封面式樣

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行政院衛生署疾病管制局九十三年度科技研究發展計畫

Human Metapneumovirus 在台灣兒童急性呼吸道疾患中的角色：為期一年的前瞻性研究並同時監測其他呼吸道病毒

研 究 報 告

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＊＊本研究報告僅供參考，不代表本署意見＊＊

Abstract

Human metapneumovirus (hMPV) virus is a newly discovered respiratory pathogen since 2001. Cohorts of young children were prospectively evaluated for hMPV infection during Sep 2003 to Sep. 2004. Patients hospitalized for acute respiratory tract infection (ARTIs) under 15 year-old during this period were enrolled. Real-time reverse-transcriptase polymerase chain reaction was used to detect viral nucleic acid. 17 (4.6%) patients with ARIs were documented for the presence of hMPV from 369 cases. 15 patients (88%) were younger than 2 year-old. Co-infection with other respiratory pathogens was observed in 5 patients. No significant difference was observed between the six patients (6/17, 35%) with underlying disease and those without. MPV infection seems not prolonged their hospitalized course, nor increased oxygen supplement nor increased necessary of intensive care in patients with underlying disease. To compared with those cases of un-identified airway pathogen, no significant difference was found except age (1.5 y/o v.s. 2.7 y/o, p=0.02).

In conclusion, the clinical manifestations were non-specific compared with other respiratory viral infections and the clinical outcomes were excellent in our group. The clinical presentation and outcome were described.

Keywords: metapneumovirus, airway infection, children, real time RT-PCR

中文摘要

Human metapneumovirus 在西元 2001 年首次由荷蘭科學家發表,可在幼兒導致常見呼吸道疾病如急性細支氣管炎,肺炎等 ,是過去人類所不知道的病毒。本計畫針對年齡小於 15 歲患有急性呼吸道疾病的住院孩童,進行呼吸道病原,為期一年前瞻性的篩檢(民國 92 年九月至 93 年九月),採取呼吸道檢體,利用細胞培養檢測流感病毒,副流感病毒,呼吸道融合病毒,及人類 metapneumovirus 的感染。並利用 RT-PCR 偵測 metapneumovirus,以了解急性呼吸道疾患中各病毒分布比率。並分析臨床病程,瞭解這些病毒在台灣引起的臨床疾病,流行季節等,並建立實驗室診斷方式以提供未來臨床上診斷的應用。在收集到的 369 個有效檢體中,有十七位(4.6%)病患的喉頭拭子或咽喉抽吸液可偵測出 metapnuemovirus 陽性的結果. 其中 15 位(88%)病童年齡小於兩歲. 有五位病童的檢體同時被偵測出其他的呼吸道傳染病原. 在十七位 metapnuemovirus 陽性的病童中,有六位(35%)是原本有其他的 underlying disease 的病童, 他們臨床上的表現及嚴重度與沒有其他疾病的健康孩子比較起來, 並未觀察到統計上有意義的不同.Metapnuemovirus 似乎並不會造成有 underlying disease 的病童延長住院天數, 也不會增加氧氣須要量及重症加護照顧的

須要．與那些無法找到呼吸道感染原因的孩童比較起來，除了平均發病年紀在 metapneumovirus 這一組是 1.5 歲，比無法找到呼吸道感染原因的孩童的 2.7 歲來得小以外 (p=0.02),其他在臨床上的表現及嚴重度,兩組病童沒有統計上顯著的差異.

整體來說 ，Metapneumovirus RT-PCR 陽性的病童與其他病因未知的兒童呼吸道感染比較起來，臨床上的表現是不具特異性，而癒後良好的．臨床的表現及預後在本文中有詳細的說明.

中文關鍵詞(至少三個)：metapneumovirus ，急性呼吸道疾病,兒童, real time PCR.

Introduction

Human metapneumovirus virus is a newly recognized respiratory tract pathogen in Netherlands (1). It belongs to a new member of the family *Paramyxoviridae*, subfamily *Pneumovirus.* Disease caused by hMPV appears similar to respiratory syncytial virus (hRSV), which is the most common cause of viral respiratory tract infection in children (2). Patients with symptoms and signs ranging from a wheezing bronchilitis to require assisted ventilation (3). The virus appeared to infect the majority of the Netherlands' children by age of 5(1). From the seroepidemiology study in Japan, the children had also been exposed to hMPV by the age of 10 years (4).

Because the virus is difficult to culture, the need for a reliable and rapid diagnostic tool is significant. The virus was identified in children or adults with respiratory tract infection in Australia, Canada, United Kingdom, France and United States by reverse transcription-polymerase chain reaction (3, 5-8). The identification of the virus in several countries indicates that it is prevalent worldwide. In this study, we sought to define the role of the virus in respiratory tract infection in hospitalized children in Taiwan. Furthermore, to introduce the more sensitive and time-saving method of real-time RT-PCR for viral detection.

