

Human rhinovirus infections in hospitalized children: clinical, epidemiological and virological features

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SUMMARY

Molecular epidemiology and clinical impact of human rhinovirus (HRV) are not well documented in tropical regions. This study compared the clinical characteristics of HRV to other common viral infections and investigated the molecular epidemiology of HRV in hospitalized children with acute respiratory infections (ARIs) in Vietnam. From April 2010 to May 2011, 1082 nasopharyngeal swabs were screened for respiratory viruses by PCR. VP4/VP2 sequences of HRV were further characterized. HRV was the most commonly detected virus (30%), in which 70% were diagnosed as either pneumonia or bronchiolitis. Children with single HRV infections presented with significantly higher rate of hypoxia than those infected with respiratory syncytial virus or parainfluenza virus (PIV)-3 (12.4% vs. 3.8% and 0%, respectively, $P < 0.05$), higher rate of chest retraction than PIV-1 (57.3% vs. 34.5%, $P = 0.028$), higher rate of wheezing than influenza A (63.2% vs. 42.3%, $P = 0.038$). HRV-C did not differ to HRV-A clinically. The genetic diversity and changes of types over time were observed and may explain the year-round circulation of HRV. One novel HRV-A type was discovered which circulated locally for several years. In conclusion, HRV showed high genetic diversity and was associated with significant morbidity and severe ARIs in hospitalized children.

Key words: Children, genetic diversity, human rhinovirus, respiratory infection.

INTRODUCTION

Human rhinovirus (HRV) is the most common cause of upper respiratory infections (URIs) in humans and

frequently causes a mild, self-limiting illness often known as the common cold [1]. The development of polymerase chain reaction (PCR) methods has markedly increased the detection of HRV and revealed greater association of HRV with more severe diseases in children such as bronchiolitis, pneumonia and asthma exacerbation [2–4]. Therefore, their impact on overall morbidity and substantial cost for health-care is thought to be considerable [5]. However,

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HRV has been detected in up to 32% of asymptomatic children, which raises questions about the clinical effect of HRV-positive results [1]. HRV belongs to the genus *Enterovirus*, family Picornaviridae with high genetic diversity. More than 150 serotypes/genotypes of HRV have been described so far and classified into three main species: HRV-A, HRV-B, and the recently discovered HRV-C [6]. The association between HRV species and clinical outcome remains controversial. Initially, the new HRV-C species was reported to cause more severe diseases than HRV-A and HRV-B [6, 7], but recent data exhibited similar clinical syndromes across all species [8]. Moreover, since HRV was believed to cause only 'common colds', it is not detected routinely in clinical practice [1]. Therefore, the molecular epidemiology and clinical impact of HRV compared to other viruses such as influenza virus (Flu), respiratory syncytial virus (RSV) on hospitalized children are not well understood, especially in tropical countries.

The objectives of this study were to compare the clinical characteristics of HRV infections to those of other viral infections as well as to describe the molecular epidemiology of HRV in hospitalized children with acute respiratory infections (ARIs) in Vietnam.

METHODS

Patients and samples

From April 2010 to May 2011, nasopharyngeal flocked swabs (MicroRheologics, Italy) ($n = 1082$) were collected within 24 h after admission from hospitalized children aged <15 years with ARIs [9] at the Children's Hospital 2, Ho Chi Minh City, Vietnam. The specimens were immediately stored at -20°C until further analysis at the laboratory. Demographic and detailed clinical data were recorded using a standardized case record form. Patients with underlying chronic diseases (e.g. bronchopulmonary dysplasia, congenital heart disease, immunodeficiency and asthma) were excluded from the study to avoid confounding that may occur when comparing disease severity between different viruses. Patients with previous respiratory infection within 3 weeks from the current hospitalization were also excluded to avoid prolonged viral shedding. All patients had complete blood counts and chest X-ray (CXR) performed at admission. URI was defined as ARI without abnormalities on CXR. Pneumonia was defined as ARI with pulmonary infiltrates on CXR diagnosed by a radiologist.

Bronchiolitis was diagnosed in the presence of wheezing together with hyperaeration, atelectasis, or peribronchial thickening on CXR in children aged <2 years. Croup was characterized by hoarseness, cough, and stridor. Disease severity was classified based on the presence of hypoxia or chest retraction.

