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Human Metapneumovirus and Respiratory Syncytial Virus Detection in Young Children with Acute Bronchiolitis

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SUMMARY This study was conducted to detect human metapneumovirus (hMPV) and respiratory syncytial virus (RSV) in young children hospitalized with acute bronchiolitis, using reverse transcriptase polymerase chain reaction (RT-PCR). Nasopharyngeal secretions were collected from 170 children between 1 and 24 months of age admitted to two tertiary hospitals in northeastern Thailand, between 2002 and 2004. Acute bronchiolitis was defined as the first episode of wheezing associated with tachypnea, increased respiratory effort and an upper respiratory tract infection. Two-thirds (115/170) were positive for viral etiologies: 64.7% RSV (110/170) and 3.5% hMPV (6/170). One patient had a dual infection. hMPV was detected between August and November, while RSV was prevalent from July through March. The clinical manifestations among the 6 hMPV, RSV and non-RSV-infected children were similar. RSV was the leading cause of acute bronchiolitis in young children and hMPV had a low prevalence in northeastern Thailand

Respiratory viruses, such as respiratory syncytial virus (RSV), influenza virus, parainfluenza virus and adenovirus, are the most frequent cause of hospitalization in young children. Among them, RSV is the most common etiologic agent. Recently, human metapneumovirus (hMPV), which was first identified in 2001 in The Netherlands,[1] was reported to be one of the primary causative organisms of acute respiratory tract infection in children worldwide.[2-15] Many epidemiological studies have shown that hMPV infections seemed to be similar to that of respiratory syncytial virus (RSV), with symptoms ranging from mild to severe respiratory distress (i.e., pneumonia and acute bronchiolitis).[8,12,13,15] One large cohort study showed that hMPV was the major cause of acute bronchiolitis in otherwise healthy young children.[8] Moreover, hMPV was found to be the third most frequent virus after RSV and rhinovirus, in children under 2 years of age, hospitalized with respiratory infection, with associated bronchiolitis and recurrent wheezing.[13]

Epidemiological surveys on the prevalence of the causative viruses in young children suffering from acute respiratory tract infection in Thailand are limited. All of the studies were conducted in central Thailand and showed that RSV was the most common organism causing lower respiratory tract infection in children under 5 years of age.[16-18] Two reports from a university hospital in Bangkok indicated the prevalence of hMPV ranging between 4.2% (in

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children under 15 years of age)[19] and 5.4% (in infants and young children).[20]

To our knowledge, there has been no epidemiological study on the causative organisms of acute respiratory tract infections in northeastern Thailand. We, therefore, conducted our study (1) to detect hMPV and RSV using reverse-transcriptase polymerase chain reaction (RT-PCR) on the nasopharyngeal secretions of children under 2 years of age, hospitalized with acute bronchiolitis and (2) to compare the clinical manifestations between the two viruses.

MATERIALS AND METHODS

Data collection

Between April 2002 and August 2004, we enrolled (with informed, written, parental consent) 179 previously healthy children between 1 and 24 months of age, hospitalized with acute bronchiolitis at two tertiary hospitals in Khon Kaen, northeastern Thailand. Bronchiolitis was defined as the first episode of wheezing associated with tachypnea, increased respiratory effort and an upper respiratory tract infection.

Upon admission, a sample of nasopharygeal secretion was taken from each child's nostril by inserting a disposable suction catheter connected to a mucus extractor and applying gentle suction with an electric suction device, without inserting any solution into the nostrils. The secretion obtained was immediately put into a tube containing viral transport medium and sent to a laboratory, where the tubes for viral detection were stored at -70°C until processed.

Our study was reviewed and approved by the Ethics Committee for Human Research of Khon Kaen University and Khon Kaen Hospital, Ministry of Public Health, Thailand.

Virus detection

RNA was extracted from 140 μl of nasopharyngeal secretion using a QIAamp viral RNA mini kit (Qiagen, Germany). The extracted RNA was examined for RSV and hMPV by hemi-nested multiplex RT-PCR. Specific primers for the nucleocapsid of RSV and matrix protein genes of hMPV were used and are presented in Table 1.[21]

RNA (25 μl) was used in 25-μl reaction mixtures using the One-Step RT-PCR kit (Qiagen, Germany) with RSV specific primers (0.4 μM forward primer [vrs P1] and 0.4 μM reverse primer [vrs P2]) and hMPV specific primers (0.4 μM forward primer (hmpv 1) and 0.4 μM reverse primer (hmpv 2).

