To explore the epidemiological and clinical features of different human metapneumovirus (hMPV) genotypes in hospitalized children. Reverse transcription polymerase chain reaction (RT-PCR) or PCR was employed to screen for both hMPV and other common respiratory viruses in 2613 nasopharyngeal aspirate specimens collected from children with lower respiratory tract infections from September 2007 to February 2011 (a period of 3.5 years). The demographics and clinical presentations of patients infected with different genotypes of hMPV were compared. A total of 135 samples were positive for hMPV (positive detection rate: 5.2%). Co-infection with other viruses was observed in 45.9% (62/135) of cases, and human bocavirus was the most common additional respiratory virus. The most common symptoms included cough, fever, and wheezing. The M gene was sequenced for 135 isolates; of these, genotype A was identified in 72.6% (98/135) of patients, and genotype B was identified in 27.4% (37/135) of patients. The predominant genotype of hMPV changed over the 3.5-year study period from genotype A2b to A2bo and B1a and then to predominantly B1. Most of clinical features were similar between patients infected with different hMPV genotypes. These results suggested that hMPV is an important viral pathogen in pediatric patients with acute lower respiratory tract infection in Changsha. The hMPV subtypes A2b and B1 were found to co-circulate. The different hMPV genotypes exhibit similar clinical characteristics.
Nasopharyngeal aspirates samples were collected from 2613 children hospitalized for acute lower respiratory tract infection at Hunan provincial People’s Hospital, China, during fixed 2 days of each week between September 2007 and February 2011. All enrolled hospitalized patients were 14 years of age or younger and were admitted for acute lower respiratory tract infection (pneumonia, bronchitis, bronchiolitis, and asthma complicated pulmonary infection). Acute lower respiratory tract infection was diagnosed on the basis of clinical and radiologic findings. The enrolled children were consulting with an illness where an acute or worsened cough was the main or dominant symptom, or had a clinical presentation that suggested a lower respiratory tract infection, with a duration of ≤28 days. All nasopharyngeal aspirates were collected within 1–3 days of admission. Demographic data and details of the clinical findings were recorded from case history. Informed consent was obtained from the parents of all children who provided specimens. The study protocol was approved by the hospital ethics committee.

**Collection and Processing of Nasopharyngeal Aspirates Samples**

All nasopharyngeal aspirates were collected and transported immediately to the laboratory at the National Institute for Viral Disease Control and Prevention, China CDC, and stored at –80°C until required for further analysis. Viral DNA and RNA were extracted from 140 μl of each nasopharyngeal aspirates specimen using the QIAamp viral DNA and the QIAamp viral RNA Mini Kits (Qiagen, Shanghai, China) according to the manufacturer’s instructions.

cDNA was synthesized using random hexamer primers with the Superscript II RT– reverse transcriptase (Invitrogen, Carlsbad, CA).

**Detection of hMPV**

Screening for hMPV was conducted using traditional PCR methods. hMPV forward (5’-CCC TTT GTT TCA GGC CAA-3’) and reverse (5’-GCA GCT TCA ACA GTA GTC G-3’) primers, which target the M gene and generate a 416-bp product, were used as described previously [Bellau-Pujol et al., 2005]. All PCR products were purified using the QIAquick PCR purification kit (Qiagen). The reaction mix contained 10 pmol of each primer and 1.25 units of ExTaq DNA polymerase (Takara Bio Inc., Tokyo, Japan). Reactions were incubated at 94°C for 8 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, followed by a final extension at 72°C for 10 min.

**Nucleotide Sequence Analysis**

All PCR products were purified using the QIAquick PCR purification kit (Qiagen) and cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and then sequenced. Sequences were determined and analyzed using the DNASTAR software package. Phylogenetic analysis was performed with Mega version 5.2 by using 1,000 bootstrapped replicates and the neighbor-joining algorithm. The GenBank accession numbers of the previously published sequences are as follows: CAN98-74(AY145258), CAN98-75(AY145259), CAN99-81(AY145264), CAN97-82(AY145265), CAN00-12(AY145267), CAN00-14(AY145269), CAN00-16(AY145271), BJ1887(DQ843659) and JPS03-240(AY530095).

