Detection and Genetic Diversity of Human Metapneumovirus in Hospitalized Children with Acute Respiratory Infections in Southwest China

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ABSTRACT

Human metapneumovirus (hMPV) is a main pathogen causing respiratory tract infection in susceptible population, particularly in children and the elderly. Specimens were collected from hospitalized children with acute lower respiratory tract infections (ALRTI) and the hMPV was detected by using real-time RT-PCR. The full length G gene of hMPV was amplified by reverse transcription PCR (RT-PCR). A total of 1410 nasopharyngeal aspirates (NPAs) were collected from April 2008 to March 2011, and 114 (10.2%) were positive for hMPV. Most of hMPV positive children were aged <5 years. The hMPV infection rate peaked in spring-summer season of 2008-2009 and 2009-2010, while hMPV circulated predominantly during winter-spring season of 2010-2011. The full length G gene of 23 hMPV strains was amplified and group A and B viruses accounted for 95.7% (22/23) and 4.3% (1/23), respectively. Genotype A2b of hMPV appeared to be predominant during the study period. Three genotypes (A2b, A1 and B1) were prevalent in the epidemic season of 2008-2009, and only genotype A2b was identified in the other two seasons (2009-2010 and 2010-2011). The G gene of hMPV was predicted to encode proteins with four different lengths, in which one with 210 amino acids was firstly identified in China. These findings suggest that hMPV was an important pathogen of ALRTI in pediatrics, especially those aged <5 years. Group A2b of hMPV likely predominates in Southwest China, and other genotypes also circulate.

Keywords: human metapneumovirus; genetic variability; epidemiology; attachment protein
INTRODUCTION

Human metapneumovirus (hMPV) was first isolated in 2001 from nasopharyngeal aspirates (NPAs) of children with acute lower respiratory tract infections (ALRTI) in the Netherlands (27). Subsequently it has been identified all over the world (2, 20, 21, 24). Children aged <5 years, elderly adults and immunocompromised patients are at an increased risk for severe hMPV infection (23, 24). Morphologically, hMPV consists of a negative-sense, single stranded and non-segmented RNA that encodes at least 9 distinct proteins (26). Among them, the two major transmembrane glycoproteins, G and F proteins, can stimulate the production of protective immune responses and therefore are antigenically significant (11). Unlike the relatively conserved F protein (95% homology at the amino acid level between group A and B hMPV), the G protein is highly variable with only 53% amino acid homology between group A and B (3).

Similar to respiratory syncytial virus (RSV), hMPV strains vary genetically and antigenically and have been classified into two broad groups: group A and group B (4, 26), with each group divided into genetic subgroups 1 and 2 (12). More recently, phylogenetic analysis showed a further bipartition of subgroup A2 into two new genetic clusters designated A2a and A2b (3). Both antigenic group A and group B were noted to co-circulate in the same city during the epidemic periods and had various patterns of predominance.

Among all the sub-lineages of hMPV, the A2 sub-lineage shows the greatest diversity. Antigenic variability is thought to contribute to re-infection throughout the life and may pose a challenge to vaccine development. Future planning for vaccine development requires in depth understanding of genetic composition of the hMPV strains prevalent in target population.

However, less information is available on the distribution pattern of hMPV strains in Southwest China. We had conducted a two-year epidemiological study on hMPV in Chongqing area from April 2016 to October 22, 2017.
2006 to March 2008 and genotype A2 was found to be the most predominant one during the study period (6). Thus, ongoing epidemiological surveillance in a consecutive manner will help assess the disease burden and genetic diversity of hMPV. The aim of the present study was to evaluate the genetic diversity of the G protein gene of hMPV in hospitalized infants and young children with ALRTI in the past three epidemic periods in Chongqing. Information on distribution of hMPV genotypes in China will be beneficial for the development of hMPV vaccines.