The positive controls comprised cDNA of the viruses, provided by Professor Dr. François Freymuth (Caen University Hospital, France). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as internal control as reported previously.[1] The one-step multiplex RT-PCR program included: reverse transcription at 50°C for 30 minutes, initial PCR activation at 94°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 30

Table 1 Primers used for one-step multiplex RT-PCR and hemi-nested multiplex PCR

	Virus	Primer	Sequence	Gene	Amplicon size (bp)
One-step multiplex RT-PCR	RSV	vrs P1	5'-GGA ACA AGT TGT TGA GGT TTA TGA ATA TGC-3'	Nucleocapsid	279
		vrs P2	5'-TTC TGC TGT CAA GTC TAG TAC ACT GTA GT-3'		
	hMPV	hmpv 1	5'-CCC TTT GTT TCA GGC CAA-3'	Matrix protein	416
		hmpv 2	5'-GCA GCT TCA ACA GTA GCT G-3'		
Hemi-nested multiplex PCR	RSV	vrs i	5'-GGT GTA CCT CTG TAC TCT C-3'	Nucleocapsid	180
	hMPV	hmpv 3	5'-AGG CCA ACA CAC CAC CAG-3'	Matrix protein	410
Internal control	-	GAPDH1	5'-TCA TCC ATG ACA ACT TTG GTA TCG TG-3'	GAPDH	564
		GAPDH2	5'-CTC TTC CTC TTG TGC TCT TG-3'		

seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes.

The one-step RT-PCR products (2 μl) were subjected to hemi-nested multiplex PCR. For each virus, an internal primer was used together with the corresponding forward primer of the RT-PCR. The hemi-nested multiplex PCR was performed in a total volume of 25 μl containing 10x buffer (New England Biolabs, USA), 0.2 mM dNTPs, RSV specific primers (vrs P1) and 0.4 μM internal primer (vrs i), hMPV specific primers (0.2 μM forward primer [hmpv 1] and 0.4 μM internal primer [hmpv 3]) and 1 U of *Taq* DNA polymerase (New England Biolabs, USA). The PCR program included: initial PCR activation at 94°C for 5 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes.

The PCR products of the one-step multiplex RT-PCR and hemi-nested multiplex PCR were visualized under ultraviolet light after electrophoresis on a 2% agarose gel stained with ethidium bromide.

Statistical analysis

The chi-square, student's t test or Mann-Whitney test were carried out as appropriate, using STATA software version 8. *P*-values < 0.05 were considered statistically significant.

RESULTS

During the study period, 261 children were diagnosed with acute bronchiolitis needing hospitalization; the parents of 179 gave consent and so the children were eligible for collection of nasopharyngeal secretion; however, only 170 samples were appropriately stored for viral detection.

One hundred and fifteen patients (67.8%) were positive for 2 viruses: RSV 110 (64.7%) and hMPV 6 (3.5%). One patient had a dual infection. All hMPV detection occurred between August and November, the end of the rainy season and the early part of the cool season, while the RSV was prevalent during rainy through cool seasons of Thailand (July through March) (Fig. 1).

The clinical characteristics of the 6 hMPV-infected children are presented in Table 2. Because of the very low number of hMPV cases, the clinical manifestations of RSV and non-RSV cases were compared instead and shown in Table 3. There was no significant difference in the demographic characteristics between the RSV and non-RSV bronchiolitis groups, except for mean weight, as children with RSV had a significantly lower mean weight than those infected with other organisms ($p = 0.010$).

