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Biodiversity and clinico-demographic characteristics of human rhinoviruses from hospitalized children with acute lower respiratory tract infections in Malaysia☆

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ABSTRACT

Background: There is accumulating evidence that human rhinovirus (HRV) causes acute lower respiratory tract infections (ALRTI). Recently, HRV-C was identified as a new species of HRV, but its spectrum of clinical disease is not well understood.
Objectives: We investigated the molecular epidemiology, demographic and clinical characteristics of HRVs among hospitalized children with ALRIs.
Study design: One hundred and sixty-five nasopharangeal aspirates taken from children <5 years hospitalized with ALRTIs in Serdang Hospital, Malaysia, were subject to reverse transcriptase-PCR for HRV. Phylogenetic analysis on VP4/VP2 and 5′-NCR regions was used to further characterize HRV. Other respiratory viruses were also investigated using semi-nested multiplex RT-PCR assay. Clinical parameters were analyzed between HRV, RSV and IFV-A mono-infections and between HRV species.
Results: HRV was detected in 54 (33%) patients for both single (36 samples) and multiple (18 samples) infections, 61.1% (22/36) represents HRV-A strains while the remaining 14 HRV-C. Strain P51was the first reported representative of HRV98. The majority of the single HRV cases were in the second half of infancy; HRV-C occurred among older children compared with HRV-A. HRV children were admitted significantly earlier and less febrile than RSV and IFV-A infection. HRV-C infected children were more likely to have rhonchi and vomiting as compared to HRV-A. Pneumonia was the most common discharge diagnosis followed by bronchiolitis and post-viral wheeze in HRV patients.
Conclusion: Our study showed high prevalence of HRVs and detection of HRV-C among hospitalized children with ALRTIs in Malaysia. Analysis of clinical parameters suggested specific features associated with HRVs infections and specific HRV groups.

1. Background

Human rhinoviruses (HRVs), members of the Picornaviridae, were first isolated in 1956, are the most prevalent human respiratory pathogens [1–3]. Recent developments in HRV detection and classification methods have led to increasing accumulation of HRV sequences in genomic databases revealing greater association of HRVs with serious ALRTIs such as deadly pneumonia [3–7]. The presence of the new HRV-C strain in severe respiratory disease has further instilled research interest in the clinical impact, molecular biology and epidemiology of HRVs.

2. Objectives

As research of HRV is limited [8], especially in Asian developing countries, this study aims to examine the molecular epidemiology, the demographic characteristics and clinical features including the newly discovered HRV-C species, among hospitalized children less than 5 years of age with ALRTI in Malaysia.

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3. Study design

3.1. Clinical assessment

A study was conducted among children more than one-month-old and less than 5 years of age with the diagnosis of ALRTI to the pediatric wards at Hospital Serdang, Selangor, Malaysia, from June 16 to December 21, 2009, after obtaining approval from both the Medical Research and Ethics committee (MREC) of the Ministry of Health Malaysia and University Putra Malaysia. Selection of subjects was based on predetermined inclusion and exclusion criteria. Children who met the final diagnosis for ALRTI had at least 3 of the following: fever, tachypnoea, cough, auscultation findings indicative of lower respiratory disease (including rhonchi, crackles, or bronchial breath sounds) and/or chest retraction supported with chest radiographic changes, if available, were included and if nasopharangeal specimens were collected within 24 h of admission. Children who had one of the followings were excluded from the study: congenital or acquired immunosuppressive conditions and patients with conditions posing a potential hazard in obtaining the nasopharyngeal samples. A standardized study protocol was developed to record demographic and medical history of the patients, clinical features including symptoms and signs, outcome of the illness and hospital course, and laboratory and radiological findings. At the end of the hospitalization, the children's charts were reviewed by four of the co-investigators and the clinical diagnosis was determined by the principal investigator. The clinical diagnosis was categorized into pneumonia, bronchiolitis, and post-viral wheeze.