Methods

Patients. The hospitalized patients with acute respiratory tract symptoms and signs were recruited into the study. The defined respiratory tract symptoms included cough, rhinorrhea, sore throat, sneezing, or dyspnea. The age group was limited to the children under 15 year- old. The throat swabs or nasopharyngeal aspirations were taken after admission. Samples are labeled then frozen at –80 ° C on receipt.

Data collection. We recorded the demographic characteristics including age, sex, family history the condition of previous hospitalization, the condition of referral, and underlying disease. Clinical characteristics including initial clinical presentation with cough/rhinorrhea /fever/dyspnea/vomiting/diarrhea/decreased appetite/lethargy/conjunctiva injection were recorded and finding of physical examination and diagnosis were collected.

The laboratory data including the value of white blood cell count (WBC), differential count and C reactive protein (CRP) on admission, x-ray finding were recorded. The treatment course of each patients including the duration between onset of symptoms and relieve (e.g. cough, fever), the value of peak body temperature, oxygen requirement, the date of sampling, necessity for intensive care, oxygen supplement, ventilator support, the outcome (complication or mortality) were collected.

Methods. The throat swabs or nasopharyngeal aspirations were tested by fluorescent antigen detection, isolation in cell culture to detect human respiratory syncytial virus (hRSV), influenza viruses A and B, adenovirus, parainfluenza viruses, adenovirus, and enterovirus. Serology of mycoplasma

was performed with complement fixation method for those patients with presentation of atypical pneumonia. Urine pneumococcal Ag was performed for those with presentation of pneumonia patch, respiratory distress, and leukocytosis with high C reactive protein. The viral RNA was extracted from all samples using QIAmp viral RNA kits (Qiagen). Real-time RT-PCR was applied to the detection of viral RNA using a single tube RT-PCR kit according to the manufacture's instructions (RNA master hybridization probes; Roche). Amplification and detection of RNA from clinical specimens were performed with an Lightcycler 2.0 instrument (Roche applied science) with one step RT-PCR. Each capillary glass contained a 10 ul reaction mix which included 2.6 ul sample RNA, 0.5 uM sense primer (MPV 01.1), 0.5 uM antisense primer (MPV 02.2), 3.25mM Mn [Oac]2, and 0.2uM fluorescein hybridization probes (TIB-MOLBIOL, Berlin, Germany). The primers used in the initial test of the respiratory specimens were based on the sequence data of a Dutch strain available from GenBank (accession no. AF 371367) and targeted the hMPV N gene. The sense primers, 5'-AACCGTGTACTAAGTGATGCACTC and the antisense primer, 5'-CATTGTTTGACCGGCCCCATAA , produce an amplicon that corresponds to nt 601-813 of the hMPV N gene. hMPV negative sense RNA genome is only 20% homologous to the nucleocapsid (N) gene of hRSV [1], so it is sufficiently distinct that its sequences do not cross- react with existing nucleic acid amplification assays. Each set of RT and PCR reactions contained appropriate negative controls. The reaction mixes were exposed to a 20min, 61 °C RT step, a 30 sec denaturation at 95 °C, and then 55 PCR cycles of 95 °C for 1 sec, 60 °C for 15 sec and 72 °C for 13 sec, using a

channel setting of F2/F1. The specificity of the obtained fluorescence signal was checked by a melting-curve analysis after each run. Samples positive for initial real time RT-PCR were reconfirmed by primers designed for L gene. The sense primer, 5'- CTACATCAGATGATACCTCAATCC and antisense primer, 5'- TTCTTCTTTCCTGAATTCCAGA, yielded an amplicon that corresponds to nt 7534-7658 of the hMPV L gene (GenBank accession no. AF 371367). Each capillary glass contained a 10 ul reaction mix which included 1 ul sample RNA, 1 uM mixed primer, 3mM MgCl2, 4.6ul H2O, 0.2ul SYBR green LC-RT PCR enzyme mix, and 2ul SYBR green reaction mix (TIB-MOLBIOL, Berlin, Germany). The reaction mixes were exposed to a 20min, 55 ° C RT step, a 30 sec denaturation at 95 ° C, and then 65 PCR cycles of 95 ° C for 0 sec, 60 ° C for 30sec and 72 ° C for 5 sec, using a channel setting of F1.

