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Detection of Human Metapneumovirus in Hospitalized Children with Acute Respiratory Tract Infection Using Real-time RT-PCR in a Hospital in Northern Taiwan

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Background/Purpose: Human metapneumovirus (hMPV) is a newly discovered respiratory pathogen. This prospective hospital-based study investigated the clinical role and features of hMPV in Taiwan.

Methods: Respiratory specimens collected from hospitalized children with acute respiratory tract infection between September 1, 2003 and April 10, 2005 were screened for metapneumovirus using real-time reverse transcription–polymerase chain reaction (RT-PCR).

Results: During the study period, 930 specimens were obtained from 926 hospitalized children. After exclusion of 200 cases due to lack of clinical evidence of airway infection or diseases with known etiology, 726 were included in the analysis. Among these, 33 children had a positive result for hMPV infection. The majority of these patients were admitted during spring and early summer. Twenty-one (63.6%) were younger than 2 years of age. hMPV accounted for 13.3% of respiratory infections occurring between the ages of 18 and 24 months and was as common a respiratory pathogen as respiratory syncytial virus (RSV) in that age group. The 11 patients (33.3%) with underlying diseases had a similar disease course to those without underlying diseases. A co-pathogen was found in 11 patients (33.3%). Infected children between 2 and 5 years of age had significantly higher titers of hMPV in their respiratory specimens ($10^{3.88}$ copies/ μ L) than children younger than 2 years ($10^{2.26}$ copies/ μ L) ($p=0.013$) and children older than 5 years ($10^{2.25}$ copies/ μ L) ($p=0.005$). hMPV positive cases were significantly older than those with RSV infection ($p=0.002$) and had a shorter duration of hospitalization ($p=0.001$), fewer days of oxygen use ($p=0.001$) and higher levels of C-reactive protein ($p=0.004$).

Conclusion: Metapneumovirus circulates in children in northern Taiwan during spring and early summer. hMPV was the most common respiratory pathogen in children aged between 18 and 24 months hospitalized with acute respiratory tract infection. Real-time RT-PCR is a sensitive method for investigating the epidemiology and diseases associated with hMPV. [*J Formos Med Assoc* 2007;106(1):16–24]

Key Words: children, metapneumovirus, prospective study, real-time RT-PCR, respiratory tract infection

Human metapneumovirus virus (hMPV) is a newly recognized respiratory tract pathogen.¹ It is a new member of the family *Paramyxoviridae*, subfamily *Pneumovirus*. Diseases caused by hMPV are similar to those caused by human respiratory syncytial virus (RSV), the most common cause of viral

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respiratory tract infection in young children.² Patients infected with hMPV manifest symptoms and signs ranging from a wheezing bronchiolitis to respiratory distress requiring assisted ventilation.³ This virus has been shown to infect the majority of children in the Netherlands by the age of 5 years.¹ A seroepidemiology study in Japan revealed that the children had been exposed to hMPV by the age of 10 years.⁴

Because hMPV is difficult to culture, a reliable and rapid diagnostic tool is needed. Reverse transcription–polymerase chain reaction (RT-PCR) has been used to identify this virus in studies of children or adults with respiratory tract infection in Australia, Canada, United Kingdom, France and the United States.^{3,5–8} These studies indicate that this virus may be prevalent worldwide. This study investigated the clinical role and features of hMPV in hospitalized children in a medical center in northern Taiwan. The value of real-time RT-PCR for hMPV detection was also investigated.

Methods

Study design

This study was conducted in the general pediatric wards of a medical center in Taipei, Taiwan. Hospitalized children younger than 15 years old with symptoms and signs of acute respiratory tract infection (ARTI) were recruited. During hospitalization, the children's signs and symptoms were recorded using a standardized form. This information was reviewed and entered into a database. Throat swab/nasopharyngeal aspiration (NPA) samples were collected and cultured for viruses.