3.2. Virus detection

During the first 24 h of admission, nasopharyngeal aspirates (NPA) were taken from each patient. Viral genome was extracted using MagMAX Viral RNA Isolation Kit (Applied Biosystems, CA, USA). Detection of HRV was performed via reverse transcription on RNA extracts using RevertAid H Minus First Strand cDNA synthesis kit (Fermentase, USA). PCR was performed for all the samples using primers that amplified a fragment encompassing the VP4/VP2 region and the hyper-variable region in the 5′-NCR of human rhinoviruses [9]. Both strands of PCR products were sequenced using ABI 3730 xl DNA Analyzer (Applied Biosystems). Specimens were also being tested for respiratory syncytial virus (RSV), human metapneumovirus (HMPV), influenza virus (IFV) type A and B, parainfluenzavirus 1–4 (PIV1-4), coronaviruses (HCoV) OC43 and 229E [10], human bocavirus (HBoV) and human adenovirus (HAdV) using PCR [11,12].

3.3. Sequence analysis

Raw sequence data were analyzed using Vector NTI Suite 10 (Invitrogen Corp., Carlsbad, CA, USA), assembled using Contig Express and aligned using AlignX software. Multiple sequence alignments were made utilizing MEGA4 software [13] with default parameters followed by manual editing. Phylogenetic tree was estimated with MEGA4 using neighbor-joining method [14] with maximum composite likelihood model with 1000 bootstrap replicates [15]. The nucleotide and deduced amino acid sequences of the VP4/VP2 regions were compared with those of HRV-A, HRV-B and HRV-C strains using reference sequences available in the GenBank using BLAST (Basic Local Alignment Search Tool – http://blast.ncbi.nlm.nih.gov/). The sequences generated in this study were submitted to GenBank under accession numbers HM044162 to HM044201.

Table 1
Multiple HRV Infections ($n = 18$).

Pathogens	No.	%
Total	18	100
HRV + RSV	11	61.1
HRV + HMPV	2	11.1
HRV + PIV-2	1	5.5
HRV + RSV + HAdV	1	5.5
HRV + RSV + HBoV	1	5.5
HRV + FluA + HAdV	2	11.1

3.4. Statistical analysis

Demographic and clinical parameters were compared between HRV, RSV, and IFV-A mono infections and also among HRV species. Pearson chi-square or Fisher's exact test were used for categorical variables. The student's independent sample *t*-test or non-parametric Mann–Whitney *U* test, ANOVA tests or Kruskal–Wallis test were applied for continuous variables where it's applicable. All analyses were performed using SPSS version 16.0. *p*-Values of <0.05 were considered statistically significant.

4. Results

4.1. Occurrence of the viruses

A total of 165 children <5 years of age who fulfilled the inclusion criteria were enrolled in the study. RSV (49/165, 29.7%) was found to be the main single virus detected from the study population, followed by HRV (36/165, 21.8%) and IFV-A (10/165, 6.1%). Multiple HRV infections were found in 18 samples (10.9%), including 14 in dual and 4 in triple infections as shown in Table 1. Of these, dual infection of HRV and RSV (11/18, 61.1%) was the most prevalent multiple infection recorded. In total, 54 out of 165 (32.7%) samples were found infected with HRV.

Out of 36 HRV single infections, 25 (69.4%) were male. The mean age of HRV patients was 11.8 ranging from 2.0 to 45.1 months. They were older than children infected with RSV and younger than IFV-A patients (mean age of 9 and 12.7 months respectively). The mean age of HRV-C infections was significantly higher than HRV-A infections (16.3 vs. 7.1 months). The majority of the HRV-A infections were found in children of 6–11 months of age; while the peak age of patients hospitalized with HRV-C was 12–23 months.

