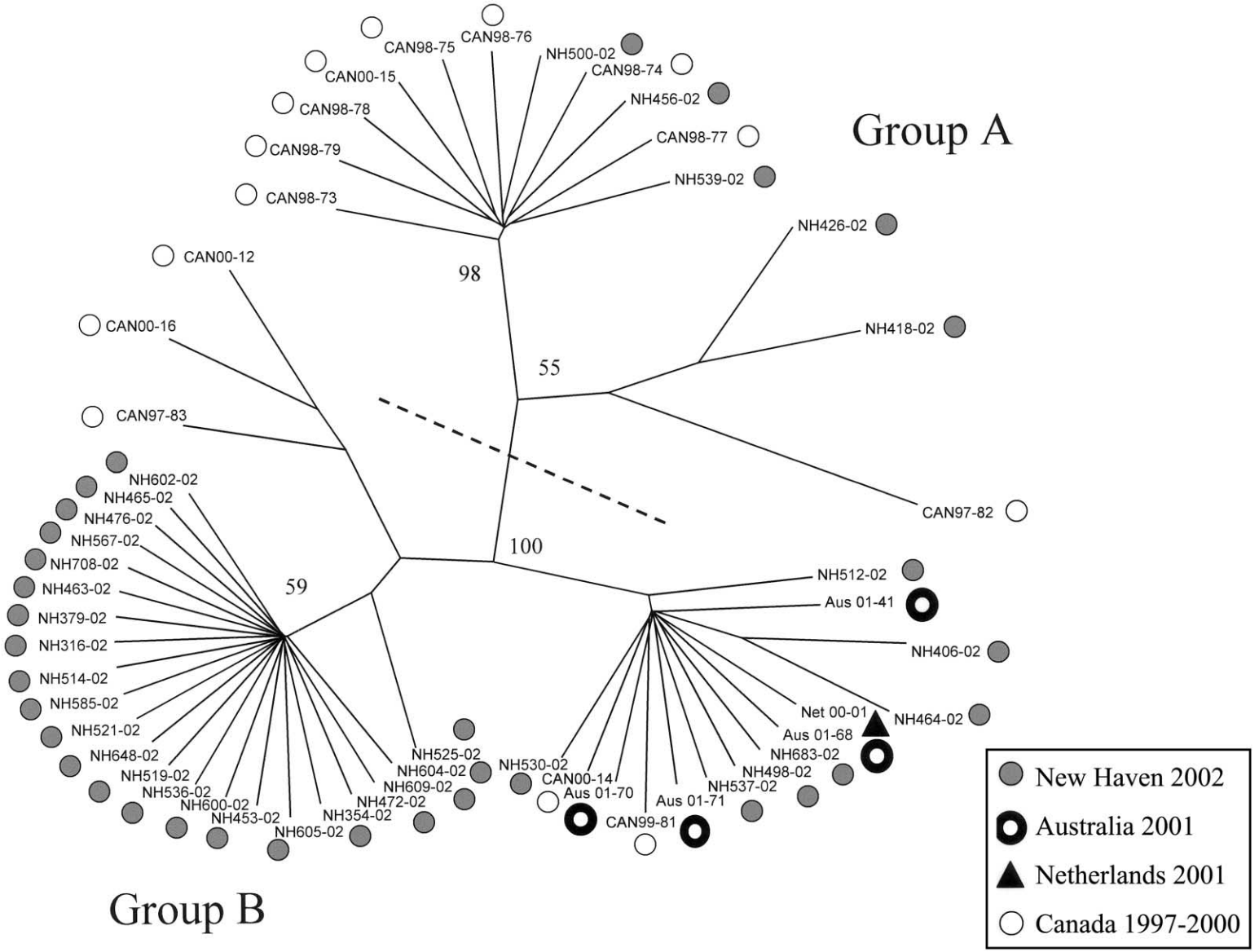