**Screening for Other Respiratory Viruses**

A standard reverse transcription-PCR technique was used to screen for respiratory syncytial virus (RSV), human rhinovirus (HRV), influenza viruses (IFVA, IFVB), parainfluenza viruses (PIV types 1–3), human coronaviruses HKU1 (HCoV-HKU1), and NL63 (HCoV-NL63), and PCR for adenovirus (ADV) and human bocavirus (HBOV) [Hierholzer et al., 1993; Bellau-Pujol et al., 2005].

**Statistical Analysis**

Inferential statistics performed included non-parametric Mann-Whitney U test for continuous variables, with data reported as medians and 25th and 75th interquartile ranges (IQR). Categorical variables were compared using the chi-square test or Fisher’s exact test. Multivariate logistic regression analysis was used to identify independent risk factors. Variables with a plausible relationship with P values less than 0.2 in the univariate analysis were entered into the multivariate analyses. Results are summarized as odds ratios (OR) and 95% confidence intervals (CI). All analyses were performed using SPSS version 20.0 software (SPSS Inc., Chicago, IL). P < 0.05 was considered statistically significant.

**RESULTS**

**Patient Characteristics**

In total, 2613 patients were included, and about 100 cases were excluded for samples missing when

**MATERIALS AND METHODS**

**Patients and Specimens**

In our research group has reported that hMPV is an important pathogen causing acute lower respiratory tract infections in children of the Hunan Province [Xiao et al., 2013]. To further explore the epidemiological and clinical features of various genotypes of hMPV, we summarized the data on the epidemiological and clinical characteristics of various genotypes of hMPV are currently limited.

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**RESULTS**

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Clinical Features of Human Metapneumovirus Genotypes

Of 2613 respiratory specimens tested in the study period, at least one respiratory virus was detected in 1629 (62.34%). The most commonly detected virus was RSV (503, 19.25%), followed by HRV (475, 18.18%), PIV-3 (317, 12.13%), HBOV (224, 8.57%), ADV (153, 5.86%), hMPV (135, 5.2%), IFV (62, 2.37%), IFVA (51, 1.95%), HCoV-HKU1 (20, 0.77%), PIV-1 (15, 0.57%), PIV-2 (11, 0.42%), HCoV-NL63 (11, 0.42%). Sixty-two of 135 (45.9%) hMPV-positive samples were found to be coinfected with other respiratory viruses, including twenty-two with HBOV, seventeen with RSV, fifteen with HRV, thirteen with PIV3, six with ADV, three with IFV, two with PIV4, two with HCoV-HKU1, and one with HCoV-NL63. HBOV was the most common coinfected virus, accounting for 22/62 (35.5%) coinfections. Among the patients with co-detections, 36.3% (49/135) were positive for hMPV plus one additional viral agent and 9.6% (13/135) were positive for hMPV plus two or three additional viruses; of these 9.6%, seven patients were positive for three and six patients were positive for four viral agents. The epidemiological and clinical characteristics of patients infected with hMPV single infection and patients with hMPV co-detections were similar. However, rhonchus were more common in patients with hMPV co-detections (χ² = 8.427, P = 0.004) (Table I). The univariate of cyanosis, crackles, and rhonchus were entered into the multivariate analyses, and rhonchus was associated with hMPV co-infection (OR = 3.057; 95% CI 1.463–6.385; P = 0.003).

**Epidemiology of hMPV**

Approximately 6.1% (103/1698) of the males and 3.5% (32/915) of the females tested positive for hMPV in this study. The male-to-female ratio in the hMPV-infected group was 3.2:1 and differed significantly between the patients with and without hMPV infections (χ² = 8.007, P = 0.005). The age of patients infected with hMPV varied from 20 days to 12 years of age (median [IQR], 10 (6–16) months), and 68.1% (92/135) were ≤ 12 months of age. Children 7–12 months of age exhibited the highest infection rate (7.2%). The hMPV infection rates among four age groups differed significantly (χ² = 12.808, P = 0.005; Fig. 1). hMPV infections occurred most frequently in spring. The positive rate of hMPV in spring reached 10.7% (65/607). The seasonal distribution exhibited statistically significant differences in the positive rates of hMPV among four different seasons (χ² = 57.483, P = 0.000; Fig. 2).