4.2. Clinical features of HRV infections vs. RSV and IFV-A infections

The clinical features of children infected with HRV, RSV and IFV-A are shown in Table 2. HRV infected patients were admitted earlier compared to RSV and influenza; children with HRV presented to the hospital after a mean duration of 1.9 days (ranged 1–9 days) as compared with HRV (4.0 days, $p = <0.001$) and IFV-A (4.8 days, $p = 0.002$). In terms of respiratory features, there were no distinctive differences among the three infections. However, fever occurred less often in HRV infections than in RSV and IFV-A. Temperature equal or above 38 °C was presented in 17% of HRV as compared with 33% of RSV and 30% of IFV-A infections. Majority of the HRV patients were hospitalized for shorter duration and received less antibiotic, particularly in comparison to RSV. Disease severity characterized by need of oxygen, admission to intensive care unit (only one case of HRV) and prolonged hospital stay did not differ among the studied virus groups. Diarrhea was less common in HRV single infections as compared with IFV-A (8.3% vs. 40%, respectively, $p = 0.031$). Leukocytosis occurred significantly more frequently in HRV single infections than in RSV ($p = 0.035$). Differential count showed neutrophilic predominance in HRV compared

Table 2
Demographic and clinical characteristics of RSV, HRV, and IFVA single infections.

Variables	RSV ($n=49$)	HRV ($n=36$)	IFVA ($n=10$)	p-Value
Demographic characteristics				
Gender, male	32 (65.3)	25 (69.4)	6 (60)	0.257
Mean age (months)	9 ± 7.3	11.8 ± 10.3	12.67 ± 8.4	
Age groups				
0–5	**21 (42.9)**	**8 (22.2)**	2 (20)	**0.047; HRV vs. RSV**
6–11	14 (28.6)	17 (47.2)	3 (30)	
12–23	11 (22.4)	7 (19.4)	4 (40)	
24–59	3 (6.1)	4 (11.1)	1 (10)	
Clinical characteristics				
DOS[a,b]	**4.0 ± 3.5**	**1.9 ± 2.1**	**4.8 ± 2.7**	**<0.001[c]; HRV vs. RSV; 0.002[c]; HRV vs. IFV A**
Fever	**44 (91.8)**	**24 (66.7)**	**10 (100)**	**0.003[d]; HRV vs. RSV; 0.044[e]; HRV vs. IFV A**
Cough	45 (100)	36 (100)	10 (100)	0.570[d]
Rhinorrhea	44 (89.8)	28 (77.8)	9 (90)	0.162[d]
Difficulty breathing	42 (85.7)	32 (88.9)	8 (80)	0.733[d]
Chest crepitation	44 (89.8)	30 (83.3)	10 (100)	0.319
Rhonchi	29 (59.2)	23 (63.9)	6 (60)	0.977
Vomiting	23 (46.9)	20 (55.6)	6 (60)	0.815
Diarrhea	7 (14.3)	**3 (8.3)**	**4 (40)**	**0.031[d]; HRV vs. IFV A**
Laboratory findings				
Hemoglobin (g/dL)	11.2 ± 1.3	11.4 ± 1.4	11.3 ± 1.2	0.934
WBC ≥ 15.0 ($\times 10^9$ cells/L)	**6 (12.2)**	**13 (37.1)**	1 (10)	**0.035[f]; RSV vs. HRV**
>7.5 ($\times 10^9$ cells/L)	**7 (14.3)**	**13 (37.1)**	2 (20)	**0.015[d]; RSV vs. HRV**
Platelet (10^9/L)	360 ± 105	398 ± 139	368 ± 142	0.488
Abnormal chest X-ray	23 (46.9)	14 (40.0)	4 (57.1)	0.747
Hospital course				
Duration of hospitalization				
1–2 days	**6 (12.8)**	**6 (17.6)**	2 (20)	**0.011; RSV vs. HRV[d]**
3–4 days	16 (34.0)	21 (61.8)	4 (40)	
≥5 days	25 (53.2)	7 (20.6)	4 (40)	
Antibiotic therapy	**41 (87.2)**	**22 (64.7)**	8 (80)	**0.016; RSV vs. HRV[d]**
Clinical diagnosis				
Pneumonia	34 (69.4)	23 (63.9)	8 (80)	0.611[d]
Bronchiolitis	13 (26.5)	8 (22.2)	2 (20)	0.853[d]
Post-viral wheeze	2 (4.1)	5 (13.9)	0	0.149[e]

