Circulation of Human Metapneumovirus Among Children With Influenza-Like Illness in Wuhan, China

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Human metapneumovirus (HMPV) is a worldwide distributed pathogen of the respiratory tract. The objectives of this study were to identify HMPV infections among children with influenza-like illness (ILI) in Wuhan and to assess circulation patterns and molecular diversity of HMPV in this area. From July 2008 to December 2013, a total of 3,883 throat swab samples were collected from ILI outpatients under 16 years old. HMPV RNA was detected in 171 samples (4.40%). All the four subtypes of HMPV were identified, among which A2 was the most common subtype (61/145, 42.1%), followed by B1, B2, and A1. During the study period, HMPV circulation presented a biennial alternation between high and low incidence in Wuhan and the seasonal peak also shift between winter and spring in two continuous seasons. Subtype A2, B1, and B2 co-circulated during the study period, with genotype A prevailing in epidemic season 2008–2009 and 2012–2013, and genotype B prevailing during other periods. This large-scale analysis of HMPV prevalence in ILI outpatient children improves the understanding of local HMPV circulation patterns and provides molecular epidemic evidence for comparative analysis of HMPV infection.

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INTRODUCTION

Discovered in the Netherlands in 2001 [van den Hoogen et al., 2001], human metapneumovirus (HMPV) has proven to be a major pathogen that causes acute respiratory illness in individuals of all ages. HMPV is an enveloped negative-sense single-stranded RNA virus, belonging to the genus Metapneumovirus, subfamily Pneumovirinae, family Paramyxoviridae [van den Hoogen et al., 2004]. Serological studies indicate that most children below the age of 5 years will already have been infected by HMPV [van den Hoogen et al., 2001; Ebihara et al., 2003]. It generally accounts for 5–15% of hospitalization for lower and upper acute respiratory infections in children and the clinical manifestations ranging from otitis media to bronchiolitis and pneumonia [Kahn, 2006; Feuillet et al., 2012]. Adults infected by HMPV usually only present with flu-like symptoms [Boivin et al., 2002; Walsh et al., 2008], but high morbidity is observed in the elderly and immunocompromised patients [Larcher et al., 2005; Kahn, 2006].

The viral RNA of HMPV is approximately 13 kb in length, including eight genes that code for nine different proteins [van den Hoogen et al., 2002; Feuillet et al., 2012]. Based on genetic differences, HMPV isolates are separated into two genotypes, A and B, with each genotype subdivided into two subtypes 1 and 2 [van den Hoogen et al., 2004]. Subtype A2 is further divided in two genetic lineage A2a and A2b [Huck et al., 2006]. HMPV has a seasonal distribution with peak incidence in winter and spring [Kahn, 2006; Mizuta et al., 2013]. Its circulation may vary each season and different...
genotypes can circulate simultaneously in variable proportions [Mackay et al., 2006; Gaunt et al., 2009; Pitoiset et al., 2010; Li et al., 2012b].

Most existing studies of HMPV infection were focused on hospitalized children [Kahn, 2006; Feuillet et al., 2012]. However, epidemiological survey of outpatients is of unique importance for a comprehensive understanding of the current circulation pattern and clinical burden and for developing control strategies. A national influenza surveillance system which tracks influenza-like illness (ILI) was launched in China in 2001 [Yang et al., 2009b]. Clinical samples of ILI patients are collected by sentinel hospitals every calendar week. In the present study, specimens from children with ILI were utilized to explore the circulation and epidemiology of HMPV in Wuhan, the largest city in central China which has a resident population over 10 million. Real-time RT-PCR assay was employed to evaluate the prevalence of HMPV infection and phylogenetic characteristics of HMPV isolates were also investigated.