Ethical standards

The Scientific and Ethical Committee of the Children's Hospital 2, Ho Chi Minh City, Vietnam (no. 25A/QD-ND2) and Nihon University School of Medicine, Tokyo, Japan (no. 25-15-0) reviewed and approved this study. Written consent was obtained from a parent or legal guardian. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Virus detection and sequencing

Viral genomes were extracted from clinical samples using the QIAamp Viral RNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Multiplex nested PCR assays were used to screen for HRV and other respiratory viruses [Flu A and B, RSV, human metapneumovirus, parainfluenza virus (PIV) types 1–4, human coronaviruses (229E and OC43), adenovirus and human bocavirus] as described previously [10]. HRV was identified by using primers, targeting the highly conserved 5'-untranslated region (5'-UTR), that can detect all known serotypes as well as the novel HRV-C species [11]. For species and type determination, another RT-PCR was performed to amplify the VP4/VP2 region on every fifth HRV-positive sample ($n = 58$) [12]. Both strains of PCR products were sequenced by a commercial company (Macrogen Japan Corp., Japan).

Phylogenetic analysis

Nucleotide sequences of the VP4/VP2 coding region (616–1004, numbered by EF582385) [13] were aligned along with reference sequences (obtained from <http://www.picornaviridae.com>). Phylogenetic trees were constructed by maximum-likelihood analysis using MEGA 5 software (www.megasoftware.net), with the most suitable model for nucleotide substitution estimated by MEGA 5, and bootstrap analysis of 1000 replicates

[14]. Types were assigned when our sequences had $\geq 90\%$ nucleotide similarity to a known prototype sequence or clustered with a reference sequence in phylogenetic analysis with a bootstrap value $> 70\%$. VP4/VP2 sequences in this study are available at GenBank (accession numbers KM676336–KM676393).

Statistical analysis

Demographic and clinical parameters of HRV mono-infections were compared to RSV, Flu A, PIV-1, and PIV-3 mono-infections and also among HRV species. Categorical variables were compared using χ^2 test or Fisher's exact test, and continuous variables were compared using the Mann–Whitney *U* test. A two sided value of $P < 0.05$ was considered statistically significant. All analyses were conducted using SPSS v. 16.0 (SPSS Inc., USA).

RESULTS

Epidemiological characteristics of HRV infections

A total of 1082 children (mean age 9 months, range 1–161 months) with ARIs were enrolled. Most (86%) patients were aged < 2 years and the male:female ratio was 1.8:1. Respiratory viruses were detected in 64.7%. HRV (234/1082, 21.6%) was the most commonly detected single virus in this study population, followed by RSV (184/1082, 17%), PIV-3 (38/1082, 3.5%), PIV-1 (29/1082, 2.7%) and Flu A (26/1082, 2.4%). Mixed infections of HRV with other viruses were found in 91 (8.4%) patients. Of these, combination of HRV and RSV was the most common co-infection recorded (48/91, 52.7%). In total, 30% (325/1082) of patients were infected with HRV. HRV could be detected throughout the year with several peaks occurring in July, November (2010) and January, April (2011). HRV-A and HRV-C were detected in almost every month with no clear seasonal distribution (Fig. 1).

Clinical characteristics of HRV infections

The demographic and clinical characteristics of patients with HRV mono-infections were compared to those singly infected with RSV, Flu A, PIV-1 and PIV-3 (Table 1). Children with HRV mono-infections were significantly older than those with RSV mono-infections (median age 9 vs. 7 months, $P = 0.021$) but younger than those with Flu A mono-infections (median age 9 vs. 22.5 months, $P < 0.001$). The

difference on prematurity and malnutrition prevalence between children with HRV mono-infection and other groups was not significant. Fever occurred less often in HRV mono-infections than in those with other pathogens ($P < 0.001$). Regarding severe symptoms, children with HRV mono-infections were more likely to have hypoxia than those with RSV (12.4% vs. 3.8%, $P = 0.002$) and PIV-3 mono-infections (12.4% vs. 0%, $P = 0.02$). Chest retraction was more common in RSV mono-infections (69.0% vs. 57.3%, $P = 0.015$) but less common in PIV-1 mono-infections (34.5% vs. 57.3%, $P = 0.028$) compared to HRV. When compared with Flu A, HRV mono-infections caused less tachypnoea (40.6% vs. 61.5%, $P = 0.041$) but more wheezing (63.2% vs. 42.3%, $P = 0.038$). White blood cell count was significantly higher in HRV mono-infections than in RSV and PIV3 infections ($P = 0.004$ and $P = 0.005$). Interestingly, the blood eosinophil count was significantly elevated in patients with HRV mono-infections compared to those infected with other viruses ($P < 0.001$). Pneumonia was the most common diagnosis in HRV patients followed by bronchiolitis.