The bold values represent significant results.
[a] Duration from onset of symptoms to admission.
[b] All the values are presented as mean ± SD.
[c] Mann–Whitney U test.
[d] Pearson χ.
[e] Fisher's exact test.
[f] The Kruskal–Wallis one-way analysis of variance was used.

with RSV patients ($p=0.015$). Pneumonia was the most common discharge diagnosis among HRV patients followed by bronchiolitis and post-viral wheeze.

4.3. Clinical features of HRV-A vs. HRV-C infections

Vomiting occurred almost two times more with HRV-C_{151} infection ($p=0.036$), as shown in Table 3. HRV-C patients were more likely to present with rhonchi in comparison to HRV-A (86% vs. 47%, respectively, $p=0.024$). The study also showed that HRV-C patients tended to have a higher neutrophil count ($p=0.032$) while lymphocytosis was associated with HRV-A ($p=0.009$). Post-viral wheeze was more common with HRV-C infections. Disease severity characterized by need of oxygen, admission to intensive care unit (only one case of HRV) and prolonged hospital stay did not differ among the studied species.

4.4. Phylogenetic analysis

HRV single infections were subjected to sequencing as the study aimed at looking into different variables of the single infections. Thirty-three of 36 HRV single samples were retrieved in sequencing. Additionally three HRV samples co-infected with other viruses were also sequenced. All 36 genetically typed HRV strains clustered close to a previously reported clinical strain in GenBank, except strain P51 which became the first reported representative of HRV98. HRV-A strains represented the majority detection (22/36, 61.1%) as shown in Fig. 1. They grouped along with prototype strains into 15 different serotypes including 12 (5), 22 (1), 24 (3), 32 (1), 34 (1), 39 (1), 41 (1), 43 (1), 46 (1), 47 (1), 56 (2), 62 (1), 89 (1), 98 (1), and 101 (1). We found that 14 (39%) of the samples were clustered in the separate clad distinct from HRV-A and HRV-B, along with representative strains of HRV-C (Fig. 2). HRV-B strains were not among the typed strains. The nucleotide similarities to closest prototype strain in HRV-A, varied from 85.1% to 95.0%, while the closest genetic relatives were observed with a variation range of 91.2% to 99.8%. The respective HRV-C strain similarities varied from 87.5% to 98.9%. Genetic analysis used for typing revealed four human enterovirus (HEV) strains in species HEV-B, HEV-C and HEV-D. However, it should be noted here that the VP4/VP2 region used for genetic typing of HRV strains does not unequivocally allow typing of HEV strains due to greater sequence similarity between strains within species.

5. Discussion

A significant burden of HRV infection, was found in 54 out of 165 patients (33%), and this concurs with previous studies with a