**MATERIALS AND METHODS**

**Patients and Clinical Specimens**

The Children's Hospital of Wuhan was selected to be the study site from July 2008 to December 2013 since it is the largest public paediatric centre in the city as well as a national influenza sentinel hospital. Young ILI patients presenting at the hospital clinic were diagnosed according to the following definition: the sudden onset of fever >38°C, with cough or sore throat in absence of other diagnoses [World Health Organization, 1999]. ILI-positive children were excluded if they: (1) were older than 15 years of age; (2) had fever for more than 3 days; and (3) had been treated with antiviral drugs.

During the 5.5 year study period, a total of 3,883 patients were recruited. About 10–15 eligible subjects were enrolled per week based on random selection of physicians, except for the period between August 2009 and September 2010, when inclusion number was doubled for the reason of 2009 influenza pandemic. After verbal informed consent was obtained from parents or caretakers, throat swabs were collected from study participants in 2.5 ml viral transport medium and were delivered to Wuhan Centers for Disease Prevention and Control for laboratory diagnosis of respiratory viruses. Since the sentinel surveillance of ILI cases is covered by Chinese legislation (Law on the Prevention and Control of Infectious Diseases, article 17) and no extra sample was obtained for the study, ethic committee approval was not required.

**Detection of Human Metapneumovirus**

Nucleic acids were extracted from 1 ml of specimen using MagNA Pure LC 2.0 (Roche Diagnostics, Rotkreuz, Switzerland) following the manufacturer's instructions. Total nucleic acids were eluted in a final volume of 100 µl. HMPV was detected by real-time RT-PCR using 7900HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA), based on the following primer/probe set: NL-N-forward (5'-CATATAAGCATGCTATATTAAAGAGTCTC-3'), NL-N-reverse (5'-CTCTGAGCATATTGGAAATCAG-3'), and NL-N-probe (5'-FAM-TGYAATGA-TGAGGTGTCACTGGGTGTTG-BHQ1-3') [Maertz-dorf et al., 2004]. High-load patient samples were applied as positive controls in the study. These controls were determined by Seeplex RV assay (Seegene, Seoul, Korea), a multiplex RT-PCR assay for the detection of 12 respiratory viruses, and confirmed by DNA sequencing. Participants' specimens were also tested for multiple other respiratory viruses using real-time RT-PCR assays [Peng et al., 2012]. Pathogens been tested included respiratory syncytial virus A and B, influenza virus A and B, parainfluenza virus 1, 2, and 3, human rhinovirus, human adenovirus, human coronavirus, and human bocavirus.

**Sequencing and Phylogenetic Analysis**

For patient samples shown to be HMPV positive, RT-PCR was employed to amplify the viral gene segments for genotyping and phylogenetic analysis. A previously described set of primers targeting at a 506 bp fragment of fusion protein (F) gene was used [Huck et al., 2006]. Retro-transcription and amplification were performed using Qiagen OneStep RT-PCR kit (Qiagen, Germany) and the amplified fragments were purified and sequenced at Sangon Biotech Co., Ltd. (Shanghai, China) using an ABI 3730XL DNA Analyzer. The GenBank accession numbers of partial F gene sequences obtained in the present study are KC350987–KC351181.

Obtained sequences were aligned with the reference sequences of 17 metapneumovirus strains (16 human and one avian strain, supplementary Table S1) using ClustalX2 [Larkin et al., 2007] and alignments were edited manually with BioEdit software. Further phylogenetic analyses were performed on a 489–bp F gene segment (nt 574–1062). Neighbor-joining (NJ) tree of F gene was estimated using MEGA software (version 5.05) [Tamura et al., 2011], with Tamura 3-parameter model of nucleotide substitution as it gave the lowest BIC (Bayesian Information Criterion) scores. Gaps were treated by pairwise deletion and substitution rates were defined as uniform among sites but different among lineages. To confirm the robustness and reliability of the branching orders, 1,000 bootstraps were performed on the neighbor-joining tree. Additional phylogenetic testing of the dataset was the maximum likelihood (ML) analysis performed by using IQPNNI (Important Quartet Puzzling and Nearest Neighbor Interchange, version 3.3.2) [Minh et al., 2005]. The Hasegawa–Kishino–Yano (HKY85) model was used for ML tree generation, while substitution rates and
base frequencies were estimated from data by the software. The numbers of iterations were twice of the sequence number of dataset.