MATERIALS AND METHODS

Collection of specimens

From April 2008 to March 2011, NPAs were obtained on three fixed days of each week from all children admitted to Chongqing Children’s hospital due to ALRTI. ALRTI was diagnosed according to the criteria developed by World Health Organization. The specimens were immediately placed at 4°C in tubes containing 3 ml of cold viral transport medium (PBS, 100 U/µL, penicillin and 100 µg/mL streptomycin) and transported to the Department of Virology within 4~6 h. Specimens were vortexed and centrifuged at 1500 g for 10 min at 4°C to separate the cells for direct immunofluorescence assay (DFA) to detect seven common viral respiratory pathogens (respiratory syncytial virus, parainfluenza virus 1, 2 and 3, influenza virus A and B and adenovirus) on the same day. Cell pellet was washed with PBS for three times and added to the cytopsion slides after being adjusted to suitable concentration. Subsequently cells were fixed with acetone for 10 minutes after being dried in room temperature. A kit from Chemicon was used to stain the cells and the manufacturer’s instructions were followed. The supernatants were stored at -70 °C for use. This study was approved by the Ethics and Research Council of Chongqing Children’s Hospital and informed consent was obtained from their parents or guardians.
RNA extraction and cDNA synthesis

RNA was extracted from 140 µl of frozen specimens with an RNeasy mini kit (QIAGEN, Germany) according to the manufacturer’s instructions. The dried RNA was dissolved in 50 µl of DNAse-free, RNase-free water. For cDNA synthesis, 100 ng of total RNA was used as a template for reverse transcription polymerase chain reaction (RT-PCR) with random primers.

Real-time PCR assays

All NPAs were tested for hMPV by real-time PCR. Real-time PCR primers and probes for hMPV F gene were used for the diagnosis of hMPV infection and designed based on the sequence in GenBank (accession number: DQ336144). Viral cDNA was amplified by using a real-time PCR with the Premix Ex Taq Kit (Perfect Real Time, TaKaRa, China) in a LightCycler instrument (Roche Diagnostics, American). Two microliters of cDNA was added to a 22uL real-time PCR master mixture containing 200 uM deoxynucleoside triphosphates, 1.5 mM MgCl2, 1.5 U of Taq DNA polymerase, and 50 pM each primer and probe. The thermocycling protocol was 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension for 7 min at 72°C. The positive was defined at cycle threshold (Ct) of ≤36. Negative and positive control was introduced in each real-time PCR. Positive control was the cDNA from a recombinant hMPV infected Vero-E6 cells. The sequence of real-time PCR primers and probes for hMPV F gene was as followed: hMPV-F(F): 5’-CGTTTCTAACATGCCGACATCTG-3, hMPV-F(R): 5’-GCTCCGGTAGACCGCCCCCTAC-3’, Probe 5’(FAM)-CCCTTTCTTCGACCACCGACACGG(Eclipse)-3’, To verify the presence and sequence of the virus, traditional RT-PCR was also used to amplify the hMPV F and G gene to ensure reproducibility. All PCR assays were verified to be able to amplify F and G gene of the recombinant hMPV positive control.

PCR amplification of full length G protein gene
We selected 23 hMPV strains by using random digits table, including seven in the epidemic season of 2008-2009, seven in 2009-2010 and nine in 2010-2011. Primers used to amplify the full length G protein were hMPVGF 5'-GAGAACATTGAGCAATAGACATG-3' and hMPVGR 5'-AGATAGACATTAACAGTGGATTCA-3'. DNA amplification was carried out by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Five microliters of PCR products were subjected to 1.5% agarose gel electrophoresis, stained with GoldView™ nucleic acid stain (SBS genetech) and visualized under UV light.

DNA sequencing

The PCR products were purified with a QIAquick gel extraction kit (QIAGEN, Germany) according to the manufacturer’s instructions. The purified PCR products were sequenced in both directions using the same PCR primers for amplification on an ABI PRISM 310 genetic analyzer (Applied Biosystems) by using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems).