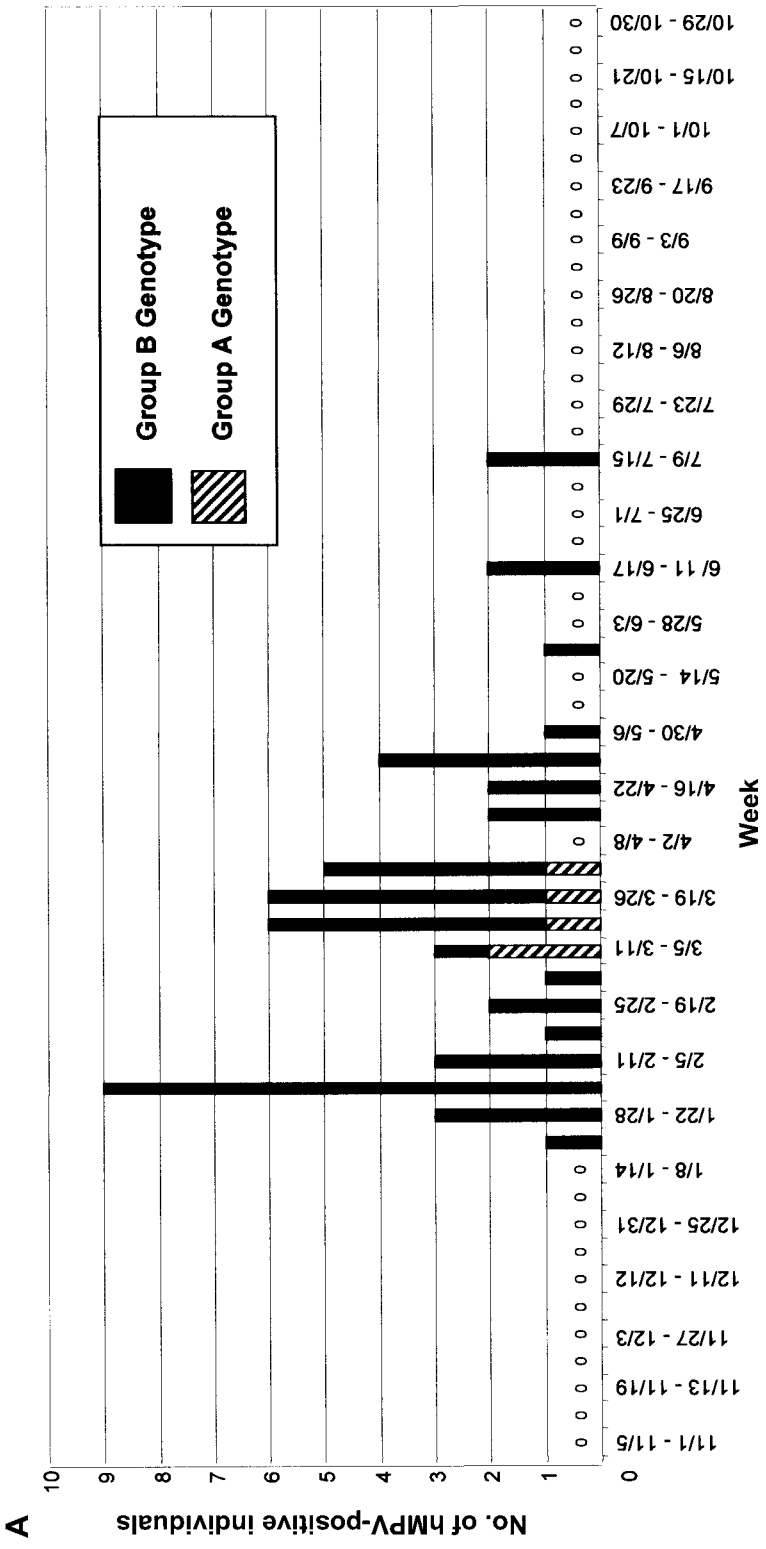