Regarding the association between co-infections and disease severity, HRV co-infections were more likely to cause chest retraction (70.3% vs. 57.3%, $P = 0.032$) than HRV mono-infections (Table 2). The clinical features of HRV-A and HRV-C mono-infections were compared but there were no significant difference (Table 3). The median age of cases with HRV-C infection was higher than those with HRV-A infection but not significant (17.5 vs. 10 months, $P = 0.192$).

Molecular characterization and phylogenetic analysis

In all, 58/325 samples were randomly selected for sequencing. By VP4/VP2 sequence analysis, HRV-A was most frequently detected (44/58, 75.9%) followed by HRV-C (14/58, 24.1%). No HRV-B was found. There were 21 different HRV-A types and 10 different HRV-C types. The most commonly detected types were A12 ($n = 9$) and C40 ($n = 3$) (Fig. 2). Some types were found across several months, such as A12, A78, and C40, suggesting that these types may circulate in the population over long time periods. On the other hand, the type distributions were almost distinct from month to month. The mean nucleotide difference between HRV-A and HRV-C was 37.2%. The mean nucleotide variability within HRV-C was greater than that within HRV-A (23.9% vs. 19.7%). One HRV-A sequence (VNM308-MAY.2010) did not

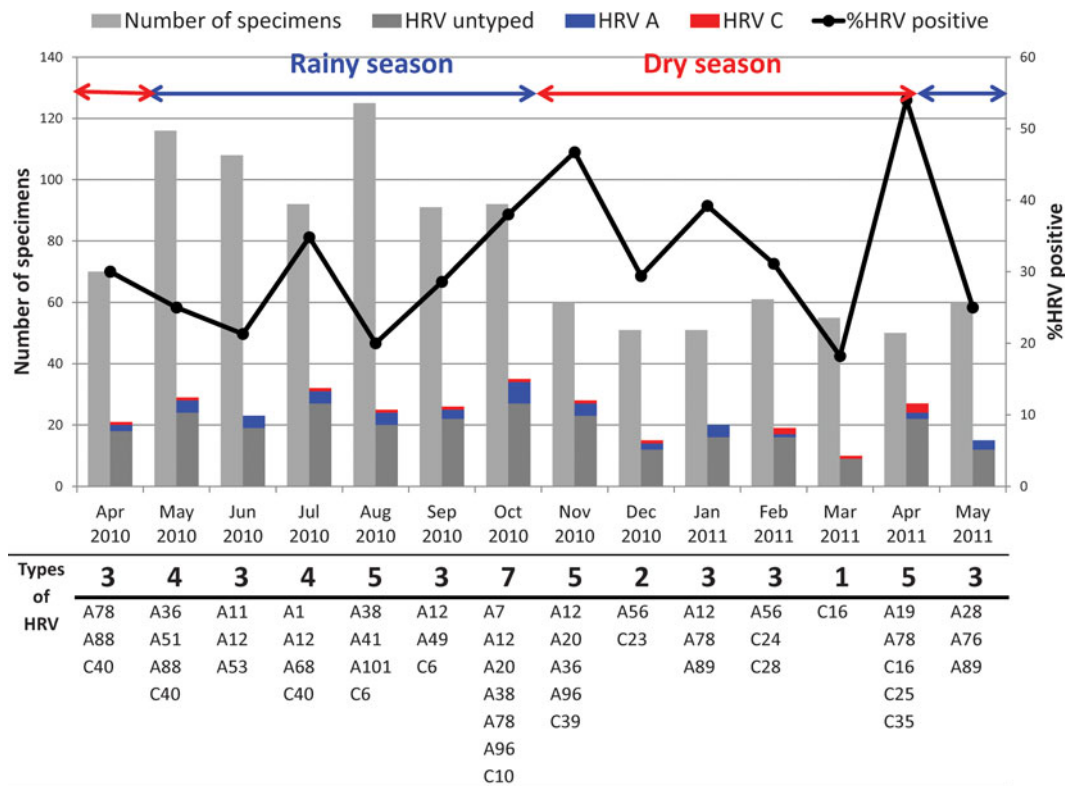


Fig. 1. Monthly distribution of HRV infections from April 2010 to May 2011.

