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ORIGINAL ARTICLE

Clinical features of different genotypes/genogroups of human metapneumovirus in hospitalized children

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Background/Purpose(s): To explore the clinical features of different human metapneumovirus (hMPV) genotypes/genogroups in hospitalized children.
Methods: From January 2005 to April 2010, 3313 children's respiratory specimens sent for the detection of respiratory syncytial virus antigen were also tested for hMPV by real time-polymerase chain reaction. Demographics, clinical presentations, and laboratory findings of patients infected with different genotypes/genogroups of hMPV were compared.
Results: A total of 725 samples were positive for hMPV (positive rate, 23%). The F gene was sequenced for 279 isolates; of these, genotype A was identified in 51% (A1, 6.1%; A2, 45%) and genotype B in 49% (B1, 19%; B2, 30%). Medical records of 152 hospitalized children were reviewed. Co-infection with other pathogens was 25.7% (39/152). Excluding co-pathogens other than respiratory syncytial virus, a total of 124 children were analyzed. The most common symptoms included cough, fever, rhinorrhea, wheezing and respiratory distress with accessory muscle usage. The main diagnosis was bronchiolitis. The most common chest radiographic findings were increased perihilar infiltrates. No significant difference was found in terms of demographics, clinical manifestations, and laboratory findings among the children infected with different serogroups of hMPV.
Conclusion: hMPV accounted for a substantial proportion of hospitalized children with lower respiratory tract infection with a high co-infection rate. The A2 subgroup was the most frequently

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observed, followed by B2. No significant difference was found among patients infected with different genotypes/genogroups of hMPV in terms of clinical manifestations.
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Introduction

Human metapneumovirus (hMPV) is a newly identified pathogen associated with acute respiratory tract infection first discovered by van den Hoogen et al[1] in 2001. Afterward, hMPV was found worldwide. Studies from Europe, North America, and Asia have indicated hMPV can cause respiratory tract infection in young children, ranging from upper respiratory tract infection to pneumonia or bronchiolitis. The clinical manifestations are very similar to those caused by respiratory syncytial virus (RSV) infection and difficult to be discriminated. According to the literature review, the positive rate of hMPV among respiratory specimens obtained from patients with respiratory illnesses ranged from 2% to 26%,[1–4] indicating hMPV an important respiratory pathogen.

Human metapneumovirus is an enveloped, single-strand nonsegmented negative-sense RNA genome of the Paramyxoviridae family. According to phylogenetic analysis of sequences obtained for part of the fusion protein gene and the complete attachment protein gene, hMPV can be classified into two major genotypes, namely A and B. Each of the genotypes can be subclassified into two minor subgroups, namely A1, A2, B1, and B2,[5,6] defined as genogroups. Different genotypes circulate worldwide each year. The role of different genogroups is not clear in human infection. The information of epidemiology, genogroup distribution and clinical characteristics of hMPV infections in Taiwan has been quite limited. Furthermore, there are scant reports focusing on the comparison of clinical characteristics among different genogroups of hMPV infection. We had surveyed and analyzed molecular epidemiology of hMPV from 2005 to 2010 in northern Taiwan.[7] To understand the clinical characteristics of different hMPV genotypes among the hospitalized children better, we conducted this study.

Materials and methods

Study design

The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital. From January 2005 to April 2010, 3313 respiratory secretion specimens (nasopharyngeal secretions, bronchoalveolar lavage, or sputum) from children, age <6 years, with suspected RSV infection sent for RSV antigen detection in CGMH were additionally tested for hMPV by real time-polymerase chain reaction (RT-PCR). Demographic data, including age, sex, and underlying diseases, were collected. Clinical information, such as fever, cough, rhinorrhea, vomiting, diarrhea, pharyngitis, decreased appetite, wheezing, respiratory distress, laboratory data, chest radiograph, hospital duration, and ventilator usage, were retrospectively reviewed from patients' medical records. The radiological finding was reported by a pediatric radiologist and was further reviewed by one of the authors (H.Y. Wei). The clinical diagnosis was reclassified according to the chest radiograph: pneumonia was defined as lower respiratory symptoms with at least one focal patch density; bronchopneumonia was defined as increased infiltration on both perihilar and peribronchial areas, especially for those older than 24 months; bronchiolitis was defined as negative finding of the chest X-ray or only slightly increased infiltration among patients younger than 24 months. Patients co-infected with bacteria or respiratory viruses other than RSV, such as influenza virus, and parainfluenza viruses (PIVs), were excluded.

