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BRIEF REPORT

Prevalence of human metapneumovirus in hospitalized children with respiratory tract infections in Tianjin, China

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Abstract Human metapneumovirus (hMPV) has recently been recognized as an important respiratory pathogen, especially in children. At present, our understanding of the characteristics of hMPV from China is very limited. Nasopharyngeal aspirates were taken from 310 hospitalized pediatric patients. Twenty (6.5%) of them were infected with hMPV, and they all developed pneumonia. Sixty five percent (13/20) of the cases were under 12 months. Phylogenetic analysis of F gene fragments indicated that three sub-genotypes of hMPV(A2a/A2b, B1,B2) circulated in Tianjin and A2b was the predominant subtype. The Vero-E6 cell line was better than LLC-MK2 for hMPV isolation. Three hMPV strains were successfully isolated using the Vero-E6 cell line.

Introduction

Acute respiratory tract illness (ARTI) is the most common disease experienced by people of all ages worldwide, especially young children. About 4,000,000 children die of pneumonia caused by ARTI [1]. About 90% of pathogens causing respiratory infection are viruses. Previous researches have demonstrated that human respiratory syncytial virus (RSV), influenza virus types A and B, and parainfluenza virus types 1–3, adenoviruses, coronaviruses, rhinoviruses and enteroviruses are important causes of ARTI. However, about 30% of ARTI pathogens remain unidentified [2].

In 2001, a new respiratory virus, hMPV, was first isolated from nasopharyngeal aspirate (NPA) specimens from children with ARTI [3]. Based on genetic and phylogenetic analysis, hMPV was categorized as a member of the genus *Metapneumovirus* of the subfamily *Pneumovirinae* of the family *Paramyxoviridae* [4]. Subsequently, the prevalence of hMPV has been reported worldwide [5–10]. hMPV infections have been reported in all age groups, with more severe disease occurring in young children under 2 years, immunocompromised individuals, and the elderly [3, 11]. Several seroprevalence studies have shown that 90–100% of children are infected with hMPV by the age of 5–10 years, and 5–7% of children are hospitalized for severe ARTI [12].

The Chinese mainland covers a vast territory. Tianjin is located in the northeastern part of China, which has a high prevalence of respiratory infections during the winter and spring seasons, but the prevalence of hMPV in this area is still unclear. To our knowledge, in mainland China, successful hMPV isolation has been reported only in Chongqing, a southwestern city. The objective of this study was to investigate the prevalence of hMPV in infants and young children who presented with ARTI in Tianjin, to identify the molecular characteristics of this virus, and to isolate hMPV in our laboratory.

Materials and methods

Three hundred and ten patients hospitalized for ARTI from February to May 2006, March to April 2008 and September

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2008 to February 2009 in Tianjin children's hospital were enrolled in the present study. The mean age was 9.7 months (ranging from 3 h after birth to 12 years). Among them, 264 (85.2%) were ≤1, 29 (9.3%) were 1–2, 12 (3.9%) were 2–5, and 5 (1.6%) were >5 years old. Two hundred and eighteen (70.3%) were male. Two hundred and eighty-two (91.0%) were pneumonia cases, 15 (4.8%) had bronchitis, and 13 (4.2%) had other respiratory infections. NPA samples were collected and rapidly transported on ice to the laboratory.

RNA was extracted from 200 µl specimens using NucliSENS easyMAG (BioMerieux, France) according to the manufacturer's instructions. The nucleoprotein (N) and fusion (F) fragments of hMPV were detected by nested RT-PCR method. Specific primer sequences and PCR amplification conditions have been described before [13, 14]. hMPV N-fragment-positive PCR specimens were detected by multi-PCR using Seeplex^{TM}RV Detection Kit-1 (Seegene, Korea) for other respiratory viruses according to the manufacturer's instructions. First-strand of cDNA was synthesized using a RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, USA). hMPV F-fragment-positive products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced by Invitrogen Biotechnology Co. Ltd., in Shanghai, China, using an ABI 3730 DNA Analyzer (Applied Biosystems, USA). Nucleotide sequence alignments were generated using the Clustal W algorithm of the MEGA 4.0.1 (http://www.megasoftware.net/mega.html) software. The nucleotide sequences identities were calculated by using DNAStar (version 5.01). Phylogenetic trees were constructed by MEGA, using the neighbor-joining method. Bootstrap proportions were plotted at the main internal branches of the phylogram to show support values. Other hMPV F gene sequences used in this study and their GenBank accession numbers are as follows: CAN00-14 (AY145299) and NL/1/00 (AF371337) for the A1 subtype; CAN97-83 (AY145296) and NL/17/00(AY304360) for the A2a subtype; JPS03-240 (AY530095) and BJ1887 (DQ843659) for the A2b subtype; CAN97-82 (AY145295) and NL/1/99 (AY525843) for the B1 subtype; CAN98-76 (AY145290), NL/1/94 (AY304362), and BJ1816 (DQ843658) for the B2 subtype; and APV-C (AY590688).