Standard curve. Quantification of hMPV RNA was performed with five 10-fold serial dilutions of a plasmid containing the primer-spanning region. The viral RNA was transformed to cDNA (complementary DNA) via Thermoscript RT-PCR system (Invitrogen). Then the cDNA was transfected into the plasmid of yT&A with 2728 bps by kit (Invitrogen). The Top-10 strain competent cells were added to the plasmids containing the hMPV cDNA then sub in ampicillin (40 ug/mL) containing LB. The LB was incubated in 37 ° C for 11-16 hours. The single colony was selected then the restriction enzyme of Eco RI and Bam HI were used for digestion. The plasmid DNA concentration was calibrated by spectrophotometry at 260 nm. Ten-fold serial dilution with RNAse free water was done then real-time RT-PCR was performed. The cycle numbers correlated to the lowest

detectable viral copies (10 copy numbers/uL) was around 40 by estimation with N gene hybridization prob.

Biostatistic *P* value was calculated with student t test or Chi-square or Fisher exact test for statistic significance (Version 10.0, SPSS, SPSS Inc. Illinois).

Results

The Cohort. A total of 460 clinical specimens were obtained corresponding to 460 admissions, and 91 cases who had no clinical evidence of airway infection but their specimens were sent for other diseases like, herpangina, hand-foot-mouth-disease, encephalitis, acute lymphadenitis, or acute gastroenteritis were excluded. No positive PCR result was detected by hMPV real time RT-PCR among these excluded cases, either. The illegible cohort included 369 different admissions/specimens (from 353 children). 17 (4.6%) patients with ARIs were documented for the presence of hMPV.

Real-time RT-PCR. To further reduce the turn-around time of RT-PCR, we converted the conventional RT-PCR to a real-time RT-PCR. During the LightCycler instrument continuously monitored amplicons accumulation by detecting the proportional increased fluorescence emitted as a result of a loss of quenching of the hybridization probes were hydrolyzed, assay reproducibility, based on analysis of the fractional cycle number at which the fluorescence generated by the accumulating amplicon exceeds the threshold cycle number, was determined after examining replicate samples, using different vials of RT-PCR master mix.

To study the efficiency of oligoprobe hydrolysis and, by extrapolation, the efficiency of the real-time, we applied fluorescent melting curve analysis to the amplicon. This resulted in a peak that was specific for the hMPV oligoprobes' nucleotide sequence and approximated its expected melting temperature.

Seasonal Distribution of hMPV Positive cases. hMPV cases appeared whole year round except July, August and November. Peak

incidence was around late spring to early summer (Fig1). Seasonality of hMPV, influenzae virus (A&B), hRSV, Adenovirus, enterovirus, mycoplasma, and parainfluenzae type 1 and 3 infections were shown in Fig2 and 3. Each curve shows the case numbers with the infected virus during the 13-month period. hRSV, parainfluenzae type 3 and mycoplasam infections persisted throughout the year. A sharp increase of influenzae A infection was seen in January, which is the coldest month in Taiwan (influenzae B was detected later in May and even July). Parainfluenzae type 1 infection seemed to company the peak incidence of hRSV infection which is also a whole-year round pathogen in Taiwan. Enterovirus related ARTIs seemed to be peak in June though enterovirus related herpangina or hand-foot-mouth disease persisted throughout the year.

Clinical Manifestation of Infected Children. Clinical characteristics of 17 children (M:F= 11: 6, male dominant, $p<0.05$) who had hMPV infection were listed in table 1. The mean age of admission was 17.4 month-old (95% CI: 10.1- 24.7) and 15 patients (88%) were younger than 2 year-old. 11 (61.7%) of them had fever during the disease course and only 5 had fever at admission. None of them needed intensive care and the mean duration of hospitalization was 4.5 days. Half of them needed oxygenation such as O2 hood, O2 tent or O2 nebulizer and the mean duration for oxygenation was 1.8 days. Mean duration of cough was 11 days. Specimens were obtained at the mean of 6.4 days after onset of diseases (0-26 day). 64.7% of children had family history of adult illness and 86.7% of them had abnormal chest x-ray such as emphysematous change, infiltration, patch or steeple signs, etc. Antibiotics were prescribed in 41.2% of them for clinical evidence of otitis media, suspicions of bacterial pneumonia, or sepsis for

young infant fever.

Other clinical symptoms were listed in table 2. Cough was the leading symptoms with subsequent fever and rhinorrhea. Half of the children showed dyspnea. Evidence of either upper or lower respiratory tract infection or both were shown (Table3). Rhonchi, rales and wheezing could be noted with auscultation in half of the children and one-third of them had retraction. There were 2 patients had no remarkable lower airway signs except symptoms of upper airway infection.