Immunofluorescent assay for detecting RSV antigen

Respiratory specimens, mostly nasopharyngeal secretions, were collected by suction and transported to the laboratory on ice. The specimens were diluted with 2 ml of sterile phosphate-buffered saline (PBS), and the mucus was broken with a Pasteur pipette. The specimens were centrifuged at 1500 rpm (219*g*) for 10 minutes at 4°C. The supernatant fluid was discarded and the pellet was resuspended in sufficient PBS to yield a visibly turbid suspension. The cell suspension was spotted onto a 2-well glass slide and air dried and then fixed in cold (4°C) acetone for 10 minutes. The slides were stained with anti-RSV monoclonal antibody conjugated with FITC (D3 Ultra DFA Respiratory Virus Screening and ID Kit) at 37°C in a humidified chamber for 30 minutes and then washed three times in PBS, 10 minutes each. The slides were rinsed with PBS (pH 7.2) and then washed three times in PBS for 10 minutes, dried and mounted with mounting medium. The slides were read under a fluorescence microscope at ×400 magnification. The presence of RSV antigen was indicated by the appearance of intracellular cytoplasmic inclusion-like bodies or particles fluorescing an apple-green color, in one or more respiratory epithelial cells.

RNA extraction

Viral RNAs were extracted from 140 μl of specimens using a QIA amp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The RNA was eluted into 30 μl elution buffer. The resulting RNA (8 μl) was subjected to cDNA synthesis using 50 ng random hexamer primer, 500 nM dNTPs and performed at 65°C/10 minutes, 4°C at least 1 minute. We applied reverse transcriptase mixture with equal volume of reverse transcriptase buffer, 10 mM dithiothreitol, 40 units RNaseOut and 200 units

Table 1 Primers and probe sequence for detection of hMPV

Primer	Sequence (5′ to 3′)	Size	Position
Forward	CATAYAARCATGCTATATTAAAAGAGTCTC	162 bp	2478—2497
Reverse	CCTATYTCWGCAGCATATTTGTARTCAG		2558—2537
Probe	5′-FAM-AATGATGARGGTGTCACTG-MGBNFQ-3′		2500—2528

SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and performed at 25°C for 10 minutes, 50°C for 60 minutes, 85°C for 5 minutes on BioMetra Thermocycler (Biometra, Göttingen, Germany).

RT-PCR detection of hMPV

RT-PCR was done using the CFX96 (Bio-Rad, Hercules, CA, USA) system with iTaqSupermix. Amplification is performed in a total volume of 20 μl containing each two 10 μl of iTaqSupermix RT-PCR Master mix, 400 nM of each primer (Table 1), 1 μM probe, 3.6 μl diethylpyrocarbonate water and 4 μl cDNA template. The RT-PCR program was: 95°C for 3 minutes, and 45 cycles with 95°C for 3 seconds, and 60°C for 30 seconds.

Sequencing of fusion protein gene

The primers (Table 2) were used to generate the amplicon of fusion protein gene segments with *Pfu* DNA polymerase (Toyobo, Osaka, Japan). Briefly, the PCR master mix contained 10 μl of cDNA, 10 μl PCR buffer, 200 μM dNTPs, 1 mM $MgSO_4$, 600 nM of each primer and 1 unit *Pfu* DNA polymerase. The PCR were perform as following conditions: 94°C for 2 minutes and 40 cycles of 94°C for 1 minute, 58.7°C for 1 minute, 68°C for 2 minutes and ended with 68°C for 10 minutes. PCR products of F gene were sequenced using BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocols. The F gene sequences were edited and assembled using DNASTAR Lasergene 5.08 (Bioinformatics Pioneer DNASTAR, Inc., Madison, WI, USA). The fusion protein gene (F427 or F141) was sequenced for those with sufficient sample to determine the genogroups. Patients with genogroup typing confirmed by F427 were chosen for better specificity, because of the larger number of base pairs compared to F141 (Table 2).