**Clinical Characteristics of hMPV in Children**

Among the 135 pediatric patients with acute lower respiratory tract infections who tested positive for hMPV, the majority had developed a cough (31 patients, 97.0%), 70 experienced a fever (51.9%), 79 suffered from wheezing (58.5%), 99 (73.3%) developed moist rales, and rhonchi were audible in 56 patients (41.5%). In addition, 16 patients experienced shortness of breath (11.9%), 5 developed cyanosis (3.7%), 19 had diarrhea (14.1%), and 15 required supplemental oxygen (11.1%). Thirty-five patients developed underlying disorders or complications (25.9%), including 6 patients had thrush, 6 patients had granulocytopenia, 3 children had congenital heart defects, 3 children had cytomegavirus infection, and some other diseases or complications. All hospitalized patients exhibited good prognoses and the median length of hospital stay was 7 (IQR 6–9) days.

**Phylogenetic Analysis of hMPV**

The sequence of positive products and standard sequences from GenBank exhibited high homology (96%–100%). Phylogenetic analyses indicated that the 135 hMPV specimens were classified into the two main genetic lineages, A and B. Ninety-eight (98/135, 72.6%) hMPV strains were subgroup A2b, thirty-six (26.6%) strains were subgroup B1, one was subgroup B2, and none were either subgroup A1 and A2a. The nucleotide and deduced amino acid sequences of the M gene of 135 hMPV specimens were compared with those of hMPV strains available at the GenBank site (Fig. 3).

**Epidemiological and Clinical Characteristics of hMPV Genotypes A and B**

The epidemiological and clinical data of 135 pediatric patients infected with type A (98 cases) or type B hMPV (37 cases) were summarized and analyzed.
|                          | hMPV-A (n = 98) | hMPV-B (n = 37) | P-value | single hMPV-A (n = 51) | single hMPV-B (n = 22) | P-value | single infection hMPV (n = 73) | P-value | co-infection hMPV (n = 62) | P-value |
|--------------------------|-----------------|-----------------|---------|------------------------|------------------------|---------|-------------------------------|---------|-----------------------------|---------|
| Male gender              | 75(76.5)        | 28(75.7)        | 0.917   | 39(76.5)               | 18(81.8)               | 0.762a  | 57(78.1)                       | 46(74.2) | 0.596                       |
| Age, months, median(IQR)b | 10 (6–23.25)    | 10 (5.5–15.5)   | 0.501   | 11 (6–24)              | 7.5 (5.0–11.25)        | 0.098   | 10 (6–20)                      | 8 (5–16) | 0.599                       |
| hMPV-A                   | 12(32.7)        | 12(32.4)        | 0.598   | 16(31.4)               | 8(36.4)                | 0.349a  | 24(32.9)                       | 20(32.3) | 0.958                       |
| hMPV-B                   | 15(40.5)        | 15(40.4)        | 0.501   | 16(31.4)               | 3(13.6)                |         | 19(26.0)                       | 14(22.6) | 0.326                       |
| LOS, days, median(IQR)b  | 7 (6–10)        | 8 (7–9)         | 0.322   | 7 (6–9)                | 8 (6.75–9)             | 0.296   | 7 (6–9)                       | 8 (6–10) | 0.326                       |
| Fever                    | 50(51.0)        | 20(54.1)        | 0.753   | 31(60.8)               | 9(40.9)                | 0.084   | 40(54.8)                      | 30(48.4) | 0.458                       |
| Cough                    | 95(96.9)        | 36(97.3)        | 1       | 49(96.1)               | 21(95.5)               | 1       | 70(95.9)                      | 61(98.4) | 0.624a                      |
| Wheezing                 | 54(55.1)        | 25(67.6)        | 0.190   | 28(54.9)               | 16(72.7)               | 0.153   | 44(60.3)                      | 35(56.5) | 0.653                       |
| Shortness of breath      | 11(11.2)        | 5(13.5)         | 0.767a  | 7(13.7)                | 4(18.2)                | 0.724a  | 11(15.1)                      | 5(8.1)   | 0.210                       |
| Cyanosis                 | 2(2.0)          | 3(8.1)          | 0.126h  | 2(3.9)                 | 3(13.6)                | 0.157h  | 6(8.2)                        | 0        | 0.062h                      |
| Crackles                 | 70(71.4)        | 29(78.4)        | 0.415   | 39(76.5)               | 18(81.8)               | 0.762a  | 57(78.1)                      | 42(67.7) | 0.176                       |
| Rhonchus                 | 45(45.9)        | 11(29.7)        | 0.089   | 19(37.3)               | 3(13.6)                | 0.044   | 22(30.1)                      | 34(54.8) | 0.004                       |
| Diarrhea                 | 10(10.2)        | 9(24.3)         | 0.035   | 6(11.8)                | 5(22.7)                | 0.289a  | 11(15.1)                      | 8(12.9)  | 0.718                       |
| Supplemental oxygen      | 8(8.2)          | 7(18.9)         | 0.120a  | 4(7.8)                 | 3(13.6)                | 0.424   | 7(9.6)                        | 8(12.9)  | 0.541                       |
| Coinfection              | 47(48.0)        | 15(40.5)        | 0.440   | /                     | /                     | /       | /                            | /        | /                           |
| Underlying disease       | 22(22.4)        | 13(35.1)        | 0.134   | 11(21.6)               | 5(22.7)                | 1       | 16(21.9)                      | 19(30.6) | 0.249                       |
| Seasonal distribution    |                |                 | 0.366a  | /                     | /                     | /       | /                            | /        | /                           |
| Spring                   | 49(50.0)        | 16(43.2)        | 0.570a  | 30(58.8)               | 10(45.5)               | 40(54.8) | 25(40.3)                      |         | 0.368                       |
| Summer                   | 8(8.2)          | 6(16.2)         | 3(5.9)  | 3(13.6)                | 6(8.2)                 | 8(12.9)  | 6(8.2)                       |         | 0.210                       |
| Autumn                   | 8(8.2)          | 5(13.5)         | 5(9.8)  | 2(9.1)                 | 7(9.6)                 | 6(9.7)  | 6(9.7)                       |         | 0.210                       |
| Winter                   | 33(33.7)        | 10(27.0)        | 7(31.8) | 20(27.4)               | 23(37.1)               |         |                              |         | 0.210                       |