Majority of hMPV positive cases were younger than 2 year-old, and most of them were 12 to 18 month-old (Fig4). Further, 15% of children who had ARTIs at this age in our cohort were hMPV positive. It appeared to be an important etiology ranging from 4 to 15% for ARTIs of hospitalized children under 2 year of age.

Six (6/17, 35%) of them had underlying diseases (One biliary atresia with liver cirrhosis, one with intestinal lymphangiectasis with protein losing enteropathy, one prematurity with pulmonary stenosis, one corrected transposition of great arteries with pulmonary stenosis, one ventricular septal defect with pulmonary stenosis, and one VACTERAL association).

The detailed information was listed on table 4 for all 17 cases of hMPV. Among these hMPV positive samples, the virus was a unique pathogen in 12 cases (70.6%), while in five it was present together with other pathogens: One with adenovirus plus positive mycoplasma serology, one with culture positive for cytomegalovirus, one with sputum antigen positive for hRSV, one with positive mycoplasma serology, and one with positive urine pneumococcal antigen.

Comparison Between Groups.

Patients with underlying disease were compared with those without. Fever was noted in 50% (3/6) of patients with underlying disease and none of them had fever while admission. Compared to patients without underlying disease, there was no statistic significance however (p=0.6). Information collected within these 2 groups showed no significant difference. MPV infection seems not prolonged their hospitalized course, nor increased oxygen supplement nor increased necessary of intensive care.

Patients with un-identified pathogen were compared with hMPV positive cases (Table 1). None of them had complications during their ARIs. Regards of ventilator support, only one child in pathogen non-identified group received intubation for respiratory support and none was observed in hMPV group. hMPV positive children seemed to be younger than those with un-identified pathogen (1.5 y/o v.s. 2.7 y/o, p=0.02). Otherwise, no significant difference was found between these two groups. Clinical manifestations were non-specific and the clinical outcomes were excellent in our group.

Discussion

Acute respiratory tract infections are a major cause of disability in children. In most cases, the cause of illness remains unidentified in half, even after extensive laboratory investigations (9). 17 (4.6%) patients with ARTIs are documented for the presence of hMPV in our cohort, which is similar to the prevalence of other group. 4.2% (5/120) Thai pediatric patients with ARTIs showed detectable hMPV based on N-gene-specific RT-PCR (10). The prevalence of children with ARTIs in Hong Kon was 5.5% (11). 6.2% of patients younger than 18 year-old in Boston were found to have positive results of F-gene-specific RT-PCR(12).

Because it has recently been discovered, the epidemiology and clinical impact of hMPV have yet to be fully defined. The present article is the first to document its occurrence in Taiwan. We use molecular methods to document that hMPV is a major cause of respiratory disease in children which implies 8.7% (17/196) of those who have no identified pathogen for ARIs. The results from other groups ranged from 2.2% to 20% according to different age group enrolled, different countries and different definitions of "virus-negative" cases (13). PCR is more sensitive for detecting a range of respiratory viruses than culture methods (14). Our result shows hMPV with ARTIs and demonstrate that the virus is associated with at least a proportion of mild, community-acquired, self-limiting respiratory disease in children.

Real-time PCR assays are ideal for use in studying the epidemiology of a newly described virus because the systems are homogenous, combing nucleic acid amplification and detection in a single closed reaction vessel. This means that the amplicon can be discarded without ever exposing the environment to contamination. From the Mackay et al., the real time assay

did an improvement in the rate of virus detection than conventional RT-PCR (2). Although identification of viral nucleic acid in respiratory specimens dose not prove that hMPV was responsible for the patients' symptoms, the association between respiratory tract illness and presence of the virus suggests a causative role (15). hMPV dose not appear to be associated with asymptomatic carriage in nasopharynx since no hMPV was found in respiratory samples from asymptomatic children younger than 2 year-old (1). To investigate the efficiency of hydrolysis of the fluorogenic oligoprobe under PCR conditions and, by extrapolation, the efficiency of the real-time PCR assay, we applied fluorescent melting-curve analysis to the amplicon. These melting peaks are due to conformational changes occurring when intact hybridized oligoprobe is melted from its template. This reduces the leaky fluorogence which results from an incompletely quenched molecule when it is maximally separated from an intact oligoprobe. As the oligoprobe returns to a relaxed state after it has melted from its template, the fluorophores are more likely to interact by molecular collision, increasing the extent of quenching. The entire process results in decreased fluoroquence, which is presented as a melting peak when the negative derivative of the rate of change in fluorescence is calculated by the LightCycler's software (2).