Figure 2. Phylogenetic analysis of human metapneumovirus (hMPV). Sequences of the hMPV F gene from isolates originating from Connecticut, The Netherlands, Australia, and Canada were used to construct a phylogenetic tree. Bootstrap values are displayed at major branch points. A representative set of Connecticut sequences is displayed. Genotypes were arbitrarily labeled groups A and B. The dotted line separates group A from group B viruses.


A
No. of hMPV-positive individuals
0
1
2
3
4
5
6
7
8
9
10
Week
11/1 - 11/5
11/13 - 11/19
11/27 - 12/3
12/11 - 12/12
12/25 - 12/31
1/8 - 1/14
1/22 - 1/28
2/5 - 2/11
2/19 - 2/25
3/5 - 3/11
3/19 - 3/26
4/2 - 4/8
4/16 - 4/22
4/30 - 5/6
5/14 - 5/20
5/28 - 6/3
6/ 11 - 6/17
6/25 - 7/1
7/9 - 7/15
7/23 - 7/29
8/6 - 8/12
8/20 - 8/26
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10/15 - 10/21
10/29 - 10/30
Group A Genotype
Group B Genotype

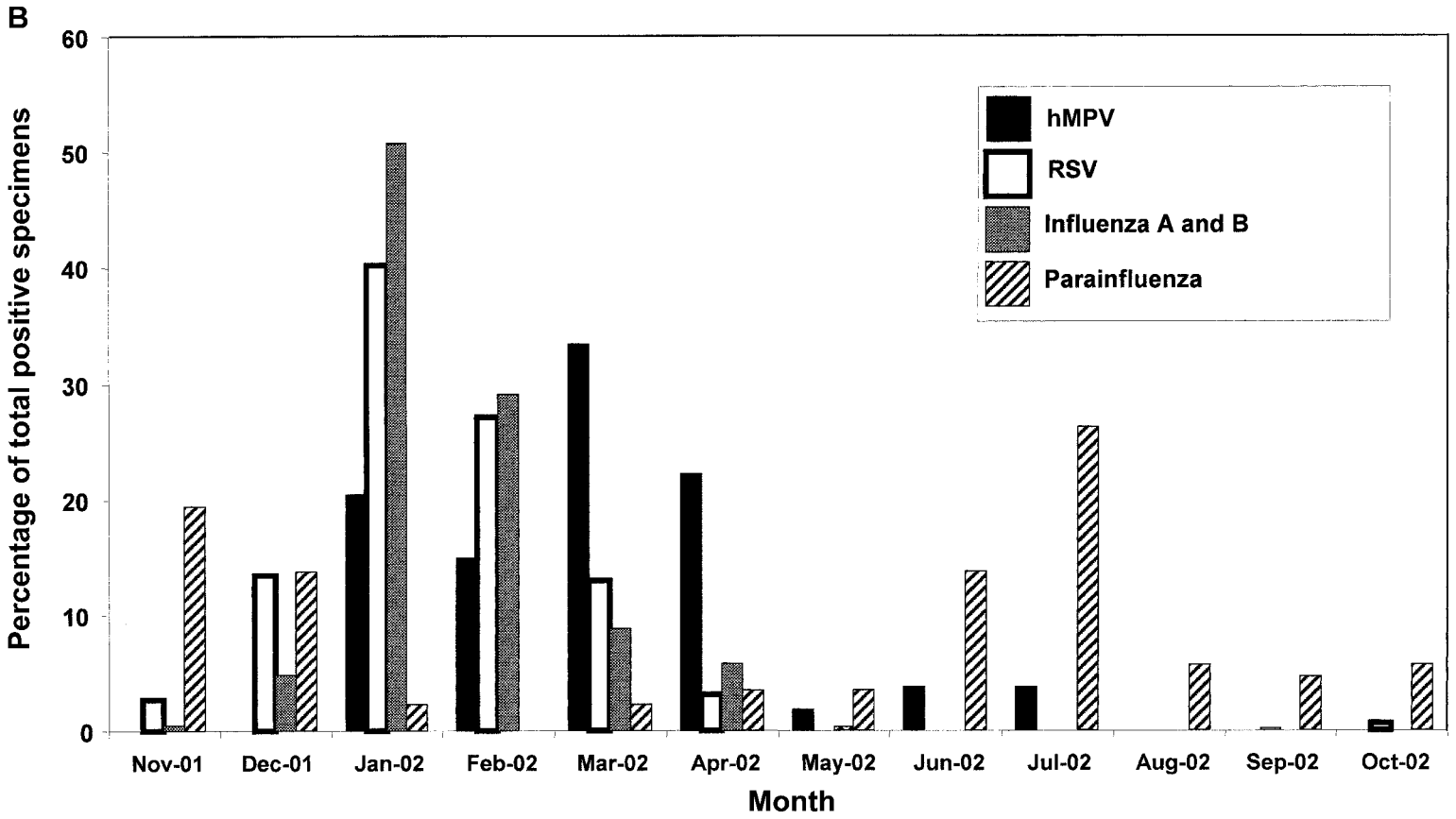

Figure 3. Distribution of human metapneumovirus (hMPV) and other respiratory viruses in New Haven, Connecticut. *A,* Weekly distribution of hMPV genotypes from 1 November 2001 to 31 October 2002. Genotype assignments are indicated in figure 2. Every other week is marked for figure clarity. *B,* Monthly distribution of hMPV, respiratory syncytial virus (RSV), influenza A and B, and human parainfluenza viruses 1–3. The percentage of total positive specimens for each virus during the 1-year period is indicated.