Case definitions

Respiratory tract symptoms assessed included cough, rhinorrhea, sore throat, sneezing, or dyspnea. Bronchiolitis was defined as an acute respiratory illness characterized by rhinorrhea, cough, and diffuse wheezes and rales, with peribronchial thickening and hyperexpansion on chest radiograph if available. Pneumonia was defined as dyspnea in a patient with focal rales or decreased

breath sounds and the presence of a focal infiltrate on chest radiograph. Laryngotracheobronchitis (croup) was defined as an acute lower respiratory tract infection characterized by hoarseness, cough, and stridor. Two sets of throat swab/NPA samples were obtained for viral culture from children with symptoms/signs of respiratory tract infection. Young infants (age <3 months) with temperature >38°C without a definite source of infection were not excluded for lack of symptoms/signs of respiratory tract infection. One set of the two specimens was promptly inoculated onto cell-culture monolayers. Aliquots of nasopharyngeal aspirates were snap-frozen and stored at –70°C for molecular analysis. The other set of nasopharyngeal aspirate was tested by direct immunofluorescence assay to detect human RSV (IMAGEN™ Respiratory Syncytial Virus; DakoCytomation, Cambridgeshire, UK), parainfluenza viruses type 1 and 3 (IMAGEN™ Parainfluenza Virus; DakoCytomation), or indirect immunofluorescence assay of antigen detection to detect *Chlamydia pneumoniae* antigen (TWAR) (DakoCytomation). Serology of mycoplasma was performed for patients who presented with atypical pneumonia (good activity, no respiratory distress, with/without pneumonia patch). Positive IgM titer (IgM-specific ELISA, SeroMP IgM; Savyon, Ashdod, Israel) or a four-fold rise of complement fixation titer (Virion, Nurzburg, Germany) in convalescent serum compared with acute serum (1–2 weeks apart) or titer 1:64 for single acute serum were defined as positive. Rapid urine pneumococcal antigen assay (Binax Now; Binax Inc., Portland, ME, USA) was performed if patients presented with pneumonia patch, respiratory distress, and leukocytosis with high C-reactive protein (CRP).

Data collection

Demographic data collected included age, sex, family history, conditions of previous hospitalization, referral or none, and underlying disease. Clinical presentations such as cough, rhinorrhea, fever, dyspnea, vomiting, diarrhea, decreased appetite, lethargy, and conjunctiva infection were recorded, as were the findings of physical

examination and diagnosis. Data including white blood cell (WBC) count, differential count, serum concentration of CRP on admission, and chest X-ray findings were recorded. The hospital course of each patient including the duration of symptoms, the value of peak body temperature, oxygen requirement, the date of specimen collection, necessity for intensive care, ventilator support, and outcome (complication or mortality) were also collected.

Molecular analysis

RNA was extracted using QIAmp viral RNA kits (Qiagen, Hilden, Germany). Real-time RT-PCR was applied to detect viral RNA using a single tube RT-PCR kit according to the manufacturer's instructions (RNA master hybridization probes; Roche Applied Science, Mannheim, Germany). Amplification and detection of RNA were performed with a Lightcycler 2.0 instrument (Roche Applied Science) with one-step RT-PCR. Each capillary glass contained a 10 μ L reaction mixture that included 2.6 μ L sample RNA, 3.75 μ L of an enzyme mixture and a reaction buffer (Lightcycler RNA master mix hybridization probes; Roche Applied Science), 0.5 μ M sense primer, 0.5 μ M anti-sense primer, 3.25 mM Mn [Oac]₂, and 0.2 μ M fluorescein hybridization probes (TIB-MOLBIOL, Berlin, Germany). The primers used in the initial test of the respiratory specimens were based on the sequence data of a Dutch strain available from GenBank (accession no. AF 371367) and targeted the hMPV N gene.² The sense primer, 5'-AACCGTGTACTAAGTGATGCACTC and the anti-sense primer, 5'-CATTGTTTGACCGCCCCATAA, produce an amplicon that corresponds to nucleotides 601–813 of the hMPV N gene. Homology between hMPV and RSV RNA genome is only 20%,¹ hence, it is sufficiently distinct that its sequences do not cross-react with existing nucleic acid amplification assays. Each set of RT and PCR reactions contained appropriate negative controls. The reaction mixtures were exposed to a 20-minute 61°C RT step, 30 seconds of denaturation at 95°C, and followed by 55 cycles of 95°C for 1 second, 60°C for 15 seconds and 72°C for 13 seconds,

using a channel setting of F2/F1. The specificity of the obtained fluorescence signal was confirmed by a melting-curve analysis after each run.

