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 *Meta pneumovirus* 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
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™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™ 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
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

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)       | 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.
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.
Vero-E6 cells are more sensitive to hMPV than LLC-MK2 culture medium are important for hMPV isolation, and Transport medium for samples and trypsin concentration in Tianjin, and A2b was the most prevalent subtype. (3)

Three subtypes, A2a/A2b, B1, B2, co-circulated in pediatric patients in Tianjin who suffered from pneumonia. hMPV was an important viral pathogen among hospitalized children usually displayed CPE on days 10–14 post-inoculation, which was longer than for other respiratory viruses. The initial hMPV isolation was reported recently that the infection efficiency of hMPV in Vero-E6 cells was better than in LLC-MK2 cells [30]. Further, it has been reported recently that the infection efficiency of hMPV in Vero-E6 cells was better than in LLC-MK2 cells [30].

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 than 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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