cluster with any previously known types and showed 13% nucleotide difference to the nearest reference types (A88, A89), suggesting a new type. By BLAST searching, only three sequences with 97–99% nucleotide similarity were found in GenBank (as of 24 September 2014), and all were from neighbouring countries (Thailand 2011, Cambodia 2013). These strains showed the pairwise-distances of at least 11.3% with all available A88, A89 sequences and formed a separate cluster with high bootstrap value (Fig. 2). Following the criteria proposed by McIntyre *et al.* (pairwise-distance >10.5%), these strains could be assigned to a new type. However, their VP1 sequences which are required for type assignment are not available, they may be designated as provisionally assigned type (numbered sequentially as HRV Apat5) [13].

DISCUSSION

This study demonstrated a significant burden of HRV infections in children with ARIs. HRV was by far the most frequently detected agent, in the majority of cases as mono-infections, in children ill enough to be hospitalized. HRV was also more common than

RSV, which is well-known to be the major cause of hospitalization in children. Comparable to the findings of previous studies worldwide [15–21], HRV was found in 30% of the patients in this study.

Similar to other studies, HRV-infected children were older than those with RSV infections but younger than those with Flu A infections [15, 19, 22]. Although traditionally associated with mild URIs, in this study HRV was responsible for about three-quarters of serious lower respiratory infections (LRIs) in hospitalized children. Interestingly, we found that HRV was able to cause more severe disease than other common viruses as determined by either higher rates of hypoxia or chest retraction. These findings are in contrast with previous studies [23–26], which reported equal disease severity between HRV and other common viral infections. Until now, most studies reported that RSV infections were more likely to have hypoxia compared to HRV infections [15, 22, 27]. However, Louie *et al.* showed that patients with HRV infections were more likely to require mechanical ventilation compared to those infected with RSV [28]. The reasons why HRV was associated with more severe disease are yet unclear. The high HRV viral load, the more pathogenic HRV serotypes, or the co-infection with other

Table 1. Demographic and clinical characteristics of hospitalized children with HRV mono-infections compared to RSV, Flu A, PIV-1 and PIV-3 mono-infections

Characteristics (%)	HRV (N = 234)	RSV (N = 184)	Flu A (N = 26)	PIV-1 (N = 29)	PIV-3 (N = 38)
Male	71.8	64.1	61.5	72.4	64.8
Age (months), median (IQR)	9 (4–19)	7 (3–13.5), <i>P</i> = 0.021	22.5 (11–32), <i>P</i> < 0.001	12.5 (9–19)	10 (6–16)
Prematurity (<37 weeks)	12.0	9.8	0	3.4	7.9
Malnutrition	9.8	6.5	11.5	3.4	7.9
Days before hospitalization, median (IQR)	3 (1–5)	2 (2–4)	3 (2–5)	2.5 (2–6)	3 (1–3)
Fever	50.0	73.9, <i>P</i> < 0.001	92.3, <i>P</i> < 0.001	89.7, <i>P</i> < 0.001	71.1, <i>P</i> = 0.022
SpO ₂ ≤ 92%	12.4	3.8, <i>P</i> = 0.002	3.8	3.4	0, <i>P</i> = 0.02
Tachypnoea	40.6	41.8	61.5, <i>P</i> = 0.041	58.6	31.6
Chest retraction	57.3	69.0, <i>P</i> = 0.015	50.0	34.5, <i>P</i> = 0.028	50.0
Wheezing	63.2	61.4	42.3, <i>P</i> = 0.038	51.7	68.4
WBC (cells/mm ³), median (IQR)	11 800 (10 000–15 200)	10 900 (8595–13 500), <i>P</i> = 0.004	11 500 (7950–13 600)	10 400 (9170–12 400)	10 100 (8530–12 200), <i>P</i> = 0.005
Neutrophil (cells/mm ³), median (IQR)	4895 (2882–7560)	3676 (2139–5888), <i>P</i> < 0.001	4344 (2372–6421)	5052 (2054–7108)	3672 (2633–5443)
Eosinophil (cells/mm ³), median (IQR)	239 (109–482)	105 (48–215), <i>P</i> < 0.001	58 (13–78), <i>P</i> < 0.001	38 (19–102), <i>P</i> < 0.001	86 (30–150), <i>P</i> < 0.001
Bronchiolitis	34.6	44.6, <i>P</i> = 0.043	15.4	13.8, <i>P</i> = 0.033	47.4
Pneumonia	35.5	35.9	46.2	31.0	34.2
Hospitalization duration (days), median (IQR)	5 (3–8)	6 (4–8)	5 (4–10)	5 (4–7)	5.5 (4–7)

HRV, Human rhinovirus; RSV, respiratory syncytial virus; Flu A, influenza A virus; PIV, parainfluenza virus; IQR, interquartile range.