*Fisher’s exact test,*
*Mann-Whitney U test, all the other: Chi-square test.

hMPV-A indicate type A hMPV, hMPV-B indicate type B hMPV, single hMPV-A indicate single type A hMPV infection, single hMPV-B indicate single type B hMPV infection.
Seasonal distribution: Spring (March-May), Summer (June-August), Autumn (September-November), Winter (December-February).
IQR, interquartile range; LOS, length of hospital stay.
The results of the distribution of epidemic seasons indicated that hMPV genotypes A and B prevailed alternately in different years. From the autumn of 2007 to the summer of 2008, hMPV genotype A2b was the predominant epidemic strain. From the autumn of 2008 to the winter of 2009, the predominant genotype of hMPV changed to combined A2b and B1 genotype. From the spring to the winter of 2010, hMPV genotype B1 became the predominant epidemic strain (Fig. 4). Comparison studies revealed that the epidemiological data and clinical characteristics were largely similar between the patients infected with genotype A hMPV and patients infected with genotype B hMPV. However, patients infected with genotype B hMPV developed diarrhea more frequently compared with pediatric patients infected with genotype A hMPV ($\chi^2 = 4.328, P = 0.035$). Excluding the factor of mixed infection, the epidemiological and clinical characteristics of patients infected with type A hMPV alone and patients infected with type B hMPV alone were also basically similar. However, rhonchus were more common in patients with type A hMPV infection ($\chi^2 = 4.072, P = 0.044$).

The univariates of fever, wheezing, cyanosis,
rhonchus were entered into the multivariate analyses, and rhonchus was associated with type A hMPV infection (OR = 0.164; 95%CI 0.036–0.751; P = 0.020).

DISCUSSION

The present study summarized the prevalence and clinical features of hMPV infections in Hunan Province between September 2007 and February 2011, including the 76 hMPV cases (Sep. 2007–Aug. 2008) reported in the preliminary study [Xiao et al., 2013]. The present study revealed that the hMPV positive rate was 5.2%, in hospitalized children with acute lower respiratory tract infections in Hunan Province, that was similar to Beijing [Zhu et al., 2011; Lu et al., 2013], higher than Southern China (2.6%) [Cai et al., 2014], and lower than Chongqing (10.2%) [Zhang et al., 2012] and Taiwan (23%) [Wei et al., 2013]. Compared with studies in other countries, that was higher than the reported rate in Japan (2.5%) [Matsuzaki et al., 2008], lower than the reported rate in Germany (11.9%) [Reiche et al., 2014], and similar to the reported rates in the United States [van den Hoogen et al., 2003; Edwards et al., 2013] and South Korea [Kim et al., 2012] (6% and 7.1%, respectively). The variation in hMPV detection rates between various regions may be related to the differences in climate, geographic locations, year and human populations.