Phylogenetic analysis

Nucleotide and amino acid sequences of group A and B hMPV were compared independently with the available hMPV sequences in the GenBank database using ClustalX version 1.81 and manually edited with BioEdit version 7.0.1. Phylogenetic trees were constructed by the neighbor-joining method in MEGA version 5. The statistical significance of the tree topology was tested by bootstrapping (1,000 replicas). The phylogenetic analysis included the hMPV sequences in GenBank belonging to four different subgroups: A1: CHN03-06(EF571502), FL-8-01(AY295989), CAN99-81(AY574224) and NL1-00(AY296035); A2a: Arg-4-00(DQ362953), CAN97-83(AY297749); A2b: CHN07-06(EF571506), BJ1819(DQ270215), BJ1887(DQ843659) and KOL-30-06(HQ599198); B1: CHN12-06(EF571511), NL-11-00(AY296035), Arg-1-00(DQ362958) and NL-1-99(AY296034); B2: CAN98-75(AY297748).
Nucleotide sequence accession numbers: The GenBank accession numbers of the nucleotide sequences obtained in the present study are JX082172-JX082194.
RESULTS

Epidemiology of hMPV

A total of 1410 specimens were collected from inpatients with ALRTI from April 2008 to March 2011, and 144 (10.2%) were positive for hMPV by real-time RT-PCR. Positive rate of hMPV was 11.8% during 2008-2009, 7.31% during 2009-2010 and 12.8% during 2010-2011. The prevalence of hMPV in Chongqing was high in April, May and June of 2008-2009 and 2009-2010 while hMPV circulated predominantly during the late winter and spring of 2010-2011 (Figure 1).

hMPV infected children were aged 1~71 months (median: 21 months). Most infected individuals were infants aged 0~6 months, accounting for 45.9% of subjects positive for hMPV infection. Infants aged 6-12 months and children aged 1~2 years accounted for 10.4% and 15.3%, respectively. Children aged 2~5 years accounted for 20.1%. (Table 1)

All 144 hMPV-positive patients had clinical symptoms. The main clinical presentations included fever (n=62; 41.5%), cough (n=142; 99.1%), wheezing (n=102; 71.1%) and cyanosis (n=110; 85.0%). The hMPV-infected children were diagnosed with bronchopneumonia (61.1%; 88/144), bronchiolitis (19.4%; 28/144), bronchial asthma exacerbation (15.9%; 23/144) and acute upper respiratory infection (3.4%; 5/144). (Table 2) Eighteen out of 144 inpatients had co-morbidities including congenital heart disease (15/144), tracheomalacia (3/144) and congenital laryngeal stridor (1/144). Eight patients developed respiratory failure and one required mechanical ventilation.

Among the eight patients with respiratory failure, congenital heart disease was identified in 3 patients and co-infection of other respiratory viruses was found in 4 patients, including 3 with RSV and 1 with PIV3.

Other respiratory viruses co-infecting with hMPV

In addition, 29.2% (n=42) of hMPV positive patients were found to have concomitant infection of
other respiratory viruses, including 19 with RSV, 11 with PIV3, 6 with ADV, 6 with InfB. Among them, 18 (42.9%) were younger than 1 year and diagnosed with ALRTI including pneumonia (n=12) and bronchiolitis (n=3).

**Phylogenetic analysis and genotype distribution patterns**

Phylogenetic analysis of 23 hMPV strains in Chongqing confirmed two main genetic lineages A and B. Interestingly, all the strains in Chongqing were clustered as A2b, A1 and B1, and subgroup B2 was not identified. During 2008-2009, subgroup A2b, A1 and B1 co-circulated, with 82% (n=9) belonging to A2b. Only one A1 and one B1 hMPV were detected in the same season. Interestingly, only subgroup A2b was identified in the next two epidemic seasons (2009-2010 and 2010-2011).

The results of phylogenetic analysis of 23 hMPV strains were showed in Figure 2.

Sequencing of the full length of G protein gene revealed the homology ranged from 55.8-58.4% at nt level and 44.1-49.7% at the aa level between groups A and B isolates. Subgroup B1 strains shared high homology with the prototype strain NL/1/99 (94.9% at nt.; 91.9% at aa), whereas subgroup A2b strains revealed homology with the prototype strain CAN97-83 ranged from 89.6-99.8% at nt level and 82.3-84.9% at aa level. Subgroup A1 strains shared high homology with the prototype strain CAN99-81 (100% in nt.). Compared with the prototype A2 strains circulating from April 2006 to March 2008 in Chongqing, 21 A2 strains prevalent from April 2008 to March 2011 revealed homology ranging from 95.7-97.7% at nt level and 94.3-96.2% at aa level. The only one subgroup B1 strains circulating in the present study shared high homology with the prototype B2 strains prevalent from April 2006 to March 2008 in Chongqing (92.8% at nt.; 90.7% at aa).