From the previous study, HMPV is known to be the leading cause of ARIs in the first years of life, with a spectrum of disease similar to that of RSV (16). Diagnosing HMPV infection and differentiating it from other respiratory viruses may be impossible on clinical grounds. Fever was a common manifestation, which is likely explained in part by enrolled children were hospitalized. Our data also showed that infected children were younger than 2 years old which was compatible to those of Freymuth et al.(7)

and Esper et al. (8), who tested for hMPV in respiratory specimens from children who were negative for other respiratory viruses. However, half of our cases have family history of adult respiratory acute illness. Though no virus isolation or RT-PCR are performed for these family members, we believe that they do have hMPV infections as their children according to the surveillance in England and Wales for adult patients of ARTIs shorter than 7 days (3). 2.2% (9/405) of them was hMPV positive in NPAs for RT-PCR.
The occurrence and severity of RSV and influenzae virus infections in children is higher in boys than girls (17) (18). Some studies have found a higher proportion of patients with hMPV infection among boys than girls(11, 19), whereas others have not(20). We found a higher percentage of patients with specimens having detectable hMPV among boys than girls (M:F= 11: 6) and it is statistically significant ($p<0.05$).

The seasonal occurrence of hMPV infection largely overlapped with hRSV infections. Although it peaks in May away from the peaks of RSV and influenzae A. Seasonal distribution has been discussed in previous reports, and the majority of them considered is a pathogen in winter in North America, United Kindom (3, 8, 15). Nevertheless, our result shows that hMPV prevalent from late spring to early summer, similar to the distributions from other reports in Hong Kong, Japan and Canada (11, 20, 21). Cases with hMPV did occur outside of the peak seasons, like the distribution happened to RSV in Taiwan. In contrast, influenzae A infections appeared to have more regular seasonal occurrence, though this was not evaluated statistically. Whether this distribution is related to the climate or population density or immunity status of different races, needs long term prospective studied to tell.

Our study was limited to screening respiratory samples collected from hospitalized patients. Although the population-based incidence and prevalence cannot be determined from these data, our findings suggest that hMPV is a major cause of ARIs. Likewise, asymptomatic infants and children were not studied, so the frequency of hMPV infection could be underestimated. Co-infection with RSV and hMPV has been described previously (22). In our cohort, there were 5 patients who had other pathogens identified during the episode of ARIs. The phenomenon observed in our group, indicates that infection with hMPV and other respiratory pathogens may occur concurrently. Viral agents recognized with hMPV symptomatic individuals include adenovirus, hRSV, influenzae A and B, and measles(5, 23).

Thanasugran et el. Reported 2 hMPV infected children developed clinical respiratory distress progressively with underlying bronchopulmonary dysplasia and truncus arteriosus status post total correction (10,24). No case with complicated admission course is observed in our series and no significant statistic difference is noted between cases with underlying disease or without. hMPV infection could develop into a life-threatening disease, particular in high-risk group or immunocompromised hosts (25). Appropriate recognition of hMPV and management when indicated should help to reduce the morbidity of the patients.

In summary, our data confirms that hMPV infections account for a portion of ARIs in hospitalized children and the outcome of the infected children are excellent without complications. No significant clinical symptoms or signs could be used to differentiated from ARTIs of other

un-identified pathogens. Real-time RT-PCR provided a sensitive diagnostic tool for emerging infectious disease.

Fig1. The seasonal distribution of cases of MPV ARIs

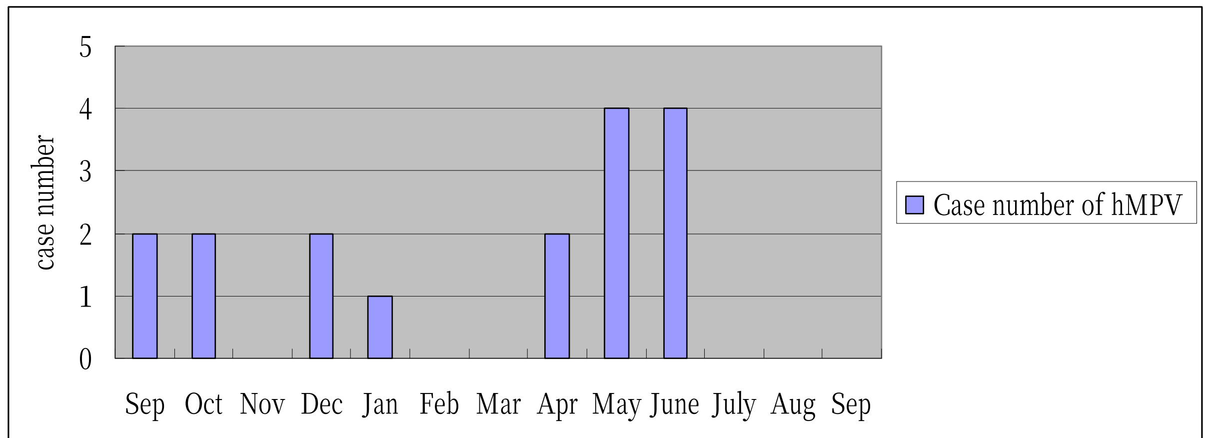


Fig2. Seasonal distribution of hMPV, hRSV, Adenovirus, enterovirus, and mycoplasma infection.