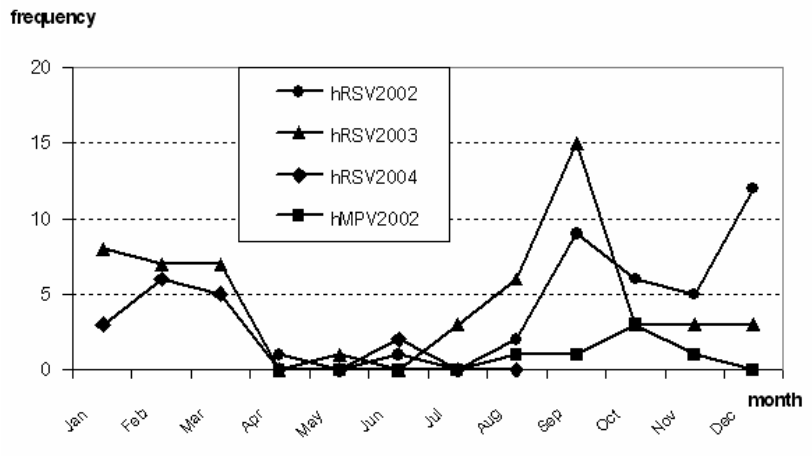

Fig. 1 Distribution of hMPV and RSV infections during the study period.

Boys and girls (mean age, 9.5 ± 5.0 months; range, 5 to 18.5 months) were equally infected with hMPV, all were breast-fed and passive smokers. A girl infected with both viruses was 18.5 months old and presented the same clinical manifestations as the other children (Table 2). All children had moderate to severe respiratory distress at admission. The youngest infant required endotracheal intubation and mechanical ventilation shortly after admission. She was extubated 14.3 hours later and her respiratory symptoms disappeared within one month. Four of the six children suffered respiratory distress for a few days but their respiratory symptoms disappeared within 11 days. One child had a longer duration of respiratory distress but all of the respiratory symptoms subsided after one month.

All six hMPV-infected children were followed-up for 3 years. Three children had subsequent wheezing at 7, 3 and 2 months after discharge, respectively, without re-hospitalization. Most of the children had no subsequent wheezing after two years. Only one child, who had a history of atopic dermatitis, had many episodes of re-wheezing: she was later diagnosed with asthma requiring inhaled corticosteroid prophylaxis.

DISCUSSION

This study confirmed previous epidemiological studies from Thailand indicating that RSV is the most common organism causing acute bronchiolitis, especially among young hospitalized children (64.7%). The incidence of RSV showed the same seasonal dependence as in other reports from Thailand; namely, a peak in the rainy season.[16-18] By contrast, the prevalence of hMPV was quite low at 3.5%, which is similar to previous studies from Bangkok (i.e., 4.2% for all ages and 5.4% in young children)[19-20] and from some other countries.[7,14]

The prevalence of hMPV, causing respiratory tract infection in children, in previous studies varied from 2 to 20 percent,[2-15] the wide variation being due to differences in the enrolled population (i.e., age, severity of disease, in-patients *vs*. all types of cases). In addition, some studies investigated the presence of hMPV solely in negative samples.[4-6] Although all our samples were tested, the prevalence in our study was low, perhaps because we used frozen specimens which might have diminished the yield.

A high prevalence of hMPV was observed in

Table 2 Clinical manifestations of hMPV children

Case number	**1**	**2**	**3**	**4**	**5**	**6**
Date	**15/08/02**	**04/09/02**	**14/10/02**	**27/10/02**	**31/10/02**	**26/11/02**
Age (months)	11.43	18.5	5.27	8.8	4.97	8
Sex	Male	Female	Male	Male	Female	Female
Breast feeding	Yes	Yes	Yes	Yes	Yes	Yes
Atopy-self	No	No	Yes	No	No	No
Atopy-parents	No	No	No	Yes	No	No
Passive smoker	Yes	Yes	Yes	Yes	Yes	Yes
O_2 saturation at admission, %	96	96	93	97	84	92
Total days of fever	3	7	2	4	7	6
Total days of dyspnea	2	4	4	2	25	18
Total days of respiratory symptoms	7	11	7	10	31	33
Length of stay, hours	25	47	74	29	179	100
Complications	No	No	No	No	ET	No
- during admission						
Readmission within one month	No	No	No	No	No	No
Re-wheezing	Yes	No	Yes	No	Yes	No
- duration after discharge	7 months		3 months		2 months	
Diagnosis asthma	No	No	Yes	No	No	No

studies that enrolled both out- and in-patients,[8-10] which might indicate that hMPV causes milder respiratory infections in children not requiring hospitalization. However, the clinical manifestations and severity of hMPV causing bronchiolitis were reportedly similar to those caused by RSV.[8,12,13,15] Unfortunately, our number of hMPV children was too few to compare clinical manifestations and seasonal distributions with those infected with RSV.