**Amino acid analysis**

The comparisons of deduced aa sequence of G protein gene of strains in Chongqing with their
prototype strains revealed that intracellular and transmembrane regions were highly conserved across the strains (supplemental data). Most of the changes in aa were observed in the extracellular domain due to nt substitution. Changes in the stop codon were observed among strains of different subgroups (nt 644 (UAG); nt 671 (UAG), nt 694 (UGA); nt 709 (UAA)), which corresponded to variable lengths in polypeptides. Strains from subgroup A2b had two different stop codons resulting in G proteins with 210aa (UAG) and 219aa (UAG), whereas subgroup B1 strains terminated at UGA stop codon and exhibited protein with 231 aa. The subgroup A1 strains terminated at UAA stop codon and exhibited protein with 236 aa.
DISCUSSION

Respiratory tract infections are the major cause of hospitalization of young children in the winter worldwide. Viruses most frequently associated with respiratory infections include rhinoviruses, coronaviruses, influenza viruses, parainfluenza viruses, RSV and adenoviruses. In 2001, a new member of the paramyxoviridae associated with respiratory infections, hMPV, was described in the Netherlands. Serological studies indicated that nearly all children had been infected by age of 5 years (26). Some previous studies have suggested that hMPV is an important causative pathogen for ALRTI in children in China (13,16), but the epidemiology of hMPV infection in countries with large population remains largely unknown due to the limitations in these studies.

In the present study, 1410 specimens were collected from pediatric patients with ALRTI from April 2008 to March 2011 and tested for hMPV by real-time RT-PCR. The enrolled patients accounted for a significant proportion of total inpatients with ALRTI in that period. Although we designed to enroll all newly admitted patients in three fixed days in every week, the results were not necessarily free from selection bias because we only collected NPAs in the daytime and some of admitted patients refused to provide NPAs. The age and sex distributions and diseases between the enrolled 1410 patients in this study and the whole 9231 inpatients in the same period were not statistically different, reflecting that the selected subjects may be relatively representative. To detect hMPV, F protein gene was chosen because it is highly conserved and has been used in previous studies (16,17).

The frequency of hMPV infection varied from 5% to 30% among hospitalized children with ALRTI in most studies (1, 18, 22, 25,) and 7.31% to 12.8% annually in this study by using real-time PCR to detect the hMPV F protein gene. The prevalence of 46% hMPV infection varied from year to year, with the lowest in the epidemic season from 2009 to 2010. The general prevalence of hMPV infection was relatively high as compared with that in some other regions, probably due to a higher population density in this city. Thus, hMPV appeared to bring a significant disease burden in Chongqing and was a common prevalent virus for ALRTI. Several seroprevalence studies have
previously shown that 90-100% of children are infected by the ages of 5-10 years. Comparable to other reports (4, 5, 20), the present study showed 71.4% of children with hMPV infection were aged <2 years. The high prevalence of hMPV infection in little children (<6 months) might be attributed to weak immune function. The symptoms of hMPV infection among children were similar to those described previously in respiratory syncytial viral infection (11, 21, 27). We found the most common clinical manifestations of hMPV-associated ALRTI were fever, cough, wheezing and cyanosis. The main diseases ranged from acute laryngotracheobronchitis, asthmatic bronchitis, bronchitis, to bronchiolitis and pneumonia. We found hMPV infection was related to wheezing, asthma exacerbations or bronchiolitis, as previous studies described (7, 8, 13). Consistent with our findings, other studies have shown that hMPV infection may lead to severe respiratory distress requiring prolonged hospitalization, mechanical ventilation, and admission to an intensive care unit (2, 11, 14). Thus hMPV infection is associated with a substantial clinical and likely economic impact.