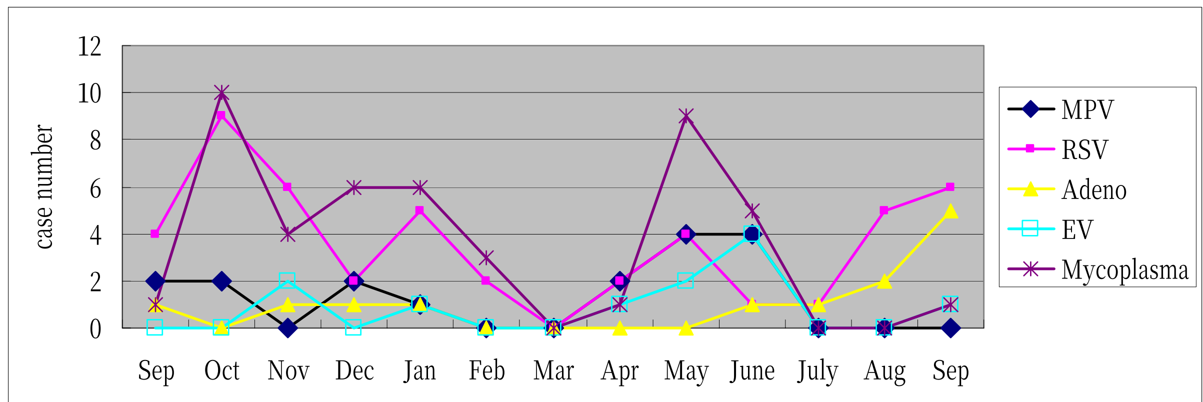


Fig3. Seasonal distribution of hMPV, parainfluenzae type 1 and 3, influenzae A and B.

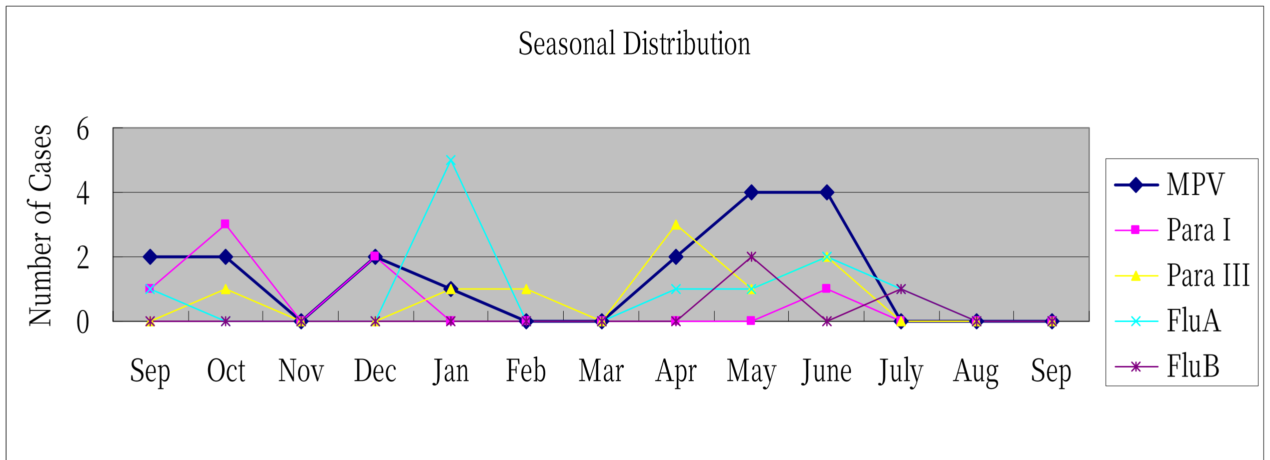


Table 1. Comparisons between MPV positive cases and pathogen non-identified group