Standard curve

Quantification of hMPV RNA was performed with five 10-fold serial dilutions of a plasmid containing the primer-spanning region. Viral RNA was transformed to complementary DNA (cDNA) using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, USA). Then, the cDNA was transfected into the plasmid with 2728 bps using yT&A TA cloning kit and ECOS™ competent cells (Yeastern Biotech Co. Ltd., Taipei, Taiwan). The top 10 strain competent cells were added to the plasmids containing the hMPV cDNA and then grown in ampicillin (50 μ g/mL) containing selective culture media (yLB agar; Yeastern Biotech Co. Ltd.). After incubating at 37°C for 11–16 hours, a single colony was selected and subjected to restriction digestion using Eco R1 and Bam H1. The plasmid DNA concentration was calibrated by spectrophotometry at 260 nm. Ten-fold serial dilution with RNase free water was done, then real-time RT-PCR was performed. The cycle number correlating to the lowest detectable viral copies (5 copies/ μ L) was around 40 by estimation with N gene hybridization probe.

Statistical analysis

Significant differences between groups were determined by Student's *t* test for comparison of means, and χ^2 or Fisher's exact test for comparison of proportions. Analyses were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA) for Windows. All reported *p* values are two-sided and *p* < 0.05 was considered statistically significant.

Results

The cohort

Between September 1, 2003 and April 30, 2005, 930 specimens were obtained from 926 hospitalized children. Specimens were obtained at a mean of 5.8 days after the onset of symptoms.

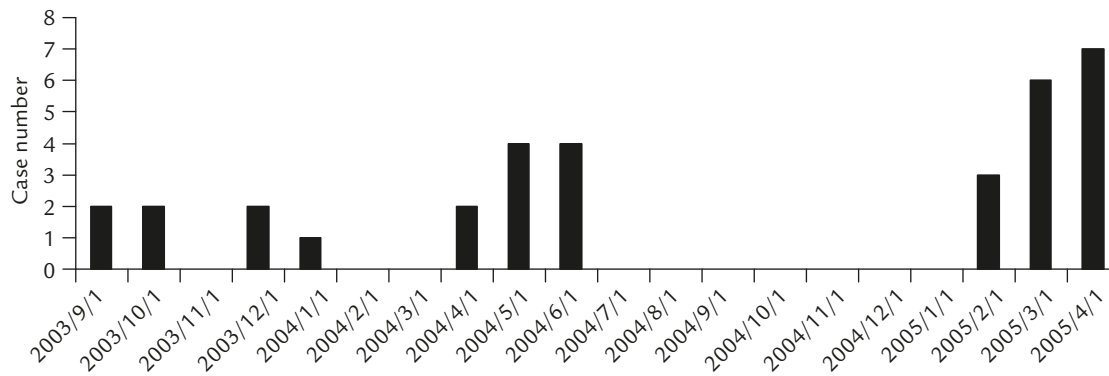


Figure 1. Seasonal distribution of cases of human metapneumovirus acute respiratory tract infection during the 20-month study period.

Two hundred cases with no clinical evidence of airway infection or diseases with known etiology were excluded from further analysis. These included patients suffering from diseases such as herpangina, hand-foot-mouth disease, encephalitis, acute lymphadenitis, or acute gastroenteritis. The PCR results for hMPV were negative for all of these excluded cases. The eligible cohort included 726 different hospitalizations/specimens (M:F=422:304). Respiratory specimens from a total of 33 (4.55%) patients with ARTIs were documented to harbor hMPV.

Seasonal distribution of hMPV infection

hMPV infections occurred year round with a peak around late spring to early summer (Figure 1) during the 20-month study period.