All results are expressed as percentages except for median (IQR) values.

Categorical variables were compared by using Fisher's exact test, and continuous variables were compared by using Mann-Whitney *U* test. Only *P* values < 0.05 are shown.

undetected respiratory viruses or bacteria may play a role [1]. HRV infection has been shown to facilitate secondary bacterial pneumonia. Bacteria are often found together with HRV in patients with pneumonia, which may influence the disease severity [1]. In this study, patients with HRV infections were admitted to the hospital later than those with RSV infections. Thus, they may be more likely to contract bacterial superinfection. Further studies to investigate these possibilities are required to clarify the role of HRV in severe LRIs. In addition, eosinophil count was significantly increased in HRV-infected children compared to other groups. In some conditions, HRV can stimulate cytokine production such as eotaxin and eotaxin-2 to attract and stimulate eosinophils [29]. Since blood eosinophil count is the predictor of reactive airway disease, our finding is consistent with previous reports indicating early infection of HRV as an important risk factor for asthma development [4, 30].

The most common co-infection was found for HRV and RSV. The high incidence and overlapping of seasonal distribution of both agents might explain this phenomenon. HRV co-infections may lead to more severe disease than HRV mono-infections as determined by higher rate of chest retraction, reflecting the decrease of lung compliance and severe dyspnoea. Similar findings were found in some studies [19], but not in others [24, 31]. Since HRV is known to have a long shedding period, co-detection may be due to successive infections. However, patients with HRV co-infection in this study were admitted to hospital earlier than those with HRV mono-infection (2 vs. 3 days), suggesting that true co-infection is more likely.

The present study indicates that HRV-A predominated over HRV-C while HRV-B was not detected. Similar findings were also reported with HRV-A being most prevalent [15, 18]. The absence of HRV-B may be due to either it being a minor species or its milder nature which does not lead to

Table 2. Demographic and clinical characteristics of hospitalized children with HRV mono-infections compared to HRV co-infections

Characteristics (%)	HRV mono-infection (N = 234)	HRV co-infection (N = 91)	P
Male	71.8	64.8	0.228
Age (months), median (IQR)	9 (4–19)	9 (3–14.5)	0.628
Prematurity (<37 weeks)	12.0	8.8	0.555
Malnutrition	9.8	6.6	0.516
Days before hospitalization, median (IQR)	3 (1–5)	2 (2–4)	0.232
Fever	50.0	71.4	<0.001
SpO ₂ ≤ 92%	12.4	12.1	1
Tachypnoea	40.6	41.8	0.9
Chest retraction	57.3	70.3	0.032
Wheezing	63.2	64.8	0.898
WBC (cells/mm ³), median (IQR)	11 900 (10 000–15 200)	11 400 (9170–15 500)	0.242
Neutrophil (cells/mm ³), median (IQR)	4895 (2883–7560)	3860 (2526–8048)	0.322
Eosinophil (cells/mm ³), median (IQR)	239 (109–482)	127 (62–268)	<0.001
Bronchiolitis	34.6	40.7	0.369
Pneumonia	35.5	37.4	0.797
Hospitalization duration (days), median (IQR)	5 (3–8)	6 (4–8)	0.078

HRV, Human rhinovirus; IQR, interquartile range.

All results are expressed as percentages except for median (IQR) values.

Categorical variables were compared by using Fisher's exact test, and continuous variables were compared by using Mann-Whitney *U* test.