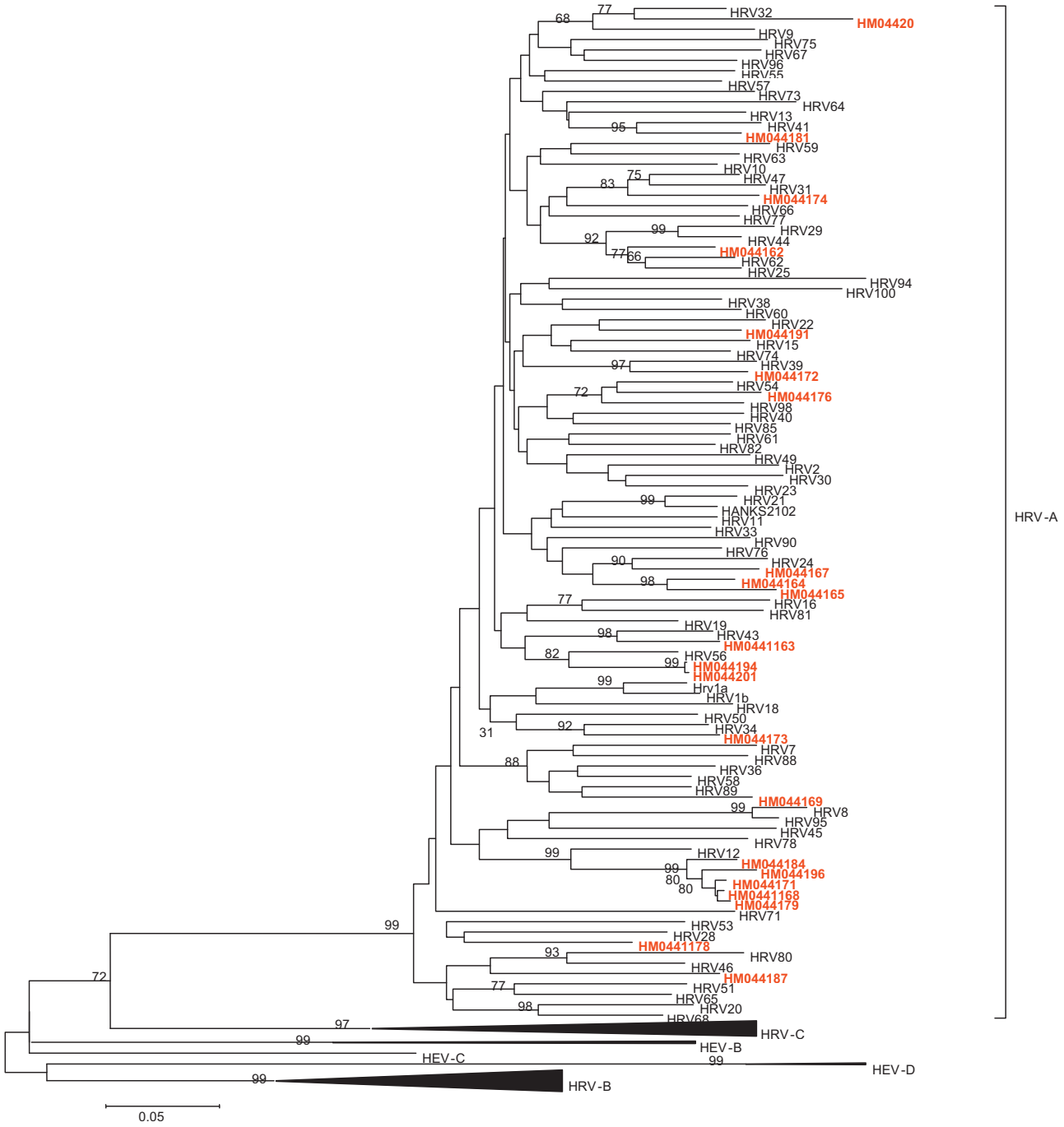

Fig. 1. Neighbor-joining dendrograms depicting phylogenetic relationships of human rhinovirus species A strains of this study in the 420 nucleotide region in VP4/VP2. Red types indicate strains of this study while reference sequences are shown in black. Branches showing >70% bootstrap support are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

rate varying from 26% to 33% in a hospitalized pediatric patient [8,16–20]. This contradicts with the traditional notion that HRV is only associated with upper respiratory tract infections. Earlier studies indicated that the clinical value of positive PCR is somewhat disputable among patients with ALRTI [3,7,19,21] as asymptomatic HRV infections is known to occur in 15–30% of individuals. Several subsequent clinical studies of small numbers of selected patients showed that HRV can replicate in lower respiratory tract. This finding concluded that detection of HRV especially in children less than 2 years does not simply represent asymptomatic infection but is related to true infection [7,22]. In addition, the ability of HRV to infect lower respiratory airways and to induce cytotoxicity to bronchial epithelium has been shown experimentally among immunocompetent individuals [18].