Statistical analysis

Significant differences between groups were determined by Kruskal-Wallis Test for comparison of medians, and χ^2 or Fisher's exact test for comparison of proportions. Analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) for Windows. All reported p values are two-sided and $p < 0.05$ was considered statistically significant.

Table 2 Primer pairs for fusion protein gene sequence

Primer	Sequence (5′ to 3′)	Size	Position
F427-forward	ATAACACCAGCAATATC	427 bp	637—653
F427-reverse	GATATGTTRATGTTGCA		1064—1038
F141-forward	GCTTCAGTCAATTCAACAG	142 bp	575—593
F141-reverse	CCTGCAGATGTTGGCATGT		716—697

Results

A total of 725 samples from 3313 samples were positive for hMPV, with a positive rate of 23%. F gene was sequenced in 279 isolates (279/725, 38%), and genotype A was identified in 51% (A1, 6.1%; A2, 45%) and genotype B in 49% (B1, 19%; B2, 30%). The medical records of 152 patients who admitted to Linko Chang Gung Memorial hospital were reviewed. Of the 152 patients, the rate of co-infection with other pathogen was 25.7% (39/152), as shown in Fig. 1. Other viruses were identified in 28 children (18.4%): RSV in 13, PIVs in two, influenza A virus in three, influenza virus B in one, adenovirus in three, rotavirus in three, and enterovirus in two. Co-infections with bacteria were documented in 13 patients (8.6%): bacteremia due to *Staphylococcus aureus* in two; clinically significant sputum culture, including *Streptococcus pneumoniae*, *Haemophilus influenza*, *Moraxella* spp., *or Klebsiella pneumoniae*, in six; *Mycoplasma pneumonia* infection proved by serology tests in two; urinary tract infection due to *Escherichia coli*, *Salmonella* enteritis and scarlet fever for one each. Two patients with coinfection of bacteria also had RSV infection; one had *Salmonella* gastroenteritis and the other had *K pneumoniae* yielded from the sputum culture. Excluding the co-infections with bacteria or

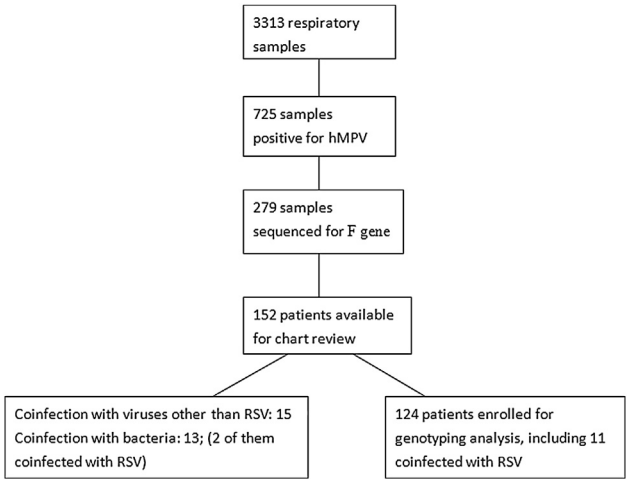

Figure 1. Flow diagram showing the sample numbers, patient numbers of hMPV identified, patient numbers of F gene successfully sequenced, the number of co-infections with other pathogens and the number of patients enrolled for analysis for different genogroups.