Clinical manifestations of hMPV infection in children

The clinical characteristics of the 33 children (M:F=23:10) with hMPV infection are listed in the Table. Cough was the leading symptom (97%) followed by fever (78.8%) and rhinorrhea (60.6%). Half of the children had dyspnea (48.5%). Diarrhea (15.2%), vomiting (12.1%), rash (6.1%), infection of conjunctiva (6.1%) and sore throat (6.1%) were also noted. Evidence of either upper or lower respiratory tract infection or both including rhonchi (48%) and rales (55%) was noted on auscultation in half of the children; 40% of the children had wheezing, one-third had chest wall retraction (33%) and stridor was audible in 21%.

The median age at admission was 20 months old and 21 patients (63%) were younger than 2 years. Other family members were also suffering from acute illness in 42.3% of the hMPV-infected children. Most patients were discharged uneventfully after a mean duration of hospitalization of 4.7 days. One 6-month-old girl with thoracic dystrophy needed intensive care for 4 days but without intubation. Nevertheless, oxygen therapy was needed in half of children (17/33, 51.5%), with a mean duration of therapy of 3.7 days. Chest X-ray was abnormal in 81.8% of the children with findings including emphysematous change, infiltration, patch and steeple signs. Antibiotics were prescribed in 57.6% of the children for clinical evidence of otitis media in five, sinusitis in two, and suspicion of bacterial pneumonia in 12.

The clinical diagnoses at the time of admission and discharge were as follows: pneumonia (45%), bronchiolitis (21%), acute otitis media (15%), nasopharyngitis (12%), asthma (6%) and sinusitis (6%). Notably, some patients had more than two diagnoses.

Age distribution of hMPV positive cases

Ninety percent of the hMPV positive children were <5 years old. Furthermore, 24.2% of hMPV positive children were aged between 18 and 24 months, while the proportion of those aged 18–24 months was only 8.1% ($p < 0.001$). hMPV was the leading pathogen among children with ARTI aged 18–24 months (13.1%, 8/61), as important

Table. Comparison of demographic and clinical characteristics of hospitalized children with human metapneumovirus (MPV) infections and those with human respiratory syncytial virus (RSV) infections*

	MPV positive (n=33)	RSV positive (n=91)	p
Age (mo)	31.8 ± 30.7	13.1 ± 13.7	0.002 [†]
Sex (M/F)	23/10	58/33	0.538
Underlying disease	12 (36.4)	30 (33.0)	0.724
Duration of hospitalization (d)	4.7 ± 2.0	6.2 ± 2.9	0.001 [†]
ICU care	1 (3.0)	13 (14.3)	0.11
O ₂ use	17 (51.5)	70 (76.9)	0.006 [†]
Duration of O ₂ use (d)	1.9 ± 2.2	3.9 ± 3.2	0.001 [†]
Intubation	0	2 (2.2)	1.000
Fever	26 (78.8)	68 (74.7)	0.641
Fever duration (d)	3.6 ± 3.0	4.2 ± 3.7	0.441
Cough duration (d)	14.5 ± 7.1	14.0 ± 8.5	0.765
Adult family history of ARIs	10/27 (37.0)	44/78 (56.4)	0.083
Abnormal chest X-ray	27 (81.8)	72 (79.1)	0.487
Co-pathogen identified	12 (36.4)	20 (22.0)	0.106
Differential count (%)			
Segment	54.1 ± 20.0	35.7 ± 19.3	0.000 [†]
Lymphocyte	31.4 ± 18.1	52.5 ± 31.5	0.001 [†]
C-reactive protein (mg/dL)	2.73 ± 3.62	0.76 ± 1.2	0.004 [†]

*Data presented as mean ± standard deviation or n (%); [†]p < 0.05 (χ^2 test). ICU = intensive care unit; ARIs = acute respiratory infections.