In this study the most prevalent combination was found between HRV and RSV. The combination of HRV and RSV as a main double infection has been reported by others [16,18,23,24]. High incidence of HRV coupled with RSV infection could be explained by the substantial overlapping of monthly distribution observed for these 2 viruses during the study period [23].

The relatively recent application of molecular study of PCR based methods for HRV has identified several novel HRVs, including HRV-C. Our study showed that HRV-B was absent while HRV-A predominates from HRV-C. This finding seems to reinforce that

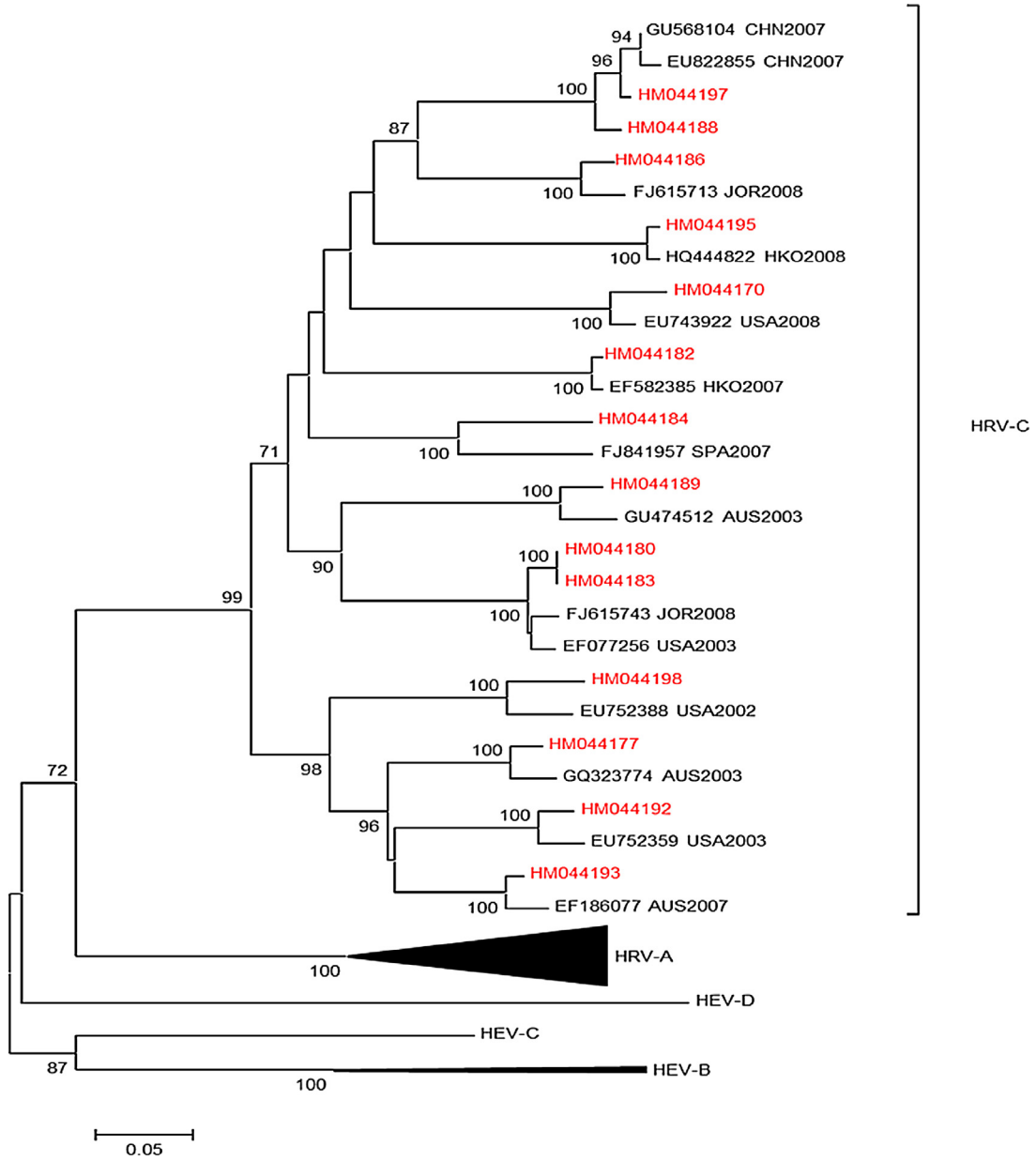

Fig. 2. Neighbor-joining dendrograms depicting phylogenetic relationships of human rhinovirus species C strains of this study in the 420 nucleotide region in VP4/VP2. Red types indicate strains of this study while reference sequences are shown in unbold. Branches showing >70% bootstrap support are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

HRV-B is a minor species worldwide; however it concurs with most findings by other investigators as HRV-A was the most prevalent compared to the other species. This may reflect apparent seasonality or yearly variation of circulating HRV. On the other hand, it may be associated with HRV-B of a milder form of disease presentation and consequent of lesser rate of hospitalization.

Consistent with other findings, our study showed that HRV infected children are older than those with RSV infections and younger than those with IFV-A infections [25,8]. The results support the importance of HRV infections among infants with acute respiratory infections [3,22] especially in its second half. HRV-A infections were more likely to occur during infancy as compared with HRV-C, further corroborating the findings that HRV-A infections are less prevalent in older age group as compared with HRV-C [26,27].

Few studies have described the clinical characteristics of HRV ALRI in infants and young children. Our study revealed that HRV infected children were hospitalized earlier in the course of their disease and were less febrile on presentation as compared to RSV and IFV-A infections. They were also more likely to be discharged earlier and tend to receive lesser