Table 3 Clinical comparisons of the four genogroups of human metapneumovirus infection

Genotypes/genogroups Number (%)	A			B			*p* value	
	A ($n = 61$)	A1 ($n = 14$)	A2 ($n = 47$)	B ($n = 63$)	B1 ($n = 19$)	B2 ($n = 44$)	A vs. B	A1/A2/B1/B2
Male gender	39 (63.9)	10 (71.4)	28 (59.6)	44 (69.8)	12 (63.2)	32 (72.7)	0.312	0.571
Median age (mo)	13.5	14.1	13.5	12.9	14.1	11.3	0.457	0.529
Fever	52 (85.2)	12 (85.7)	40 (85)	51 (81.0)	18 (94.7)	33 (75.0)	0.819	0.469
Fever (d)	3.9	3.7	3.8	3.9	4.0	3.8	0.920	0.868
Temperature (median, °C)	39.03	38.92	39.04	39.3	39.00	38.81	0.359	0.682
Cough	60 (98.4)	14 (100)	46 (98)	61 (96.8)	18 (94.7)	43 (97.7)	1.000	0.460
Rhinorrhea	41 (67.2)	10 (71.4)	31 (67)	50 (79.3)	16 (84.2)	34 (77.3)	0.074	0.142
Vomiting	19 (31.1)	3 (21.4)	16 (35)	26 (41.3)	6 (31.6)	20 (45.5)	0.514	0.289
Diarrhea	15 (24.6)	3 (21.4)	12 (25)	14 (22.2)	3 (15.8)	11 (25.0)	0.787	0.776
Pharyngitis	27 (44.3)	6 (42.8)	21 (46)	33 (52.4)	12 (63.2)	21 (47.7)	0.516	0.601
Wheezing	34 (55.7)	10 (71.4)	24 (50)	33 (52.4)	13 (68.4)	20 (45.4)	0.127	0.116
Respiratory distress	18 (29.5)	4 (28.5)	14 (29)	23 (36.5)	9 (47.4)	14 (31.8)	0.711	0.619
Intubation	0 (0)	0 (0)	0 (0)	1 (1.6)	0 (0)	1 (2.3)	1.000	0.641
ICU admission	2 (3.3)	0 (0)	2 (4)	3 (4.8)	1 (5.3)	2 (4.5)	1.000	0.738
Leukocyte ($\times 10^9$ cells/L)	10.14	8.39	10.78	10.52	11.72	10.04	0.660	0.561
CRP (mg/L)	23.13	28.08	22.08	16.19	18.17	15.50	0.456	0.426
Admission (d)	4.98	4.21	5.24	4.98	4.94	5.00	0.211	0.607
Focal consolidation on CXR	10 (16.4)	1 (7.1)	9 (19)	5 (7.9)	2 (10.5)	3 (6.8)	0.201	0.528
RSV co-infection	4 (6.6)	1 (7.1)	3 (6.4)	7 (11.1)	1 (5.3)	6 (13.6)	0.202	0.081
Diagnosis								
Bronchiolitis	42 (68.9)	12 (85.7)	30 (63.8)	53 (84.1)	15 (78.9)	38 (86.3)	0.056	0.062
Bronchopneumonia	8 (13.1)	1 (7.1)	7 (14.9)	4 (6.3)	1 (5.3)	3 (6.8)	0.224	0.493
Pneumonia	10 (16.4)	1 (7.1)	9 (19.1)	6 (9.5)	3 (15.8)	3 (6.8)	0.284	0.304
URI[a]	1 (1.6)		1 (2.1)					

[a] There was only one patient in the URI group, so no *p* value is shown.
The statistical comparison between genotypes A and B, as well as among genogroups A1, A2, B1 and B2 were determined by the χ^2 test.
CRP = C-reactive protein; CXR = chest X-ray; ICU = intensive care unit; RSV = respiratory syncytial virus; URI = upper respiratory tract infection.

viruses other than RSV, clinical data from a total of 124 children were collected and analyzed.

The clinical characteristics of the 124 children with hMPV infection are shown in Table 3. The median age was 13.0 months (ranging from 8 days to 5 years and 9 months) and 105 patients (84.7%) were younger than 2 years. The male:female ratio was 2:1. Underlying diseases were noted in 21 (16.9%) children and included prematurity with gestational age <32 weeks in eight patients, congenital heart disease in six (also had chromosome anomaly with Dandy walker variant and mitochondrial encephalopathy, complete agenesis of *corpus callosum*, Pierre Robin syndrome, and DiGeorge syndrome for one each), gastroesophageal reflux disease in five, asthma in two, and cerebral palsy and infantile spasm in one.