hMPV-positive NPA samples identified by RT-PCR were inoculated onto LLC-MK2 and Vero-E6 monolayer cells as reported previously [15]. After adsorption for about 1 h, the monolayer cells were incubated at 37°C in MEM containing 2% fetal bovine serum (FBS) and 0.025% or 0.013% trypsin (Salabrio, Beijing, China), the cytopathic effect (CPE) was observed two or three times per week, the medium was changed once per week. Total RNA of each CPE-positive culture was extracted, the hMPV N and F gene fragments were then amplified by RT-PCR using the outer primer sets, and positive products were sequenced. Samples without CPE were passaged blindly three times.

Results

Of the 310 specimens tested, 20 (6.5%) were positive for hMPV N gene fragments. Two (10%) of the hMPV-positive samples (TJ08-07, TJ06-06) also were positive for rhinovirus and adenovirus, respectively. All 20 hMPV-positive patients suffered from pneumonia, accounting for 7.1% (20/282) of all pneumonia cases tested in this study. The mean age of 20 cases was 25 months (ranging from 16 days to 9 years), namely, 13 cases (65.0%) were ≤1 year, 5 (25%) were 1–2 years, 1 (5%) was 2–5 years, 1 (5%) was >5 years. Among them, 12 (60%) were male. Nearly all months tested in the study had hMPV-positive samples except March 2008, October 2008 and January 2009. The monthly distribution is shown in Fig. 1.

Eighteen hMPV F gene sequences (681 nt) obtained from 20 N-gene-positive samples were used to construct a phylogenetic tree (Fig. 2).The tree showed two genetic types, A and B, two subtypes within type B and three subtypes within type A. A2a/A2b, B1, B2 subtypes of hMPV co-existed in Tianjin. No significant seasonal distribution was observed. Of the 18 strains, 5% (1/18) was A2a subtype, 65% (13/18) were A2b subtype, 5% (1/18) was B1 subtype, and 15% (3/18) were B2 subtype. A2b was the most prevalent subtype in Tianjin, China, accounting for 44.4% (4/9) in 2006, 87.5% (7/8) in 2008, and 66.7% (2/3) in 2009. It should be noted that the A2b subtype in Tianjin had a few differences compared with the same subtype in Beijing (BJ1887) and in Japan (JPS03-240). Compared with BJ1887, 13–17 nucleotides were

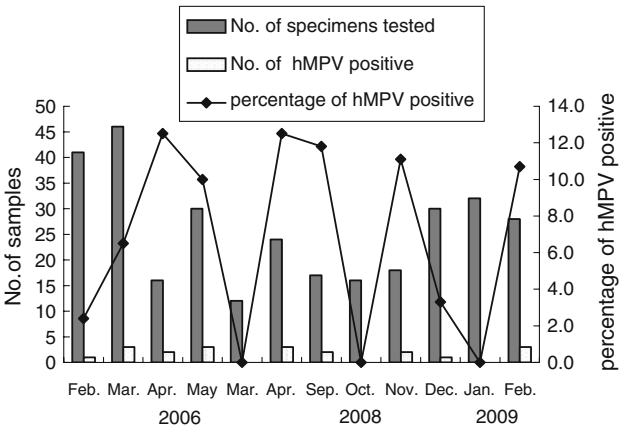

Fig. 1 Monthly distribution of hMPV-positive samples among respiratory specimens submitted to the laboratory in 2006, 2008, and 2009