antibiotics as compared to the other two infections, especially to RSV. HRV is known to have a shorter incubation period and hence presented earlier as compared with HRV or occasionally with IFV-A especially with H1N1 infection. This could be explained further in terms of the cytopathology on the respiratory epithelial cells as HRV is the least invasive with minimal cell damage and secondary bacterial infection is far less a complication compared to RSV and IFV. As a result, patients were subjected to lesser antibiotics and stayed shorter in the hospital as seen in this study. However, neutrophilia as documented in the present study may not reflect secondary bacterial infection in HRV. Several studies in the past, acknowledged the increase infection in both peripheral and airway neutrophils as a risk to development of asthmatic exacerbations. On the other hand, IFA-A infection tended to be associated with diarrhea, as the infection usually resulted in

Table 3
Clinical characteristics of HRV-A and HRV-C single infections.

Variables	HRV-A ($n=19$)	HRV-C ($n=14$)	p-Value
Demographic characteristics			
Gender, male	15 (78.9%)	8 (57.1%)	0.178
Mean age (months)			
Age groups	7.1 ± 3.8	16.3 ± 11.0	**0.01**
0–5	6 (31.6)	2 (14.3)	**0.007**
6–11	11 (57.9)	4 (28.6)	
12–23	2 (10.5)	5 (35.7)	
24–59	0	3 (21.4)	
Clinical characteristics			
Fever	14 (73.7)	8 (57.1)	0.459
Cough	19 (100)	14 (100)	0.67
Rhinorrhea	15 (78.9)	10 (71.43)	0.695
Difficulty breathing	17 (89.5)	13 (92.9)	1
Vomiting	8 (42.1)	11 (78.6)	**0.036**
Chest recession	16 (84.2)	13 (92.9)	0.62
Chest crepitation	15 (78.9)	13 (92.9)	0.366
Rhonchi	9 (47.4)	12 (85.7)	**0.024**
Laboratory findings			
WBC ≥ 15.0 ($\times 10^9$ cells/L)	6 (31.6)	5 (38.5)	0.721
ANC ($\times 10^9$ cells/L)	4.6 ± 2.5	7.3 ± 4.3	**0.032**[c]
ALC ($\times 10^9$ cells/L)	7.3 ± 3.8	4.5 ± 1.8	**0.009**[d]
Platelet (10^9/L)	417 ± 135	348 ± 126	0.157[c]
Hospital course			
Mean DOH[a,b]	4.18 ± 2.430	4.07 ± 1.439	0.554[d]
Antibiotic therapy	10 (58.8)	10 (71.4)	0.707[e]
Supplemental O_2	14/17 (82.4)	11/13 (84.6)	1.000[e]
Underlying disease			
Asthma	3 (15.8)	1 (7.1)	0.451
Clinical diagnosis			
Pneumonia	12 (63.2)	9 (64.3)	0.947[e]
Bronchiolitis	6 (35.3)	2 (14.3)	0.241[e]
Post-viral wheeze	1 (5.9)	3 (21.4)	0.158[e]