Evolutionary distances within and between different clades were calculated by using the MEGA 5.05. In addition, sequence identity within HMPV samples identified in this study and between samples and contemporary circulating strains all over the world were estimated. Genomes of 23 HMPV strains collected after 2002 were obtained from NIAID Virus Pathogen Database and Analysis Resource (ViPR) [Pickett et al., 2012] and served as reference sequences of contemporary circulating strains (Supplementary Table S2). Sequence homology percentages of nucleotide level and amino acid level were calculated by using MegAlign programme of Lasergene package.

Statistical Analysis

Data were analyzed using PASW Statistics (version 18.0; SPSS, Chicago, IL). Categorical variables were compared by the \( \chi^2 \)-test. For continuous variables, Kruskal–Wallis test were employed after determining the normality of their distributions by Kolmogorov–Smirnov test. A \( P \)-value of less than 0.05 was considered statistically significant.

RESULTS

Prevalence of HMPV Infection

A total of 3,883 ILI patients ranging from 1 month to 15 years old (median age 34 months, mean age 45 months) were enrolled in this study, of whom 53.1% were male and 46.9% were female (Table I). HMPV RNA was detected in 4.40% (171/3883) of all patient specimens and in 7.37% (146/1981) of specimens that tested negative for other respiratory viruses. The whole detection rate of HMPV was lower than that of influenza virus (23.2%), rhinovirus (8.27%), parainfluenza virus (6.21%), respiratory syncytial virus (5.74%), and adenovirus (5.15%), but higher than that of human coronavirus (3.37%) and bocavirus (0.95%). Co-infection was observed in 25 HMPV RNA positive samples, among which three samples have been shown to be positive for three different respiratory viruses. Pathogens most frequently co-infected with HMPV were influenza virus (nine cases) and rhinovirus (six cases).

The HMPV RNA positive patients included 99 males and 72 females, with a male:female ratio of 1.375:1 (\( \chi^2 = 1.649, P = 0.199 \), chi-square test). The age range of those patients was 6 months to 13 years (median age 28 months, mean age 32 months). Over 90% of HMPV RNA positive children were under 5 years old (\( \chi^2 = 32.32, P < 0.001 \), chi-square test) and the highest prevalence rate occurred in the group aged 3 years old, which was 7.91% (45/569) (Fig. 1A).

Seasonal Circulation of HMPV Infection

HMPV was detected in every season during the study period. As expected, the virus circulated predominantly in winter and spring, with 80.1% of the identified infections occurring between December and May (Fig. 1B). A biennial alternation between high and low incidence was observed during the study (Fig. 1C). HMPV presented a high-level activity in the first two epidemic seasons. Of 431 ILI patient samples collected in the epidemic season 2008–2009, 31 (7.19%) were identified as HMPV RNA positive. Seasonal peak occurred from November through February and the highest detection rate was 22.2% (6/27, February 2009). The year-round positive rate of the epidemic season 2009–2010 was 6.28% (68/1082). Peak circulation of HMPV in this season delayed to spring and early summer (March to June) with a highest monthly positive rate of 32.5% (26/80) in April 2010. In the next two epidemic seasons, however, the prevalence of HMPV became much lower. The detection rates in seasons 2010–2011 and 2011–2012 were 1.27% (11/863) and 2.52% (13/516), respectively. HMPV-positive cases were distributed sporadically with no notable circulation peak. A rise of HMPV detection occurred in the last period of study. The year-round positive rate of epidemic season 2012–2013 soared up to 7.41% (45/607) with a highest detection rate of 22.7% (10/44) in January 2013.