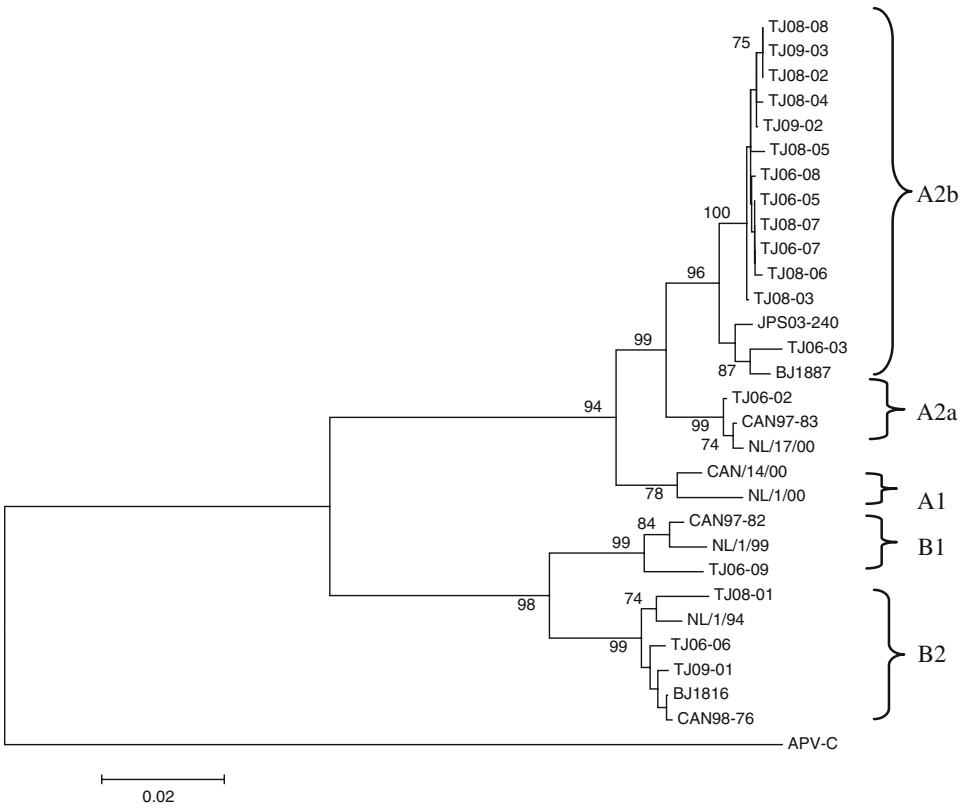

Fig. 2 Phylogenetic analysis of partial sequences of the F gene of hMPV (681 nt; 79–759 nt). hMPVs was designated by an abbreviation of their place of origin (TJ = Tianjin), followed by the year of isolation and unique strain number. The tree was constructed by the neighbor-joining method with 100 bootstrap replicates. Isolates from the Netherlands (NL/1/00, NL/1/99, NL/1/94, NL/17/00), Japan (JPS03-240), Canada (CAN00-14, CAN97-82, CAN97-83, CAN98-76), and Beijing (BJ1887, BJ1816) and avian pneumovirus subgroup C (APV-C) were included in the analysis

Table 1 Nucleotide and amino acid identities of hMPV F gene fragments of Tianjin strains between different subtypes and within the same subtype

Subgroup (no. of strains)	Nucleotide and amino acid identity (%)		
	A2	B1	B2
A2 (14)	95.0–100.0 (97.3–100.0)	84.1 (95.5)	82.5–85.7 (93.8–96.5)
B1 (1)		100.0 (100.0)	93.5–93.9 (98.7–99.1)
B2 (3)			97.2–99.6 (99.6–100.0)

Numbers before parentheses are nucleotide identities. Numbers in parentheses are amino acid identities

different, while with JPS03-240, 10–13 nucleotide differences were found, but among the A2b subtype strains obtained in the present study, at most, 5 nucleotides varied. Analysis of F gene fragments indicated that nucleotide and deduced amino acid sequence identity between types A and B was 82.5–85.7% and 93.8–96.5%, respectively, whereas between subtypes B1 and B2, it was 93.5–93.9% and 98.7–99.1%, respectively. The sequences within A2 shared a higher nucleotide identity of 95.0–100.0%, while within B2 they shared 97.2–99.6% identity, as shown in Table 1.