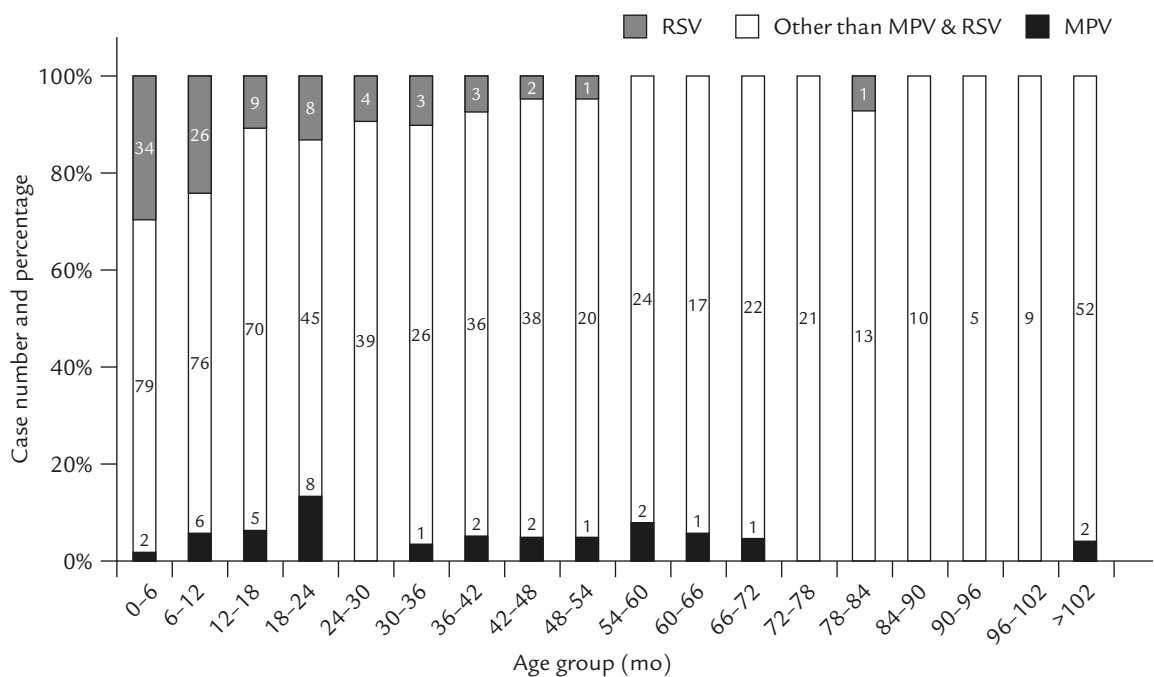


Figure 2. Proportional distribution of human metapneumovirus (MPV) and human respiratory syncytial virus (RSV) infections in different age groups.

as RSV (13.1%, 8/61) (Figure 2). In contrast to hMPV, RSV was more prevalent in infants (0–12 months old, $p < 0.001$); 26.9% of infants with ARTIs were RSV positive. hMPV was an important etiology in hospitalized children < 2 years of age, accounting for 5.7% of ARTIs.

Underlying diseases in hMPV positive cases

Eleven (33.3%) children infected with hMPV had underlying diseases, including biliary atresia with liver cirrhosis in one, intestinal lymphangiectasis with protein losing enteropathy in one, corrected transposition of great arteries with pulmonary stenosis in one, ventricular septal defect with pulmonary stenosis in one, vertebral, anorectal, cardiac, tracheal, esophageal, renal, limb (VACTERAL) association in one, hepatomegaly in one, Prader-Willi syndrome in one, Jeune thoracic dystrophy in one and prematurity in three. There was no significant difference between hMPV infected children with or without underlying disease with regard to hospitalization course, duration of oxygen requirement, duration of fever, and CRP level.

Pathogens other than hMPV

hMPV was the sole pathogen in 22 (66.7%) cases. Other pathogens were detected in 11 cases, including adenovirus, RSV, enterovirus, coronavirus NL-63, *M. pneumoniae* and *S. pneumoniae*. Co-infection did not significantly impact the hospitalization duration, fever duration, WBC count, or CRP levels of patients with hMPV infection.