### TABLE I. Demographic Characteristic of Patients in This Study

| Characteristic | All cases tested (n = 3883) | HMPV RNA positive cases (n = 171) | Subtyped cases (n = 145) |
|----------------|----------------------------|----------------------------------|-------------------------|
|                | Gender                     |                                  | A1 (n = 1) | A2 (n = 61) | B1 (n = 42) | B2 (n = 41) |
|                | Male                       | 2062 (53.1)                      | 99 (57.9)  | 35 (57.4)  | 23 (54.8)  | 22 (53.7)  |
|                | Female                     | 1821 (46.9)                      | 72 (42.1)  | 1 (100.0)  | 26 (42.6)  | 19 (46.3)  |
|                | Age group (years)          |                                  |            |            |            |            |
|                | <1                         | 481 (12.4)                       | 17 (9.94)  | 0 (0.0)    | 10 (16.4)  | 1 (23.8)   | 3 (7.32)   |
|                | 1–2                        | 1484 (38.2)                      | 84 (49.1)  | 1 (100.0)  | 21 (34.4)  | 28 (66.7)  | 20 (48.8)  |
|                | 3–5                        | 1109 (28.6)                      | 63 (36.8)  | 0 (0.0)    | 27 (44.2)  | 13 (31.0)  | 16 (39.0)  |
|                | 6–15                       | 809 (20.8)                       | 7 (4.09)   | 0 (0.0)    | 3 (4.92)   | 0 (0.0)    | 2 (4.88)   |

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Genetic Diversity of HMPV in Wuhan

Phylogenetic analysis was conducted on the 489 bp amplicons of HMPV F gene (amino acid position: 192–354) of 145 samples identified in this study. The other 26 specimens were not subtyped because of the lack of sample, unsuccessful amplification or low quality of sequencing. As shown in the NJ tree, all four subtypes of HMPV were detected and the clustering was supported by high bootstrap values (>85%) of major intersects (Fig. 2). Genotype A included 62 specimens. Except one belonged to

Fig. 1. Circulation of HMPV among ILI children in Wuhan, July 2008 to December 2013. A: Prevalence of HMPV in different age groups. B: Seasonal distribution of HMPV cases during observation. Each column in chart stood for the cumulative number of positive cases in one certain month (January, February, March, etc.) during the 5.5-year study period. C: Monthly detection rate of HMPV subgroups.

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Fig. 2. Phylogenetic analysis of partial F gene fragments of HMPV strains in Wuhan. The tree was constructed using neighbor-joining algorithm with Tamura 3-parameter method through MEGA 5.05. Analyses were performed on a 489 bp F gene segment (nt 574–1062) and had involved 162 nucleotide sequences. HMPV strains from this study were identified by the place of origin (WH for Wuhan), followed by sampling number started with two-digit year code (from 08 to 13). Sixteen reference sequences representing various HMPV lineages were included in the analysis, as well as avian metapneumovirus C (AMPV) as outgroup. Bootstrap values greater than 80% were shown (1,000 replicates). Scale bar is on the unit of average nucleotide substitutions per site.

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subtype A1, all the other 61 samples were classified as subtype A2, and further, lineage A2b. Among genotype B, subtype B1 included 42 strains and B2 included 41 strains. Notably, sequences of consecutive epidemic seasons were clustered in the clade of B1. Also there were two clusters in B2 clade that each was comprised by multiple identical or closely related sequences of one epidemic season (2009–2010 and 2012–2013, respectively). ML analysis revealed similar tree topology with the NJ tree (data not shown).

Sample sequences identified in this study shared high homology with sequences of contemporary circulating strains from all over the world at both nucleotide level and amino acid level (Table II). Evolutionary distances within groups suggested B2 was more heterogeneous than other HMPV subtypes (nucleotide distance: 0.011, amino acid distance: 0.004) and the nucleotide distance between groups showed that clade B2 was slightly more distant from genotype A than clade B1 (0.163 vs. 0.158).

### Distribution of HMPV Subgroups

Except subtype A1 that only one case had been identified, all other HMPV subtypes were found to share similar population distribution: more male patients than female, and the majority of patients were under 3-year old. There was no significant difference among four subtypes in their gender (x² = 5.320, P = 0.504, chi-square test) and age distribution (x² = 0.495, P = 0.920, Kruskal–Wallis test).