Children with evidence of hMPV had symptoms consistent with either upper or lower respiratory-tract disease or both (table 2). The most-common symptoms included fever (77%), cough (67%), and rhinorrhea (64%). Findings on physical examination included wheeze (51%), retractions (53%), and hypoxemia (38%). Twenty-six patients (49.1%) were admitted to the hospital. A chest radiograph was obtained for 36 patients, 20 (55.5%) of which showed abnormal results. Abnormal findings included focal infiltrates (27.8%), hyperinflation (13.9%), and peribronchial cuffing (11.1%). Nineteen (35.2%) of 54 children had associated comorbidities that included a history of prematurity (<36 weeks gestation), chronic lung disease, complex congenital heart disease, or compromised immunity.

Phylogenetic analysis of hMPV. Phylogenetic analysis based on the hMPV F gene sequences was performed and revealed that at least 2 distinct hMPV genotypes circulated during the study period (figure 2). hMPV F gene sequences from Connecticut were similar to those of strains identified from individuals from The Netherlands, Australia, and Canada [2, 4, 7]. A third potential genotype, which included New Haven isolates 426 and 418, may represent a third distinct genotype. The phylogenetic analysis of the Canadian and Dutch strains was consistent with results published elsewhere [2, 7]. All individuals in whom group A virus was identified were hospitalized.

Seasonal distribution of hMPV. The weekly distribution of hMPV-positive specimens is shown in figure 3*A*. The majority of hMPV-positive individuals were detected during January–April (49 [91%] of 54). Eighteen (17.6%) of 102 specimens tested positive for hMPV in March 2002, and 12 (25.0%) of 48 specimens tested positive for hMPV in April 2002. Two individuals tested positive for hMPV in both June and July. Group B was the predominate genotype observed during the study period. Group A isolates were clustered in March. The monthly distribution of hMPV was compared with that of RSV, influenza (both A and B), parainfluenza, and adenovirus detected during the study period. Figure 3*B* shows the percentage of total specimens with positive test results for each virus by month. RSV and influenza activity peaked in January, before the peak of hMPV activity. Parainfluenza viruses (types 1–3) were detected throughout the year. Data for RSV, influenza, parainfluenza, and adenovirus represent the monthly distributions of specimens obtained from all age groups.

DISCUSSION

We have established that hMPV circulated in Connecticut during 2001–2002. Our data indicate that hMPV has a seasonal distribution. Most of the hMPV-positive individuals were identified during the winter and spring seasons, with the peak of hMPV activity occurring in March and April. These findings are consistent with those of a study from The Netherlands that demonstrated that hMPV had a seasonal distribution [15]. The Dutch study revealed a peak of hMPV activity during December and January, which suggests that the circulation of this virus, like RSV, can vary from location to location [17]. Several other studies have documented the detection of hMPV during the winter and spring months. These studies were limited, however, because respiratory specimens from only a limited number of months were tested [9–12, 14]. The peak of hMPV activity followed the peaks of both RSV and influenza activity, although it is unclear whether this seasonal distribution varies from year to year. The monthly distribution for each seasonal epidemic of RSV can vary within a community [18].

In our study, we chose to test respiratory specimens obtained from children aged <5 years. We chose this age group because the initial reports of hMPV suggested that, by age 5 years, nearly all individuals had serological evidence of hMPV infection. Although our testing was limited to young children, the circulation of the virus in this age group likely parallels its circulation in the community. Other studies of hMPV, which included both children and adults, did not reveal a significant difference in the monthly distribution between the 2 groups [9, 15]. Furthermore, the circulation of respiratory viruses in the pediatric population is often a marker for the circulation within the community [19, 20]. The identification of hMPV during the summer months suggests that low-level circulation of the virus in the community may occur throughout the year. When sensitive methods, such as genome amplification techniques, have been applied to the study of viruses with an apparent seasonal distribution, year-long circulation of the virus often is observed [21].

At least 2 distinct genotypes of hMPV were detected during the study period. Group A isolates were clustered in March. This may represent an outbreak of this genotype in the community. It is unclear whether these genotypes represent distinct serotypes of hMPV or genetic variants of a prototype strain. Isolates NH 462 and NH418 in group A may represent a third distinct genotype. Further analysis with additional hMPV isolates will be required to determine whether these strains represent a distinct genotype. Two genotypes of hMPV have also been found elsewhere [2, 6–8, 12]. The similarity among the hMPV genotypes found in Connecticut, Canada, The Netherlands, and Australia suggests that the epidemiology of hMPV may be similar to that of other paramyxoviruses. Genetically diverse strains of RSV circulate within communities during the same year, and genetically similar strains circulate in different locations in different years [22]. However, the worldwide distribution of several distinct strains of hMPV, as is observed with influenza, cannot be ruled out by the current epidemiological data.