Three hMPV-positive specimens identified by RT-PCR were inoculated onto LLC-MK2 and Vero-E6 cells, and no CPE was observed on day 16 after inoculation. Then, we extracted RNA from cultures of the two cell lines. The N and F genes were positive in Vero-E6 cultures, but negative in LLC-MK2 cultures by RT-PCR as described above. Then, the PCR-positive Vero-E6 cell cultures were inoculated onto another flask of LLC-MK2 or Vero-E6 cells. Six days later, CPE (focal round cells, increased granules, cell disruption, and subsequent detachment) was observed in Vero-E6 cells. On day 9, most Vero-E6 cells were detached from the cell monolayer, but LLC-MK2 only showed mild CPE on day 10 after inoculation, which was easily confused with cell aging, as shown in Fig. 3. The F fragment nucleotide sequences obtained from the passage-2 cultures were identical to those from the corresponding samples. It may be concluded that hMPV strains were successfully isolated in Vero-E6 cells and that hMPV could also be propagated in LLC-MK2 cells, but Vero-E6 cells are more susceptible to hMPV than LLC-MK2 cells are. We also found that hMPV isolation is dependent on the concentration of trypsin. Cells could not be maintained for

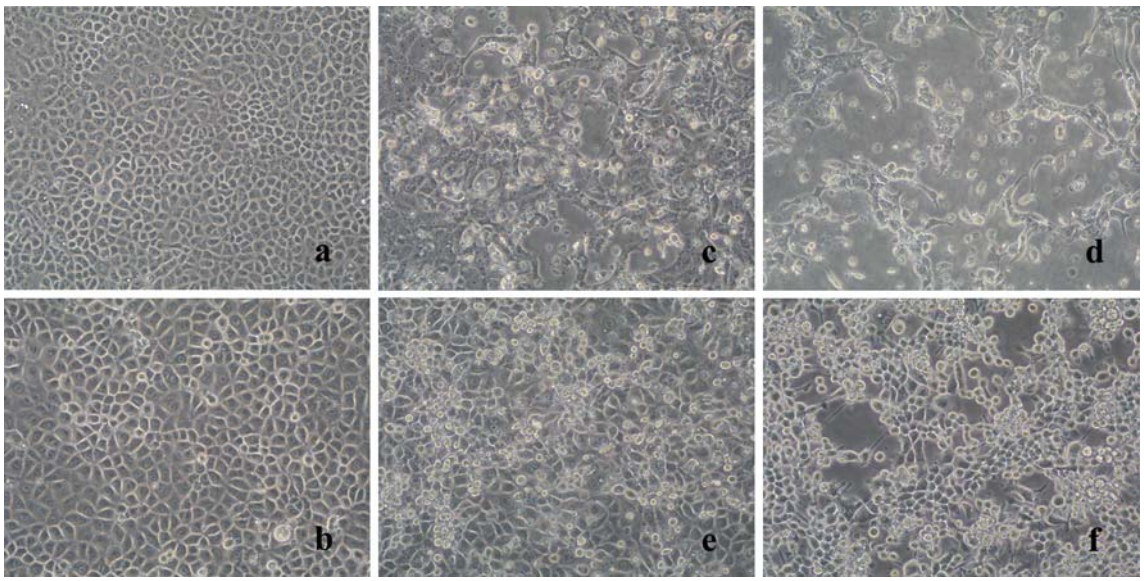

Fig. 3 Micrographs of hMPV uninfected **a**, **b** and infected **c**–**f** Vero-E6 cells and LLC-MK2 cells. **c**, **d** Vero-E6 cell culture 6 and 9 days after inoculation with sample TJ09-01 in passage 2. Early CPE was recognized as granular and roundup cell formation, cell destruction, then detachment from the cell monolayer. **e**, **f** LLC-MK2 cell culture 10 and 15 days after inoculation with Vero-E6 cell culture of TJ09-01. LLC-MK2 cells CPE was not as clear as in Vero-E6. Magnification, ×200

a long period when 0.025% trypsin was used in the culture medium, whereas when using 0.013% trypsin, cells could grow well and be maintained for about 1 month.