Genotypes A and B circulated simultaneously during the observation except the last half year, when only subtype B2 was detected. Changes in the predominant genotype occurred in 1–3 years’ interval (Table III). Subtype A2 was prevailed in epidemic seasons 2008–2009 and 2012–2013 and B1 was the predominant subtype during epidemic seasons 2009–2010 to 2011–2012, following by B2. The single A1 strain was detected in epidemic season 2012–2013.

### TABLE II. Homology Level of HMPV Subgroups

| HMPV Subgroups | Sequence identity within samples (%) | Sequence identity between samples and contemporary circulating strains (%) |
|----------------|-------------------------------------|---------------------------------------------------------------|
|                | Nucleotide sequences | Amino acid sequences | Nucleotide sequences | Amino acid sequences |
| A1             | N/A                   | N/A                | 97.5–98.5           | 100                |
| A2b            | 97.9–100              | 99.2–100           | 95.2–99.8           | 99.3–100           |
| B1             | 97.4–100              | 100                | 97.7–99.7           | 100                |
| B2             | 95.3–100              | 96.9–100           | 97.0–100            | 97.9–100           |

*Reference sequences of contemporary circulating strains from the world were listed in Table S2.

### DISCUSSION

To discover the epidemiological and molecular features of HMPV infection in Wuhan, we screened 3,883 respiratory samples from ILI outpatients under 16 years of age that were collected over a period of five and half consecutive years. HMPV was detected in 171 samples, giving a positive rate of 4.40%. Similar frequencies have been reported among the German paediatric outpatients with ILI (3.99%) [Reiche et al., 2014] and ARTI children in Beijing, China (4.02%) [Zhu et al., 2011], whereas influenza surveillance system in northern Greece has detected a higher incidence in ILI outpatients during season 2005–2008 (12.8% in age group 0–5 years, 7.36% in age group 0–18 years) [Gioula et al., 2010].

The present study was very much facilitated by the existing ILI surveillance which continuously collects clinical samples from outpatients with respiratory issues. However, it also caused potential sampling bias, as fever is not a typical clinical manifestation of HMPV in children [Reiche et al., 2014]. According to the surveillance definition of ILI, only febrile patients were sampled, thereby increasing the likelihood of underestimating HMPV circulation.

No gender preference was found in the study. Interestingly, although HMPV infection in Wuhan occurred more frequently in children under the age of five as expected, the highest prevalence was in children aged between 2 and 4 years, not in younger infants like many previous studies described [van den Hoogen et al., 2001; Williams et al., 2004; Lambert et al., 2007; Zhu et al., 2011]. One explanation is that the risk of exposure is higher among children between 2 and 4 because they are just entering kindergartens in this age. The observation might also be related to the higher virulence of HMPV in infants and young children [Boivin et al., 2002]. The virus has been demonstrated to be second only to RSV as a cause of bronchiolitis in early childhood [Kahn, 2006], which leads to substantial proportion of hospitalization of younger patients. Children above 2 years old, on the other hand, are more immunocompetent and are more likely to become outpatient rather than be hospitalized during a HMPV infection.
The majority of HMPV positive cases were diagnosed at the end of winter and early spring, which is the typical epidemic season of this virus in temperate zone [Kahn, 2006]. However, the seasonal distribution of HMPV in Wuhan varied in different study years. Three main epidemic peaks occurred first in the winter-spring season in year 2008–2009 (November to February), then shifted to the spring-summer season in year 2009–2010 (March to June), and finally back to the winter-spring season in year 2012–2013 (November to March). Multiple researchers from Europe and Asia also observed similar biennial shifts between winter and spring seasonality of HMPV. A significantly higher prevalence was identified in summer peak in Austria [Aberle et al., 2010], in winter peak in Japan [Mizuta et al., 2013], but not in Beijing [Zhu et al., 2011], or Wuhan as shown in this study. Besides, the incidence of HMPV was not even in each season through the study. Two epidemic seasons with high viral activity were followed by two low activity epidemic seasons and again by a high activity epidemic season. The cause of different seasonality and yearly incidence is unclear. Our data did not support the assumption that circulation of different genotypes resulted in the change of temporal distribution, since no correlation was found between a certain HMPV genetic subgroup with high prevalence. But the possibility cannot be ruled out that environmental factors and circulation of other respiratory pathogens (such as the pandemic of A/H1N1/2009) have affected the epidemical dynamics of HMPV.