	MPV positive (N=17)	pathogen non-identified (N=179)	P value
Age (year)	1.5 ± 1.2	2.7 ± 3.0	0.002＊
Sex (M/F)	11/6	103/76	0.567
Underlying disease	6 (35.3%)	58 (32.4%)	0.808
Hospitalized duration (day)	4.5 ± 2.0	5.0 ± 3.3	0.527
ICU care	0	5 (2.8%) (0.1 ± 0.7)	1.000
O2 use	7(52.9%)	45 (41.9%)	0.379
O2 use duration (day)	1.8 ± 2.2	1.6 ± 2.6	0.759
Fever	11 (61.7%)	145/179(81%)	0.111
Fever duration (day)	3.1 ± 4.8	3.6 ± 4.5	0.697
Peak BT	39.0 ± 0.6	39.1 ± 0.8	0.847
Cough duration (day)	11.0 ± 6.6	10.3 ± 9.2	0.772
Sampling day after onset (day)	6.4 ± 6.0	6.3 ± 6.6	0.987
Adult family history of ARIs	7/15(46.7%)	69/140 (49.3%)	0.847
Abnormal chest x-ray	13/15(86.7%)	119/169 (70.4%)	0.239
White blood cell count(x 10^9/L)	12.224 ± 6.503	11.607 ± 6.799	0.72
C reactive protein (mg/dL)	2.477 ± 3.059	2.086 ± 3.201	0.629
Antibiotics	7(41.2%)	107 (59.8%)	0.137
Bronchodilator inhalation	2(11.8%)	32 (17.9%)	0.742
Steroid use	0	10(5.6%)	1.000

＊ p value <0.05 revealed statistic significance

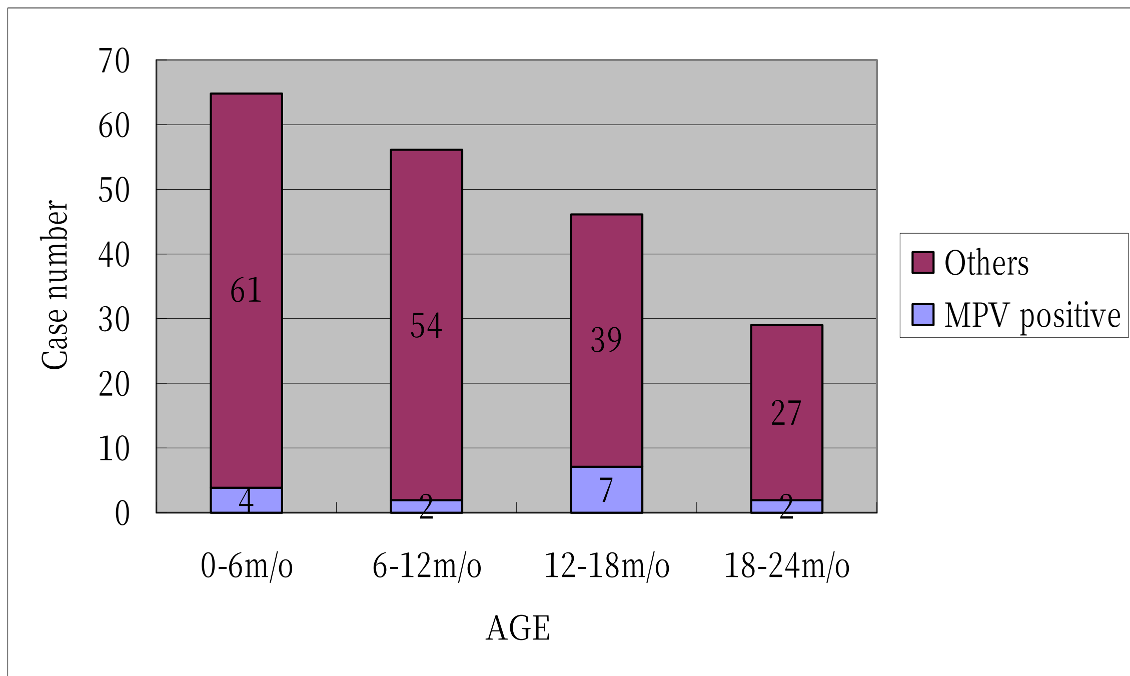
Table 2 . Clinical symptoms of MPV patients

Clinical symptoms	Patients No. (Total=17)	%
cough	15	88
fever	11	65
rhinorrhea	11	65
dyspnea	8	47
decreased appetite	5	29
diarrhea	4	24
rash	2	12
sorethroat	1	6
vomiting	1	6
oral ulcer	1	6

Table 3. Clinical signs of MPV patients

Clinical signs	Patients No. (total=17)	%
rhonchi	9	53
rales	8	47
wheezine	7	41
retractions	6	35
stridor	4	24
Nil	2	12

Fig 4. Proportional distribution of MPV positive cases (younger than 2 year-old)