The present study found that the hMPV positive rate was higher in male pediatric patients, which is consistent with the most of the previous studies [Peiris et al., 2003; van den Hoogen et al., 2003; Wei et al., 2013], but different from a study in the United States which found hMPV infections were equally distributed between males and females [McAdam et al., 2004]. Furthermore most previous studies have indicated that the majority of hMPV-positive patients are children of young ages [McAdam et al., 2004; Vicente et al., 2006; Lu et al., 2013; Wei et al., 2013; Reiche et al., 2014], which is similar to the findings of the present study. The peak of hMPV infections was found in the spring in the present study, which is similar to that of Beijing [Zhu et al., 2011], but different from that of Chongqing where the peak of hMPV epidemics are observed in the spring/summer and the winter/spring seasons [Zhang et al., 2012]. In the United States, hMPV infections have been reported to occur predominately in the winter and spring seasons [McAdam et al., 2004]. These unique characteristics of hMPV infections may be related to the geographic region and climate, and requires the further study for verification.

In the present study the most common clinical symptoms of hMPV-positive patients included coughing, wheezing and fever, which in some pediatric patients, were accompanied by shortness of breath, cyanosis and moist rales heard in pulmonary auscultation, which were similar to those reported previously [Zhang et al., 2012; Lu et al., 2013; Wei et al., 2013]. The present study revealed a rather high mixed infection of hMPV with other viruses (45.9%), and HBOV was the most frequently detected virus with hMPV. The rates of hMPV coinfection of previous studies range from approximately 9.4% to 70.8% [Zhu et al., 2011; Fathima et al., 2012; Kim et al., 2012; Zhang et al., 2012; Lu et al., 2013; Wei et al., 2013; Cai et al., 2014], and the commonly co-detected viruses include RSV, HRV and PIV-3. These discrepancies are most likely due to the differences in the types of viruses examined and the test methods employed in the studies. However, the discrepancies may also be related to the selection of research objects, geographic regions and research periods. Previous studies have indicated that mixed infection may aggravate the diseases and affect prognosis [Greensill et al., 2003]. In the present study, lung auscultation did reveal that rhonchi were more common in patients with mixed hMPV infection compared with patients with single hMPV infection. However, no other significant differences were found between the patients, including the requirement of supplemental oxygen and duration of hospitalization.

Consistent with the findings reported by Xiao et al. [2013], the present study revealed that both hMPV genotype A and genotype B were prevalent in the Changsha area from September 2007 to February 2011. hMPV genotypes A2 and B1 were the most
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predominant genotypes. The annual distribution indicated that genotype A2 and genotype B1 prevailed alternately. Previous studies have reported the prevalence of certain predominant hMPV genotypes in a given year. hMPV sub-genotypes may vary in different years and alternative predominance of hMPV sub-genotypes has been observed [Matsuuzaki et al., 2008; Pitoiset et al., 2010; Kim et al., 2012; Lu et al., 2013; Wei et al., 2013]. The present study found that the epidemiological characteristics and clinical features were fundamentally similar among patients infected with different genotypes of hMPV, which is consistent with the results of some studies [Bosis et al., 2006; Wei et al., 2013]. Diarrhea was a clinical manifestation that occurred more frequently in patients infected with type B hMPV compared with patients infected with type A hMPV in this study. However, there are different epidemiological and clinical findings in other two studies [Matsuuzaki et al., 2008; Kim et al., 2012]. Furthermore, a study indicated that patients with type A hMPV infection are often associated with more severe symptoms [Vicente et al., 2006]. However, there is no apparent link between the genotype of hMPV and the severity factors of the disease in other studies [Agapov et al., 2006; Pitoiset et al., 2010]. Certainly, more data are needed to elucidate this issue.

In conclusion, the present study explores the epidemiological and clinical manifestations of infections with various genotypes of hMPV and especially the predominant prevalence of hMPV genotypes in different years. However, retrospective analysis was conducted in the present study without control samples, and the sample sizes of certain sub-genotypes of hMPV were small. These are the major limitations. And a long period of data accumulation with a larger sample size and control is required in the future study.

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