Quantification of MPV in respiratory specimens

The mean viral titer of the 33 positive specimens was $10^{2.65}$ copies/ μ L (range, $10^{0.5}$ to 10^5). The mean titer of the 22 specimens for which hMPV was the only respiratory virus detected was $10^{2.66}$ copies/ μ L compared to $10^{2.64}$ copies/ μ L in the 11 dually infected samples ($p = 0.968$). Viral loads of hMPV in respiratory specimens were not significantly associated with factors including sex, underlying disease, fever, family history, and oxygen therapy (Figure 3). Children between 2 and 5 years of age had significantly higher hMPV titer in their respiratory specimens (mean, $10^{3.88}$ copies/ μ L) than children < 2 years old (mean,

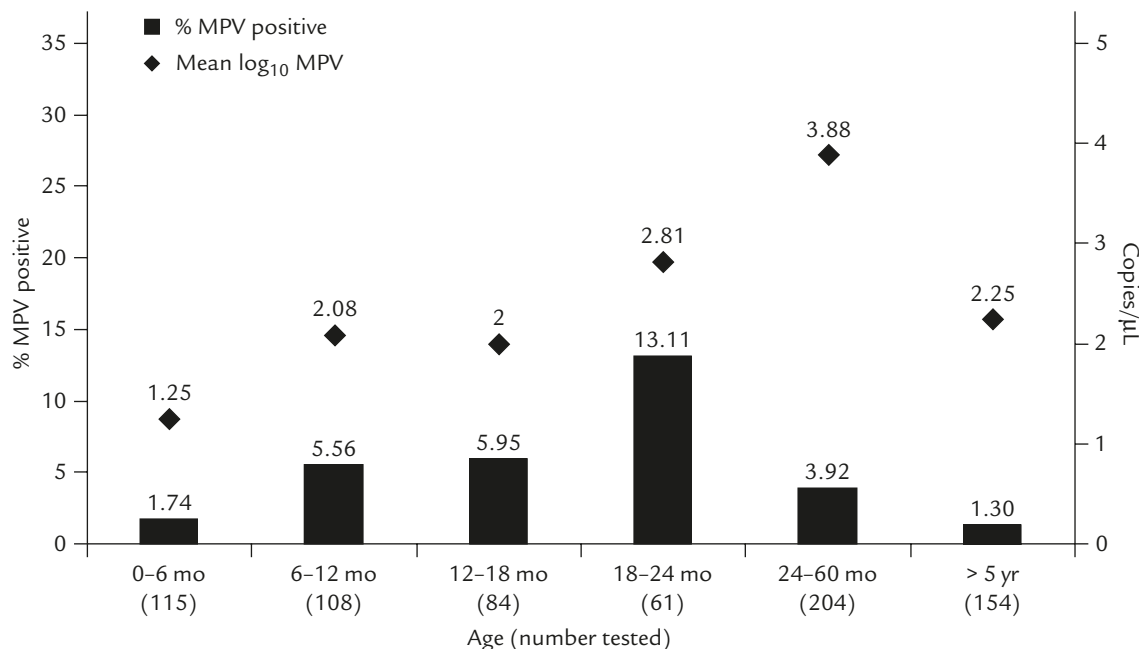


Figure 3. Proportion of specimens positive for metapneumovirus (MPV) and the mean number of MPV copies, expressed as \log_{10} copies/ μ L, were determined among respiratory specimens collected from patients belonging to each of six age groups.

$10^{2.26}$ copies/ μL) ($p=0.013$) and children > 5 years old (mean, $10^{2.25}$ copies/ μL) ($p=0.005$).

Comparison with RSV infection

Two children with RSV infection required intubation and respiratory support while none of the hMPV infected children did (Table). No other complications were observed in these two groups. Compared to RSV infected children, hMPV infected children were older ($p=0.002$), had shorter duration of hospital stay ($p=0.001$), shorter duration of oxygen requirement ($p=0.001$) and higher level of CRP ($p=0.004$). Similar rates of identification of co-pathogens were observed in these two groups.