Table 3. Demographic and clinical characteristics of hospitalized children with HRV-A mono-infections compared to HRV-C mono-infections

Characteristics (%)	HRV-A (N = 28)	HRV-C (N = 12)	P
Male	64.3	75.0	0.716
Age (months), median (IQR)	10 (6–20)	17.5 (8–31.5)	0.192
Prematurity (<37 weeks)	17.9	0	0.298
Malnutrition	7.1	8.3	1
Fever	64.3	50.0	0.49
SpO ₂ ≤ 92%	7.1	0	1
Tachypnoea	35.7	50.0	0.49
Chest retraction	57.1	50.0	0.738
Wheezing	57.1	75.0	0.477
WBC (cells/mm ³), median (IQR)	12 500 (10 500–14 700)	13 900 (10 500–16 300)	0.738
Neutrophil (cells/mm ³), median (IQR)	4334 (2859–7805)	6036 (5328–8935)	0.457
Eosinophil (cells/mm ³), median (IQR)	195 (105–317)	383 (187–437)	0.086
Bronchiolitis	32.1	16.7	0.451
Pneumonia	46.4	50.0	1
Hospitalization duration (days), median (IQR)	5 (3.5–7.5)	4.5 (3.5–7)	0.85

HRV, Human rhinovirus; IQR, interquartile range.

All results are expressed as percentages except for median (IQR) values.

Categorical variables were compared by using Fisher's exact test, and continuous variables were compared by using Mann-Whitney *U* test.

hospitalization. In recent studies, HRV-A was reported more commonly during infancy, while HRV-C was the most common species in older children [1, 8]. Since our patients were predominantly

young infants, this should be seen as why we found HRV-A more frequently than HRV-C. Comparable with previous studies, we did not find significant differences between both species in terms of severity