Discussion

The presence of hMPV has been reported worldwide [5–10, 16, 17]. Data from the present study showed the incidence of hMPV in Tianjin was 6.5%, similar to 8.9% in Japan [5], 6.4% in America [8], 6.6% in France [9], and 5.5% in Hong Kong [10], but lower than 30% in Beijing, China [16].

Clinical manifestations associated with hMPV infections in young children range from common colds and mild upper respiratory tract infections to more severe lower respiratory tract infections, such as bronchiolitis and pneumonia. Several studies have demonstrated that hMPV is more likely to be associated with bronchiolitis in early childhood [17, 18]. However, other investigations, including the present study, showed that hMPV is more frequently associated with pneumonia than with bronchiolitis [19–21]. Data obtained from the present study indicate that 7.1% of children hospitalized with pneumonia in Tianjin suffer from hMPV infection. Some research has demonstrated that hMPV is involved in cases of coinfections, mainly with RSV, but the frequency and severity of symptoms vary greatly between reports [21, 22]. In this study, two hMPV-positive patients were simultaneously infected with adenovirus and rhinovirus, but no coinfection with RSV was found.

hMPV infections seem to have a seasonal distribution (i.e., winter and spring) in temperate regions [6, 8, 23, 24]. However, hMPV outbreaks have occurred during the spring–summer seasons in Hong Kong [10]. Although most studies have limited their surveillance to the typical respiratory season, other reports suggest that hMPV may also be circulating throughout the year [11, 25]. Data from the present study show that there was no significant seasonal distribution in Tianjin. Notably, three hMPV-positive specimens in 2006 and two in 2008 were collected in May (spring) and September (summer), indicating that hMPV could be prevalent in summer.

Primary hMPV infections seem to occur at a young age (<1 year), and the incidence is the same for males and females [21]. Seroprevalence studies have shown that 90–100% of children are infected by the age of 5 [3, 26]. Our results agree with those of Samransamruajkit et al. [7], that more (65.0%) hMPV-infected cases were less than 1 year old, and males were more (60%) susceptible to hMPV.

Currently, four distinct major hMPV phylogenetic subtypes, A1, A2, B1, and B2, have been described [26–28]. In 2006, Huck et al. [29] described a novel subtype within the A2 subtype, after which the A2 subtype was divided into A2a and A2b. In the present study, we found that 70% (14/20) of hMPVs detected in Tianjin were of the A2 subtype, and the F fragment nucleotide sequences shared an identity of 95.0–100.0%. Among them, 92.9% (13/14) were A2b

subtype, and the nucleotide sequence identity within the A2b subtype was 99.4–100%, except for an A2b strain (TJ06-03) that shared a lower identity of 96.1–96.6% with other A2b subtype strains (data not shown). We also found that A2b (65%) was the predominant subtype circulating in Tianjin. It was observed that there was no correlation between the prevalent type and the season. To our knowledge, this may be the first report of the A2b subtype circulating in mainland China and becoming the most prevalent subtype in Tianjin. In the report by Huck et al. [29], approximately one-third of all genotypes detected in Germany during two consecutive seasons were classified as A2b. Phylogenetic analysis in our study showed BJ1887, detected in Beijing, China, which had six nucleotide differences in the F gene fragment compared to the Japanese strain JPS03-240, should also be classified as A2b.

Van den Hoogen et al.[3] demonstrated that hMPV had been circulating among humans for at least half a century , but then why was it not identified up to 2001? The main reason is that it cannot replicate in the common cell lines, MDCK, HeLa, and RD, which are usually used to isolate respiratory viruses. The initial hMPV isolation was obtained in tertiary monkey kidney (tMK) cells [3], and the cells usually displayed CPE on days 10–14 post-inoculation, which was longer than for other respiratory viruses. Subsequently, several groups have reported hMPV isolation in LLC-MK2 cells [11, 23, 24]. Further, it has been reported recently that the infection efficiency of hMPV in Vero-E6 cells was better than in LLC-MK2 cells [30]. At present, the only successful isolation of hMPV in mainland China was in Chongqing. In this study, we tried to culture hMPV in LLC-MK2 and Vero-E6 cells. The results showed that Vero-E6 cells were more sensitive than LLC-MK2 for hMPV, and Vero-E6 can be maintained for a longer period compared to LLC-MK2, which is more important for hMPV replication. We must point out that three aspects were ‘a priori’ for improved isolation of hMPV. First, the medium used to transport specimens, MEM without FBS, is superior to Hanks’ or PBS. Second, specimens should be inoculated onto cells as soon as possible. Finally, our results indicate that the concentration of trypsin (0.13%) was better suited for hMPV isolation than 0.025%, which could be destructive to the cells during prolonged incubation.