In previously healthy children we found that hMPV infected children mainly in the first year of life, as reported previously,[7,13] but contrary to a previous report from Thailand that reported infections of older children with underlying diseases.[20] Dual infection of hMPV and RSV might increase the risk of disease severity necessitating mechanical ventilation, especially in children under 3 years of age.[9] The only child who had a dual infection in our study did not require intensive care; however, one of our hMPV-infected children developed respiratory failure requiring mechanical ventilation shortly after hospitalization. This child was infected very early in life and had a low birth weight (2,600 g), which might have precipitated respiratory failure.

Although the clinical presentation of hMPV infection is similar to that of RSV in young children, the pathogenesis of hMPV disease requires further clarification since the results of different studies are contradictory. Jartti *et al.*[22] found two cytokines in the respiratory secretion of hMPV infected children linked to RSV disease, namely: interleukin-8 (a chemotactic factor mainly for neutrophils) and RANTES, regulating activation, normal T-cell expression and secretion (a chemotactic factor for eosinophils). However, Laham *et al.*[23] found lower levels of respiratory cytokines in hMPV-infected children than in RSV-infected children.

Several studies have demonstrated an association between RSV bronchiolitis early in life and development of asthma later on.[24-26] In contrast, little data are available to demonstrate an association between hMPV infection and the future development of asthma, perhaps because of the small number of patients at each center.[7,22] Notwithstanding, 3 of 6 children in our study had subsequent wheezing and

Table 3 Clinical manifestations of acute bronchiolitis: RSV and non-RSV children

Characteristics	**Total** N = 170	**RSV** N = 110	**Non-RSV** N = 60	***p-value***
Mean (SD) age, months	10.7 (5.7)	10.2 (5.7)	11.7 (5.7)	0.062
Median (quartile range)	9.7 (6.4-14.7)	9.2 (6-14.1)	10.0 (7.9-16.2)	
Age < 6 months, n (%)	36 (21.2)	25 (22.7)	11 (18.3)	0.503
Males, n (%)	107 (62.9)	65 (59.1)	42 (70.0)	0.159
Mean (SD) weight, kg	8.3 (0.1)	8.1 (0.2)	8.7 (0.2)	0.010
Breast feeding, n (%)	160 (94.1)	102 (92.7)	58 (96.7)	0.297
Mean (SD) breast feeding, months	4.3 (0.3)	4.4 (0.3)	4.1 (0.5)	0.703
History of atopy (parents), n (%)	49 (28.8)	34 (30.9)	15 (25.0)	0.416
Passive smokers, n (%)	102 (60.0)	63 (57.3)	39 (65.0)	0.326
History of fever or fever at admission, n (%)	155 (91.2)	99 (90.0)	56 (93.3)	0.464
O_2 saturation < 95 % at enrollment, n (%)	83 (48.8)	57 (51.8)	26 (43.3)	0.298
Mean (SD) duration of respiratory distress, days,	5.1 (4.4)	4.9 (3.9)	5.4 (5.2)	0.478
95% CI	4.4-5.7	4.2-5.6	4.1-6.7	
Mean (SD) duration of O_2 administration, hours,	32.4 (34.0)	34.7 (36.3)	28.3 (29.4)	0.245
95% CI	27.3-37.6	27.8-41.5	20.7-35.9	
Mean (SD) length of stay, hours,	61. 3 (36.9)	62.8 (39.2)	58.6 (32.3)	0.485
95% CI	55.7-66.9	55.3-70.2	50.3-67.0	
Mean (SD) duration of respiratory symptoms, days,	11.2 (6.9)	11.3 (6.6)	10.9 (7.3)	0.777
95% CI	10.1-12.2	10.0-12.5	9.1-12.8	

one had an atopic history diagnosed as asthma, but wheezing in all children disappeared after two years of follow-up.

Although many other respiratory viruses can be detected by RT-PCR, owing to budget restrictions our study was limited to detect only (1) RSV, the most common pathogen causing wheezing in young children with a high association of asthma development, and (2) the prevalence of the novel hMPV.

In conclusion, among the two causative viruses, this study showed that RSV was the major causative organism of acute bronchiolitis in previously healthy, hospitalized, young children (64.7%) from Khon Kaen, northeastern Thailand, while hMPV showed a significantly lower prevalence (3.5%). Large-scale and long-term follow-up epidemiological studies of all respiratory viruses causing wheezing in young children are needed.

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