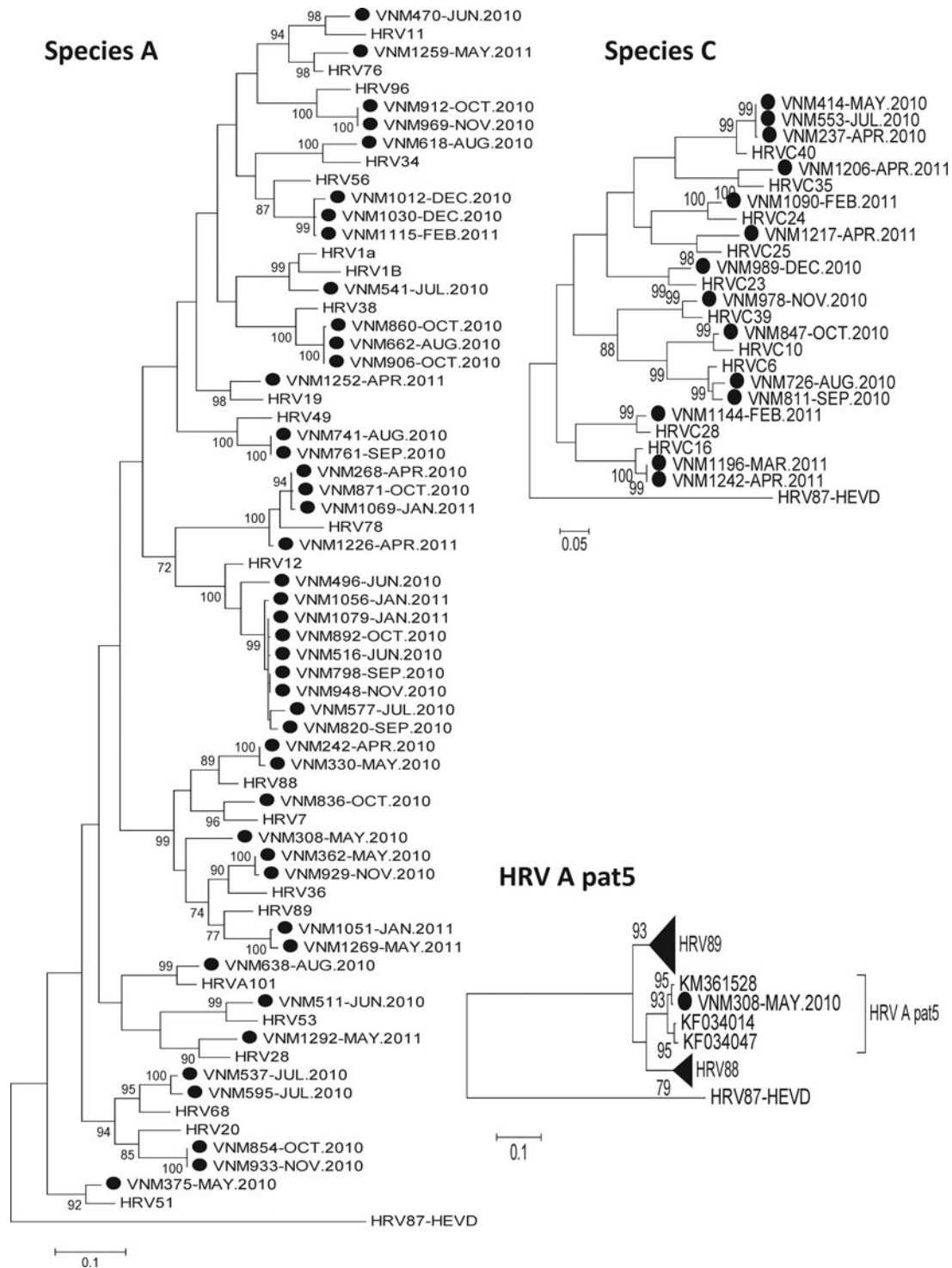


Fig. 2. Diversity of HRV types detected in the study. Phylogenies of the VP4/VP2 sequences of HRV-A, HRV-C, HRV-Apat5 detected in this study and reference strains were constructed with MEGA 5 software using the maximum-likelihood method. Data were bootstrap re-sampled 1000 times to assess the robustness of branches, values >70% are shown at the branch nodes. The HRV strains in this study are indicated by a solid round symbol (●). HEV-D (HRV-87) (GenBank accession no. AY040243) sequence was used to root the trees.

[8, 23, 24]. By contrast, other authors have suggested HRV-C might be responsible for more severe disease [6, 7, 15]. The variations could be attributed to the

difference either in viral load or in studied populations and study designs. Therefore, these data should be treated with caution.

Consistent with other studies, HRV was circulating throughout the year with several peaks of activity in our study [6, 18, 21, 32]. Although seasonal variation is a common characteristic of many respiratory viruses, its causes are largely unknown. To some extent, the year-round circulation of HRV may be explained by the great genetic diversity of HRVs. This speculation was supported by the rapid changing of HRV types from month to month. Even in some types that lasted for several months (e.g. HRV-A12), the strains of the same type were also different, which proves the diverse nature of HRV populations (Fig. 2). In addition, although only a part of the samples were typed, the peaks of HRV activity are likely due to the introduction of more HRV types into the community at the same time (July 2010: four types; October 2010: seven types, November 2010: five types; April 2011: 5 types). These findings imply that HRV strains may have a time-limited circulation manner.

The identification of a new HRV type which was circulating only within Southeast Asia for several years is interesting since HRV is known to circulate freely worldwide [13]. However, the underlying reason remains unknown. Although using VP4/VP2 sequences for classification and typing has been shown to agree well with the more reliable typing method using VP1 sequences, sequencing the VP1 of the novel type HRV Apat5 identified here is required for confirmation [13].

This study has several limitations. First, the lack of a control group of healthy children is the major limitation. Since HRV has been frequently identified from asymptomatic children, the causative role of HRV in hospitalized ARI cases is questionable. Second, due to the possibly prolonged shedding from previous infection, the detection of viral RNA in respiratory samples may not always reflect a current illness. By excluding patients with previous infection within 3 weeks and comparing only single infections with each other, we tried to limit the effect of prolonged viral shedding as much as possible. Third, the study only included hospitalized children so that the results do not reflect the overall burden of HRV in the general paediatric population. However, the severity of HRV infection compared to other viral infections may still be valid since the groups being compared share the same selection bias of being hospitalized. Fourth, a small number of Flu A and PIV cases may hamper the ability to reveal the significant differences compared to HRV cases individually.

According to the hospital's triage process, patients with suspected influenza infection will be referred to an Infectious Diseases ward for isolation and treatment. As only samples from Respiratory wards were collected in this study, the number of influenza cases may be underrepresented. Fifth, due to limited resources only 20% of HRV-positive samples were sequenced, which may be insufficient to see the association of a specific species or type with particular disease manifestation. Finally, viral loads were not measured which may affect the evaluation of the association between HRV infections and disease severity.

In conclusion, this study shows HRV as an important pathogen associated with significant morbidity and severe LRIs in hospitalized children. A high genetic diversity and changing of HRV types over time were observed and may be responsible for the year-round circulation of HRV infections. Improved definition of the clinical impact of HRV infections may support better guidance of preventive and therapeutic interventions in clinical practice.

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DECLARATION OF INTEREST

None.

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