Reference

1. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 2001;7:719-24.
2. Mackay IM, Jacob KC, Woolhouse D, Waller K, Syrmis MW, Whiley DM, et al. Molecular assays for detection of human metapneumovirus. J Clin Microbiol 2003;41:100-5.
3. Stockton J, Stephenson I, Fleming D, Zambon M. Human metapneumovirus as a cause of community-acquired respiratory illness. Emerg Infect Dis 2002;8:897-901.
4. Ebihara T, Endo R, Kikuta H, Ishiguro N, Yoshioka M, Ma X, et al. Seroprevalence of human metapneumovirus in Japan. J Med Virol 2003;70:281-3.
5. Nissen MD, Siebert DJ, Mackay IM, Sloots TP, Withers SJ. Evidence of human metapneumovirus in Australian children. Med J Aust 2002;176:188.
6. Peret TC, Boivin G, Li Y, Couillard M, Humphrey C, Osterhaus AD, et al. Characterization of human metapneumoviruses isolated from patients in North America. J Infect Dis 2002;185:1660-3.
7. Freymouth F, Vabret A, Legrand L, Eterradossi N, Lafay-Delaire F, Brouard J, et al. Presence of the new human metapneumovirus in French children with bronchiolitis. Pediatr Infect Dis J 2003;22:92-4.
8. Esper F, Martinello RA, Boucher D, Weibel C, Ferguson D, Landry ML, et al. A 1-year experience with human metapneumovirus in

children aged <5 years. J Infect Dis 2004;189:1388-96.

9. Garibaldi RA. Epidemiology of community acquired respiratory tract infections in adults: incidence, etiology and impact. Am J Med 1985; 78: 32-7
10. Thanasugarn W, Samransmruajkit R, Vanapongtipagorn P, Propphal N, Van den Hoogen B, Osterhaus A.D.M.E, Poobvorawan Y. Human metapneumocvirus infection in thai children. Sacnd J Infect Dis 2003; 35:754-6
11. Peiris JS, Tang WH, Chan KH, Khong PL, Guan Y, Lau YL, et al. Children with respiratory disease associated with metapneumovirus in Hong Kong. Emerg Infect Dis 2003;9:628-33.
12. McAdam AJ, Hasenbein ME, Feldman HA, Cole SE, Offermann JT, Riley AM, et al. Human metapneumovirus in children tested at a tertiary-care hospital. J Infect Dis 2004;190:20-6.
13. Mejias A, Chavez-Bueno S, Ramilo O. Human metapneumovirus: a not so new virus. Pediatr Infect Dis J 2004;23:1-7.
14. Stockton J, Ellis JS, Clewley JP, Zambon MC. Multiplex PCR for typing and studying influenza and respiratory syncytial viruses. J Clin Microbiol 1998; 36: 2990-512.
15. Esper F, Boucher D, Weibel C, Martinello RA, Kahn JS. Human metapneumovirus infection in the United States: clinical manifestations associated with a newly emerging respiratory infection in children. Pediatrics 2003;111:1407-10.
16. Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, et al. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children.

N Engl J Med 2004;350:443-50.

17. Liese JG, Grill E, Fischer B, Roeckl-Wiedmann I, Carr D, Belohradsky BH. Incidence and risk factors of respiratory syncytial virus–related hospitalizations in premature infants in Germany. Eur J Pediatr 2003; 162:230–6.
18. Quach C, Piche-Walker L, Platt R, Moore D. Risk factors associated with severe influenza infections in childhood: implication for vaccine strategy. Pediatrics 2003; 112:e197–201.
19. van den Hoogen BG, van Doornum GJ, Fockens JC, Cornelissen JJ, Beyer WE, de Groot R, et al. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. J Infect Dis 2003;188:1571-7.
20. Boivin G, De Serres G, Cote S, Gilca R, Abed Y, Rochette L, et al. Human metapneumovirus infections in hospitalized children. Emerg Infect Dis 2003;9:634-40.
21. Kashiwa H, Shimozono H, Takao S. Clinical pictures of children with human metapneumovirus infection: comparison with respiratory syncytial virus infection. Jpn J Infect Dis 2004;57:80-2.
22. Greensill J, McNamara PS, Dove W, Flanagan B, Smyth RL, Hart CA. Human metapneumovirus in severe respiratory syncytial virus bronchiolitis. Emerg Infect Dis 2003;9:372-5.
23. Boivin G, Abed Y, Pelletier G, Ruel L, Moisan D, Cote S, et al. 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 2002;186:1330-4.

24. Van den Hoogen B, Osterhaus A.D.M.E, Poobvorawan Y. Human metapneumocvirus infection in thai children. Sacnd J Infect Dis 2003; 35:754-6.
25. Pelletier G, Dery P, Abed Y, Boivin G. Respiratory tract reinfections by the new human Metapneumovirus in an immunocompromised child. Emerg Infect Dis 2002;8:976-8.