The three most common symptoms were cough (97.6%), fever (83.9%), and rhinorrhea (73.4%). The mean duration of fever was 3.9 days. Half of the children had wheezing (54.0%) and one-third had respiratory distress with accessory muscle usage (33.1%). The most common chest radiologic findings were increased perihilar/peribronchial infiltration (76.6%) and focal patch were seen in 15 (12.1%) patients. The major clinical diagnosis was bronchiolitis/bronchopneumonia (86.3%). Oxygen therapy given by a hood was routine in our hospital and was applied to almost all children (86.3%). Most patients were discharged uneventfully after a mean duration of 4.98 days.

Compared with those without RSV co-infection, clinical characteristics of the patients with RSV co-infection were not significantly different, including demographics and clinical manifestations (Table 4).

Of the 122 children with complete blood counts measurements, leukopenia (white blood cells $<5 \times 10^9$/L) was noted in 6 patients (4.9%) and leukocytosis (white blood cells $>15 \times 10^9$/L) in 16 patients (13.1%). Of the 120 children with serum C-reactive protein (CRP) level measurements, 72 (60.0%) had a serum CRP level within normal range (<10 mg/L) and 14 (11.7%) had a CRP level >50 mg/L.

Five children, three girls and two boys, were ever admitted to the intensive care units. The median age was 12.18 months. Three of them have underlying disease and included DiGeorge syndrome with ventricular septum defect in one, Dandy walker variant with mitochondrial encephalopathy, heart structure anomaly (coarctation of aorta, patent *ductus arteriosus*, atrium, and ventricular septum defect) in one and perinatal asphyxia sequelae in one. The main reason for these five patients requiring intensive care was respiratory distress with hypoxemia under a 35% to 40% oxygen hood. One patient needed ventilator support and was intubated. Respiratory distress was improved by high flow mask or hood for the other four patients. The median duration of ICU stay was 6 days.

Table 4 Comparisons of clinical characteristics between human metapneumovirus-infected children with and without respiratory syncytial virus co-infection

Characteristics	Total ($n = 124$)	%	Without RSV ($n = 113$)	%	RSV co-infection ($n = 11$)	%	p
Male gender	82	66.1%	76	67.3%	6	54.5%	0.506
Median age (mo)	13.0		12.9		16.37		0.461
Fever	104	83.9%	95	84.1%	9	81.8%	0.789
Fever (d)	3.9		3.9		3.44		0.983
Temperature (median, °C)	39.00		39.00		38.99		
Cough	121	97.6%	111	98.2%	10	91.0%	1.000
Rhinorrhea	91	73.4%	82	72.7%	9	81.8%	1.000
Vomiting	45	36.3%	38	33.6%	7	63.6%	0.120
Diarrhea	29	23.4%	28	24.8%	1	9.1%	0.443
Pharyngitis	60	48.4%	57	50.4%	3	27.3%	0.307
Wheezing	67	54.0%	64	56.6%	3	27.3%	0.156
Respiratory distress	41	33.1%	36	31.9%	4	36.4%	0.726
Intubation	1	0.8%	0	0%	1	9.1%	0.087
ICU admission	5	4.0%	4	3.5%	1	9.1%	1.000
Leukocyte ($\times 10^9$ cells/L)	10.33		10.37		9.95		0.172
CRP (mg/L)	19.86		20.20		8.12		0.560
Admission (d)	4.98		4.92		5.64		0.309
Focal consolidation on CXR	15	12.1%	13	11.5%	2	18.2%	0.623
Diagnosis							
Bronchiolitis	95	76.6%	87	77.0%	8	72.7%	0.7161
Bronchopneumonia	12	9.7%	11	10%	1	9.1%	0.9521
Pneumonia	16	12.9%	14	12%	2	18.2%	0.8321
Upper respiratory infection	1	0.8%	1	1%	0	0%	0.7551

CXR = chest X-ray; CRP = C-reactive protein; ICU = intensive care unit; RSV = respiratory syncytial virus.