Discussion

ARTIs are major causes of morbidity and mortality in children. Even after extensive laboratory investigations, about half of ARTIs have no identifiable etiologic agent.^{9,10} hMPV is a newly discovered viral pathogen and its clinical epidemiology and impact have yet to be fully defined. This virus has been shown to be circulating in many Asian and Western countries.^{3-8,11-13} The present study found that 4.6% of children hospitalized due to ARTIs at a medical center in northern Taiwan were infected with hMPV. This rate is similar to what has been reported in other countries, including 3.9% in the United States,¹³ 5.5% in Hong Kong,¹² and 6.2% in Italy.^{10,11}

Real-time PCR has become an important tool in the study of infectious diseases, especially for a newly identified virus that is difficult to grow in cell culture. A study by Mackay et al showed that real-time RT-PCR improved the detection of virus compared with conventional RT-PCR.² Although identification of viral nucleic acid in respiratory specimens does not prove that hMPV is responsible for patients' illnesses, the association between respiratory tract illness and presence of the virus suggests a causative role.¹³ Furthermore, hMPV does not appear to be associated with asymptomatic carriage in the nasopharynx since

no hMPV was found in respiratory samples from asymptomatic children < 2 years old.¹ In addition to its improved sensitivity and specificity, RT-PCR specific for the N gene has been shown to be more sensitive than RT-PCR for genes L, F, M and P of hMPV.^{9,14}

The seasonal occurrence of hMPV infection largely overlaps with that of RSV infections. hMPV is considered to be a pathogen from December till May in North America and the United Kingdom.^{3,8,15} In the present study, hMPV infection peaked in late spring and early summer. Similar seasonal patterns have been observed in Hong Kong, Canada and Japan.^{12,16,17} A study from Tennessee noted that infections began in March and peaked in May, with some disease activity in June and September, which was quite similar to our results.¹⁸ In the present study, sporadic hMPV infection also occurred outside of the peak seasons during the 20-month study period. Possible reasons for the seasonal pattern may include multiple circulating strains of hMPV, climate change, and demographic factors. Phylogenetic study and long-term prospective study are needed to clarify the responsible factors.¹⁰

Previous studies showed that co-infection of RSV or other respiratory pathogens with hMPV occurred in 10–75% of subjects.^{10,19-21} Co-infecting viral agents found in hMPV-infected symptomatic individuals include adenovirus, RSV, influenzae A and B, and measles.^{5,22} Among these, dual infections with hMPV and RSV seem to increase disease severity.^{19,20} In this study, one-third of the hMPV-infected cases had other pathogens identified during the episode of ARTIs. There was no significant difference in severity of infection, however, between patients with dual infections and single hMPV infection. Furthermore, the viral titers of hMPV in specimens from patients with single infection were equal to those of patients with dual infection. We could not confirm whether dual infection with hMPV and RSV is more severe because there was only one such case in our cohort. Nevertheless, comparison of characteristics between patients with single RSV and hMPV infections showed that RSV infected younger children,

led to a more prolonged hospitalized course and higher oxygen demand.

Kuypers et al reported that children aged 7–12 months were most likely to be infected with hMPV and had significantly higher titers of hMPV in respiratory specimens.²¹ However, in the present study, children aged 18–24 months had the highest prevalence, but children aged 2–5 years had the highest viral load. The present study and Kuyper et al's study are not directly comparable since their series included both inpatients (76%) and outpatients (24%), while we studied inpatients only. Overall, the data suggest that hMPV may behave differently in different regions.

Thanasugarn et al reported progression to respiratory distress in two hMPV-infected children who had pre-existing bronchopulmonary dysplasia and truncus arteriosus status post total correction.²³ These cases suggest that hMPV can cause life-threatening disease, particularly in high-risk groups or immunocompromised hosts.²⁴ Nevertheless, among the one-third of cases with underlying diseases in the present study, no severe complications were noted.

This study was limited to screening respiratory samples collected from hospitalized patients. Hence, we could not determine the population-based incidence and prevalence. Likewise, sensitivity and specificity of real-time RT-PCR could not be analyzed as virus culture was not used as a gold standard. Recently, four distinct genetic lineages (A1, A2, B1 and B2 lineages) of hMPV have been described.²⁵ Each year, the prevalences of infections caused by these four lineages of hMPV have varied.¹⁰ The primers used in this study might miss some viruses of the hMPV B lineage. Hence, this study may have underestimated the prevalence of hMPV infection.

In summary, this study has confirmed that hMPV does circulate in Taiwan, accounting for 4.6% of ARTIs in hospitalized children. The outcomes of the infected children were good without complications. Real-time RT-PCR is a sensitive and effective diagnostic tool for identifying hMPV infection.

Acknowledgments

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