In conclusion, our study demonstrated that: (1) hMPV was an important viral pathogen among hospitalized pediatric patients in Tianjin who suffered from pneumonia. (2) Three subtypes, A2a/A2b, B1, B2, co-circulated in Tianjin, and A2b was the most prevalent subtype. (3) Transport medium for samples and trypsin concentration in culture medium are important for hMPV isolation, and Vero-E6 cells are more sensitive to hMPV than LLC-MK2 cells.

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References

1. Zar HJ (2002) Respiratory infections in children in developing countries. Pediatr Ann 31:133–138
2. Ruiz M, Ewig S, Marcos MA, Martinez JA, Arancibia F, Mensa J, Torres A (1999) Etiology of community-acquired pneumonia: impact of age, comorbidity, and severity. Am J Respir Crit Care Med 160:397–405
3. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD (2001) A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 7:719–724
4. Lamb RA, Kolakofsky D (2001) Paramyxoviridae the viruses and their replication. In: Knipe DM, Howley PM (eds) Field’s virology, vol 1, 4th edn. Lippincott Williams and Wilkins, Philadelphia, pp 1305–1340
5. Ebihara T, Endo R, Kikuta H, Ishiguro N, Ishiko H, Hara M, Takahashi Y, Kobayashi K (2004) Human metapneumovirus infection in Japanese children. J Clin Microbiol 42:126–132
6. Bastien N, Ward D, Van Caeseele P, Brandt K, Lee SH, McNabb G, Klisko B, Chan E, Li Y (2003) Human metapneumovirus infection in the Canadian population. J Clin Microbiol 41: 4642–4646
7. Samransamruajkit R, Thanasugarn W, Prapphal N, Theamboonlers A, Poovorawan Y (2006) Human metapneumovirus in infants and young children in Thailand with lower repiratory tract infections; molecular characteristics and clinical presentations. J Infect 52:254–263
8. Esper F, Boucher D, Weibel C, Martinello RA, Kahn JS (2003) Human metapneumovirus infection in the United States: clinical manifestations associated with a newly emerging resp iratory infection in children. Pediatrics 111:1407–1410
9. Freymouth F, Vabret A, Legrand L, Eterradossi N, Lafay-Delaire F, Brouard J, Guillois B (2003) Presence of the new human metapneumovirus in French children with bronchiolitis. Pediatr Infect Dis J 22:92–94
10. Peiris JS, Tang WH, Chan KH, Khong PL, Guan Y, Lau YL, Chiu SS (2003) Children with respiratory disease associated with metapneumovirus in Hong Kong. Emerg Infect Dis 9:628–633
11. Boivin G, Abed Y, Pelletier G, Ruel L, Moisan D, Côté S, Peret TC, Erdman DD, Anderson LJ (2002) Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-tract infections in all age groups. J Infect Dis 186:1330–1334
12. Deffrasnes C, Hamelin M, Boivin G (2007) Human metapneumovirus. Semin Respir Crit Care Med 28:213–221
13. Viazov S, Ratjen F, Scheidhauer R, Fiedler M, Roggendorf M (2003) High prevalence of human metapneumovirus infection in young children and genetic heterogeneity of the viral isolates. J Clin Microbiol 41:3043–3045
14. Ludewick HP, Abed Y, van Niekerk N, Boivin G, Klugman KP, Madhi SA (2005) Human metapneumovirus genetic variability, South Africa. Emerg Infect Dis 11:1074–1078
15. Mao H, Yang X, Zhao X (2008) Characterization of human metapneumoviruses isolated in Chongqing, China. Chin Med J 121:2254–2257
16. Zhu RN, Qian Y, Deng J, Wang F, Hu AZ, Lu J, Cao L, Yuan Y, Cheng HZ (2003) Human metapneumovirus may associate with acute respiratory infections in hospitalized pediatric patients in Beijing, China. Zhonghua Er Ke Za Zhi 41:441–444 (in Chinese)