The comparison of demographics and clinical characteristics of the patients with different genotypes as well as genogroups is shown in Table 3. No statistically significant difference was found among the children with different genotypes as well as genogroups of hMPV infection in terms of demographics, clinical manifestations, and laboratory data, although focal patch on chest radiographs was more frequently seen in patients with genotype A than those with genotype B (16.4% vs. 7.9%).

Discussion

Results from the present study indicate that between 2005 and 2010, hMPV accounted for nearly 25% of the hospitalized children with lower respiratory tract infection (LRTI) in northern Taiwan. Co-infections with bacteria or other viruses were frequently seen, with a rate of at least 25.7%. The most common diagnosis of LRTI caused by hMPV among hospitalized children was bronchiolitis and accounted for 76.6%. The A2 subgroup was the most frequently observed, accounting for more than 40%, followed by B2, which was consistent with the previous report from Taiwan.[8] No significant differences were found among patients infected with different genotypes in terms of demographics and clinical symptoms and signs.

There have been four previous studies focusing on the correlation between genotypes and disease severity in young children.[9–13] Similar to the current study, none of the investigators identified any significant difference among patients infected with different genotypes in terms of clinical characteristics. Vicente et al[12] indicated that hMPV genotype A may be more virulent than genotype B. Pitoiset et al[11] found that more abnormal findings on chest X-ray, such as bronchial wall thickening, alveolar consolidation, perihilar haziness, or thoracic distension, were noted in patients infected with serotype B than A (44% vs. 15%, $p < 0.01$), while in the present study, we found more focal consolidation in patients infected with serotype A than B. The difference between each genotype as well as genogroup was believed to be subtle, unless the case number is large enough. Compared to other studies, the percentage of fever in the present study (83.9%) was apparently higher, which may be explained by the different inclusion criteria and only hospitalized children were included in the present study.

The viral co-infection rate in the present study was 22.5%. Since virus isolation as well as molecular diagnostic methods for respiratory viruses other than hMPV were not performed for each patient, the viral co-infection rate was apparently underestimated. No significant difference was found between hMPV-infected children with and without RSV co-infection in term of disease severity. Even though RSV co-infection rate was higher for children with genogroup B2 infection, the clinical characteristics and disease severity was not significantly different among the children infected with different genogroups of hMPV.

Humidified oxygen is usually indicated for hospitalized infants who are hypoxic. However, the oxygen therapy is a routine treatment in our general ward for young patients with tachypnea or respiratory distress, even when mild. A

hood with oxygen supplement from 35% to 40% was commonly administered. Therefore, we did not take "oxygen supplement" into account as one different variable for comparison. According to the medical records, the proportion of oxygen hood usage in the present study was 86%, which was much higher than that in previous reports (ranging from 27–56%).[11,14] Only 10 patients (8%), who presented with marked respiratory distress, had documented saturation data, ranging from 62% to 92%. We usually administered oxygen supplement directly, instead of making a strict assessment as to who needs oxygen therapy.

The major limitation of our study was its retrospective nature. Disease severity could not be calculated, virus detection methods were not uniform, and treatment response could not be evaluated. Also, the study population was limited to hospitalized children and those with respiratory specimens sent for RSV antigen detection, making the estimation of incidence rate and the perspective of disease spectrums of hMPV infection in children unobtainable. In addition, RSV was the most common co-infection pathogen in the current study (8.6%, 13/152). The higher percentage could be explained by RSV antigen test being the only test performed for each patient. We excluded pathogens other than RSV because the samples were from patients with a clinical picture of "RSV infection-like", and thus selection bias cannot be avoided. Therefore, we further compared the variables between those with and without RSV coinfection, such as fever, wheezing, and respiratory distress, and found that there was no significant difference, regardless of dual infection of RSV/hMPV.

In conclusion, hMPV is an important pathogen causing LRTI among hospitalized children. Mix infection with other viruses or bacteria is common. Even though there are two different genogroups and four genotypes of hMPV, no statistically significant differences was noted in patients infected with different genogroups or genotypes of hMPV in terms of demographics and clinical symptoms and signs.

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