Phylogenetic analysis of partial F gene revealed that different HMPV subgroups circulated simultaneously in most time of the study. Only one genotype was predominant in each epidemic season and the dominance of a certain genotype lasted for one or three seasons. Such replacement of prevailing genotype is believed to be a random escape from the adaptive immunity of population to the circulating genotype [Williams et al., 2006; Aberle et al., 2010; Pitoiset et al., 2010; Reiche et al., 2014]. Interestingly, a group of researchers from Chongqing, a city in Southwest China, has studied the epidemiology of HMPV among hospitalized children with acute lower respiratory tract infections between July 2008 and March 2011, yet they observed quite different seasonal distribution and prevailing genotype from Wuhan: subtype A2b was predominant during the whole period and peaked in March to May 2009 and November 2010 to February 2011 [Zhang et al., 2012]. Considering the 800 km linear distance between Chongqing and Wuhan, the difference was likely to be a result of geographic isolation as well as the distinction of study subjects.

In this study, only one sample in epidemic season 2012–2013 was identified as subtype A1. As a matter of fact, a number of studies have observed the absence of A1 in recent years [Li et al., 2012a, 2012b; Velez Rueda et al., 2013; Reiche et al., 2014], indicating this subtype has been going through a global trough from 2006. The only exception was an Italian study that reported the circulation of subtype A1 was predominant in season 2009–2010 [Apostoli et al., 2012]. The analysis of sequence diversity showed that nucleotide distance within HMPV subtypes was 0.007–0.011 and the corresponding amino acid distance was 0.000–0.004. Such low diversity might be explained by functional constraints on paramyxovirus fusion proteins that prevent progressive antigen drift [Yang et al., 2009a]. It has been identified that a region between residue 260–300 on F1 subunit of fusion protein presents considerable variation [Yang et al., 2009a], but no amino acid substitution specific for Wuhan strains was noticed in this study. There were multiple closely related or even identical sequences from the same epidemic seasons or two consecutive seasons in clades B1 and B2, implicating that HMPV infections due to highly similar viruses within one period, at least in genotype B [Reiche et al., 2014].

Among all detected HMPV positive samples, 14.6% were found to be co-infected by one or two other respiratory viruses. This portion of sample was not excluded during data analysis because previous study suggested no significant difference in clinical manifestation and severity between patients with a co-infection and those without [Kahn, 2006]. The positive rate of HMPV was lower than those of several other common respiratory viruses among the ILI children in Wuhan, but given the population size represented by the study subjects, this moderate rate could mean a large number of respiratory infection cases and significant health burden.

In summary, this is the first long-term study on HMPV infection among ILI children in China. It shows that metapneumovirus is rather a rare cause of ILI in Wuhan. A higher detection rate in children aged between 2 and 4 years was observed, as well as the biennial rhythm of viral seasonality. Phylogenetic analysis revealed that all subtypes of HMPV were circulated in Wuhan, yet each genotype prevailed for 1–3 consecutive seasons. Strains in Wuhan were evolutionally conservative and multiple identical isolates were observed in one season. Since only limited information about the virus’s prevalence and lineage distribution in the outpatient, these data improve the understanding of the local HMPV circulation patterns and genetic diversity. However, further surveillance in adults, ILI outpatient and inpatient, are required to find the missing part of puzzle.

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