17. García-García ML, Calvo C, Casas I, Bracamonte T, Rellán A, Gozalo F, Tenorio T, Pérez-Breña P (2007) Human metapneumovirus bronchiolitis in infancy is an important risk factor for asthma at age 5. Pediatr Pulmonol 42:458–464
18. do Carmo Debur M, Bordignon J, Duarte dos Santos CN, Vidal LR, Nogueira MB, de Almeida SM, Raboni SM (2007) Acute respiratory infection by human metapneumovirus in children in southern Brazil. J Clin Virol 39:59–62
19. Choi EH, Lee HJ, Kim SJ, Eun BW, Kim NH, Lee JA, Lee JH, Song EK, Kim SH, Park JY, Sung JY (2006) The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000–2005. Clin Infect Dis 43:585–592
20. Abdullah Brooks W, Erdman D, Terebuh P, Klimov A, Goswami D, Sharmeen AT, Azim T, Luby S, Bridges C, Breiman R (2007) Human metapneumovirus infection among children, Bangladesh. Emerg Infect Dis 13:1611–1613
21. Camps M, Ricart S, Dimova V, Rovira N, Muñoz-Almagro C, Garcia JJ, Pons-Odena M, Marcos MA, Pumarola T (2008) Prevalence of human metapneumovirus among hospitalized children younger than 1 year in Catalonia, Spain. J Med Virol 80:1452–1460
22. Greensill J, McNamara PS, Dove W, Flanagan B, Smyth RL, Hart CA (2003) Human metapneumovirus in severe respiratory syncytial virus broncholitis. Emerg Infect Dis 9:372–375
23. Regev L, Hindiyeh M, Shulman LM, Barak A, Levy V, Azar R, Shalev Y, Grossman Z, Mendelson E (2006) Characterization of human metapneumovirus infections in Israel. J Clin Microbiol 44:1484–1489
24. Sarasini A, Percivalle E, Rovida F, Campanini G, Genini E, Torsellini M, Paolucci S, Baldanti F, Marchi A, Grazia Revello M, Gerna G (2006) Detection and pathogenicity of human metapneumovirus respiratory infection in pediatric Italian patients during a winter–spring season. J Clin Virol 35:59–68
25. Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, Wright PF, Crowe JE Jr (2004) Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N Engl J Med 350: 443–450
26. Peret TC, Boivin G, Li Y, Couillard M, Humphrey C, Osterhaus AD, Erdman DD, Anderson LJ (2002) Characterization of human metapneumoviruses isolated from patients in North America. J Infect Dis 185:1660–1663
27. van den Hoogen BG, Herfst S, Sprong L, Cane PA, Forleo-Neto E, de Swart RL, Osterhaus AD, Fouchier RA (2004) Antigenic and genetic variability of human metapneumoviruses. Emerg Infect Dis 10:658–666
28. Ljubin-Sternak S, Santak M, Cepin-Bogović J, Baće A, Vojnović G, Mlinarić-Galinović G, Forcić D, Drazenović V, Falsey AR (2008) Detection of genetic lineages of human metapneumovirus in Croatia during the winter season 2005/2006. J Med Virol 80:1282–1287
29. Huck B, Scharf G, Neumann-Haefelin D, Puppe W, Weigl J, Falcone V (2006) Novel human metapneumovirus sublineage. Emerg Infect Dis 12:147–150
30. Abiko C, Mizuta K, Itagaki T, Katsushima N, Ito S, Matsuzaki Y, Okamoto M, Nishimura H, Aoki Y, Murata T, Hoshina H, Hongo S, Ootani K (2007) Outbreak of human metapneumovirus detected by use of the Vero E6 cell line in isolates collected in Yamagata, Japan, in 2004 and 2005. J Clin Microbiol 45: 1912–1919