The respiratory specimens tested were submitted to the Clinical Virology laboratory at Yale–New Haven Hospital at the discretion of each individual's medical team. Therefore, these

specimens likely represent individuals with symptomatic hMPV infection. Our study was not designed to evaluate asymptomatic infection or mild disease. However, asymptomatic hMPV infection in infants and young children may be rare. In a study of 400 respiratory specimens from infants without symptoms of respiratory-tract disease in The Netherlands, none tested positive for hMPV [15].

Curiously, all patients in whom genotype group A was identified were hospitalized. Although these individuals represented a small fraction (9.3%) of the hMPV-infected population, this finding may suggest a difference either in the virulence of the 2 genotypes or in the immunity to this genotype within the community. The possibility that genotypes of hMPV differ in virulence is intriguing. We have previously demonstrated that specific RSV genotypes within subgroup A are associated with severity of disease [23]. Study of these viruses in the natural human host has distinct advantages over animal studies and may lead to significant insights into viral pathogenesis.

The clinical features associated with hMPV appear to be similar to those observed with other respiratory viruses, such as RSV and parainfluenza. Symptoms of lower respiratory-tract infection, such as tachypnea, wheezing, and chest-wall retractions, were observed in more than one-half of the children infected with hMPV. Hypoxemia was observed in more than one-third of the children. These findings are consistent with the clinical features in children described elsewhere [2, 7, 11–13]. For children who had a chest radiograph, over one-half had abnormalities. Chest hyperinflation, which reflects small-airway inflammation and air trapping and is frequently observed in RSV disease, was a relatively uncommon finding. This clinical observation suggests that RSV and hMPV may have distinct pathological mechanisms.

Our study was limited to the testing of specimens submitted to a clinical diagnostic laboratory and individuals aged <5 years. Therefore, it is unclear whether the high proportion of disease in children aged <12 months or the high proportion of symptomatic disease in children with underlying comorbidities represents a true epidemiological phenomenon. Active, prospective, population-based testing will be required to further define the epidemiology and spectrum of illness related to hMPV.

To determine the clinical features associated with hMPV, we limited our testing to respiratory specimens in which respiratory pathogens—RSV, influenza A and B, parainfluenza, and adenovirus—were not detected. The role that hMPV plays as a copathogen is unclear. Recent findings about the role of hMPV in RSV disease are conflicting. Greensill et al. [8] reported a high frequency of hMPV in children with severe RSV disease. Because the seasonal distribution of hMPV and RSV appears to overlap, coinfection may be common. However, data from other studies have suggested that dual infection with RSV and hMPV is rare. Defining the length of viral shedding and determining the frequency of asymptomatic hMPV infection is essential in establishing the role that this virus plays in RSV disease. Recently, hMPV has been identified in several individuals with severe acute respiratory syndrome (SARS) [24]. The possibility that hMPV exacerbates disease caused by the SARS coronavirus also requires further investigation.

hMPV-positive specimens were collected from 1 child 54 days apart. This individual had an acute respiratory decomposition at the time both specimens were obtained and had resolution of symptoms after the initial illness. This individual had no known primary immunodeficiency, which is associated with the prolonged viral shedding of other respiratory viruses. Therefore, we considered this patient to have been reinfected with hMPV. However, this individual had a history of prematurity, which may favor the possibility of prolonged viral shedding. Although we cannot discount the possibility of prolonged hMPV shedding, this individual may have been reinfected with a strain belonging to the same genotype as the original virus. Infection with hMPV, like RSV, may not induce protective immunity. Reinfection with RSV in childhood occurs frequently [25]. Further studies are needed to determine the length of viral shedding in hMPV infection and the frequency of reinfection with hMPV.

In conclusion, we have demonstrated that hMPV circulated in Connecticut and is associated with respiratory-tract disease in infants and young children. hMPV activity peaked during the late winter and early spring. Our data strongly suggest that hMPV is a significant pathogen in children aged <5 years. Additional studies are needed to further define the epidemiology of this newly emerging respiratory pathogen.

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