Although the winter-spring season is traditionally regarded as the typical epidemic season of hMPV infection in the temperate regions, our results showed that the hMPV infection peaked in the spring-summer season (April-June) of 2008-2009 and 2009-2010 and winter-spring season (November-February) of 2010-2011. The hMPV seasonality observed in this study was comparable to that reported in Japan, which showed a biennial hMPV infection, with a peak between November and March in odd years and between March and June in the successive even years (19). The difference in the hMPV seasonality between our study and other studies is unclear. Previous studies have shown that climate can affect the seasonality of hMPV infection (14, 18, 22). However, the climate records for Chongqing fail to indicate significant variability in the same months of different years. Apparently, more studies with long period are needed to elucidate the real pattern of hMPV circulation in this region.

Because of the similar seasonal distribution of hMPV and other respiratory viruses, the potential co-infection likely existed. Some studies have found a co-infection rate of 10%-75% in hMPV and
other respiratory virus (10, 15, 23). In this study, co-infection of hMPV with other respiratory viruses was detected in 29.2% of hMPV-infected patients, and the co-infected viruses included respiratory syncytial virus, influenza virus, parainfluenza virus and adenovirus. Of 25 patients identified as a co-infection with RSV, 3 developed respiratory failure, indicating that hMPV and RSV co-infection may lead to a more severe disease than infection alone.

Phylogenetic analysis of G protein gene sequences in the present study showed both the group A and B hMPV circulated in Chongqing. Generally, group A hMPV is reported more common than group B (9, 14, 16). In the present study, among 23 hMPV strains, 22 were grouped as A and 1 as B (subgroup B1). HMPV group A was a likely predominant virus circulating in Chongqing, which was in consistent with other studies (8, 17, 20). Genotype A2b, A1 and B1 viruses were detected in the epidemic season of 2008-2009, of which A2b were more common. In 2009-2010 and 2010-2011, only genotype A2b viruses were identified. Genotype B2 viruses were not identified in the present study. Indeed, circulation of hMPV of different genotypes has been reported to vary annually, with replacement of predominant genotypes every 1-3 years in a given population. Such genotype replacement is believed to result from adaptive immunity of a population to the predominant circulating genotype. However, genotype A2b viruses were predominant in the consecutive three epidemic seasons in Chongqing, which was consistent with the findings in epidemiological study from April 2006 to March 2008 (6). We found that genotype A2b viruses prevalent in the study period revealed high homology with those circulated from April 2006 to March 2008 which may be attributed to the low G gene diversity.

Although a report described that group A hMPV may be more pathogenic than group B hMPV (22), no significant difference in the severity of ALRTI between groups or subgroups because only one group B hMPV strain was detected. Further studies are required to better understand the clinical significance, seasonality, and molecular epidemiology of hMPV.
The predicted G protein of hMPV strains had different lengths which may be attributed to amino acid substitution, insertion, deletion and/or change in the stop codon. G proteins with 4 different lengths were identified in this study and this was the first report showing hMPV strain with 210 amino acids circulating in China.

In summary, hMPV subgroups are observed among inpatients in Chongqing China in the past three consecutive epidemic seasons. Genotype A2b has become a dominant genotype in the three epidemic seasons. This study thus contributes to a better knowledge on the molecular epidemiology of hMPV in China mainland. Comprehensive information on the prevalence of hMPV is important for the selection of appropriate vaccine strains and thus may contribute to vaccine development.

Acknowledgements
This work was supported by the National Natural Science Foundation of China (No. 30330590) and in part by Program for Chongqing Excellent Young Scientists Fund (CSCT, 2008BA5040).
REFERENCES


Fig. 1  Season distribution of hMPV infection in infants and children with ALRTI in Chongqing between April 2008 and March 2011.

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**Fig. 1** Season distribution of hMPV infection in infants and children with ALRTI in Chongqing between April 2008 and March 2011.
Fig. 2. Phylogenetic trees of nucleotide sequences of G protein gene of Chinese hMPV group A (panel A) and B (panel B). The numbers at the branch nodes represent the number of bootstrap probabilities. Reference sequences for each genotype (A1, A2a, A2b, B1, B2) were obtained from GenBank. Additional sequences were included for the comparison by selecting representatives of distinct clusters in previous studies and selecting isolates from GenBank that gave the best hits in blast searches with each of the Chinese clusters. The genotype assignment is indicated at the right by brackets.
### Table 1. Age distribution of 144 patients with hMPV infection

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### Table 2. Clinical features of 144 patients with hMPV infection

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