Normal ranges (Reference: International Federation of Clinical Chemistry (IFCC)): WBC: 4.5–13.5; neutrophil: 2.0–6.0; lymphocyte 5.5–8.5; platelet: 150–400. The bold values represent significant results.

[a] Duration from onset of symptoms to admission.
[b] All the values are presented as mean ± SD.
[c] *t*-Test was used.
[d] Mann–Whitney test was used.
[e] Fisher's exact test.

a more systemic involvement with direct invasion of the gastrointestine by the virus [28].

This study further distinguishes certain clinical features of the two species of HRV in ALRTI. Although in general, the clinical presentations were comparable for both HRV-A and C, rhonchi and vomiting were more common in HRV-C infected children as compared to HRV-A. In our study, the presence of rhonchi was associated with ALRTI rather than asthma, as only one had asthma as an underlying disease. As shown in previous studies, this finding further supports the role of HRV-C among patients with febrile wheeze in ALRTI. A large clinical cohort study indicated that this group of children has a preexisting predisposition to asthma in early childhood. On the other hand, vomiting has not been reported in other studies as a prominent presentation in HRV-C. Vomiting with coughing is common in children (post-tussive vomiting) and it could be related to the wheezing episodes in our study and indicate a more severe form of infection in HRV-C as compared to HRV-A. Likewise, no characteristic laboratory finding has been associated previously with any specific HRV species; the modest increase in lymphocytes in HRV-A and neutrophils in HRV-C in our study could be coincidental and probably of no clinical importance. Consistent with the study by Jin [29], there was no significant difference in terms of disease severity for both species of HRV. On the other hand, Miller [8] found that HRV-C patients were more likely to require supplemental oxygen than HRV-A. The variations could be partly attributed to bacterial co-infections, viral load and type of the studied population. Therefore, these data must be interpreted with some caution.

Several different types were detected including outbreak-like clusters, e.g. HRV12. Phylogenetic analysis confirmed global prevalence of HRV-C strains. This study showed the large variation of concomitantly circulating HRV strains as reported previously [30]. Due to increased sequence typing efforts during recent years, most of the HRV strains of this study were shown to have very close genetic relatives circulating in other geographical areas.

In this study, HRV has been implicated as a cause of hospitalization in children with ALRTI in our locality. The dominant presence of both HRV-A and C concurs with global epidemiologic studies in other parts of the world. There were also some differences between the demographic variables, clinical manifestations and laboratory findings of HRV, RSV and IFV-A infections and within the HRV species. However, corroboration of the clinical significance and its pathogenesis will require larger numbers of subjects and should include a control group with no respiratory infection. The study could not be generalized as only it involved small numbers and in-patients are involved. A prospective longitudinal multicenter population based studies, utilizing quantitative PCR methods, are needed to better explore and understand the role of HRV in ALRTIs.

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Conflict of interest

The authors involved in this study have no conflicts of interest to declare.

Ethical approval

Approval was from both the Medical Research and Ethics committee (MREC) of the Ministry of Health Malaysia and University Putra Malaysia. Informed consent was obtained from cares of the patients.

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