Original Article

Molecular Epidemiology of the Human Rhinovirus Infection in Mongolia during 2008–2013

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SUMMARY: Rhinovirus infections are common in all age groups world-wide, and they occur throughout the year. In this study, we examined 2,689 nasopharyngeal swabs collected in Mongolia during 2008–2013. Human rhinoviruses (HRVs) were detected in 295 (11.0%) samples, and 85 (28.8%) patients were co-infected with other respiratory viruses. HRV was co-detected with bocavirus, human coronavirus, and respiratory syncytial virus in 21 (24.7%), 17 (20.0%), and 14 (16.5%), respectively. We tested 170 (57.6%) of the 295 HRV-positive samples: 117 HRV strains were typed by using the VP4/VP2 method and 53 by using 5' UTR method. We found HVR-A, HVR-C, and HVR-B infections in 80 (47.1%), 76 (44.7%), and 14 (8.2%) samples, respectively.

INTRODUCTION

Human rhinoviruses (HRVs) are the most frequent causes of acute respiratory tract infections (RTIs). HRVs are small, non-enveloped, single-stranded, positive-sense RNA viruses that are now classified within the *Enterovirus* genus of the family *Picornaviridae*. Traditionally, they have been associated only with mild upper respiratory tract infections (URTIs) (1). Most “classical” HRVs have a relatively low optimal temperature for growth (33°C): this explains their residing in the human nasopharynx and association with URTIs (2).

The first HRV was discovered in 1953, and by 1987 HRVs were classified into 100 distinct serotypes based on their antigenic cross reactivity in neutralization tests (3,4). These multiple serotypes have been subdivided into two major HRV clades (HRV-A and HRV-B) (4). HRV-C was co-discovered in USA (5) and Australia (6) in 2006, and now contains 51 types (7). These viruses have been implicated as the causes of asthma exacerbation (8,9) and severe respiratory tract illnesses in children, immunosuppressed individuals, and the elderly (10–14). HRV-associated mortalities have also been recently reported (13,15–17).

In our previous orientation study (18), we selected 240 samples collected during 2008–2009 in Mongolia. Respiratory viruses were detected in 103 (43%) samples with the highest number of positive results obtained for rhinoviruses (38 samples [15.8%]). Since rhinoviruses

were found to be the most prevailing type of circulating respiratory viruses in Mongolia, we performed this study with the aim of characterizing these HRVs in detail.

MATERIALS AND METHODS

Clinical samples: Between November 2008 and May 2013, nasopharyngeal swabs (NPS) were collected from 2,689 patients (50.1% male, 49.9% female; age 0–86 years; median age, 2 years) with acute RTIs from the Influenza Sentinel Surveillance Sites in Mongolia. Of these, 1,396 (52%) samples were from hospitalized patients and 1,293 (48%) were from outpatient visitors. All samples were stored at –70°C at the Department of Virology, National Center of Communicable Diseases (NCCD) in Mongolia until the investigation. This study was evaluated and approved by Ethical Committee, NCCD, MoH Mongolia, and informed consent was obtained from each patient.

Multiple virus detection: Viral DNA and RNA were extracted from the NPS samples by using the Viral DNA/RNA extraction kit (Bioneer, Daedeok, Korea). All samples were simultaneously screened for respiratory pathogens, including human parainfluenza viruses (types 1, 2, 3, and 4), influenza virus (A, A(H1N1), and B), respiratory syncytial virus (RSV), HRV, enterovirus, human coronaviruses (229E, NL63, HKU1, and OC43), human metapneumovirus, adenovirus, bocavirus, parechovirus, and *Mycoplasma pneumoniae*. The screening was performed using the multiplex real-time PCR (FTD Respiratory pathogen 21 kit [Fast-track diagnostics Ltd., Luxemburg]) according to the instructions provided by the manufacturer.

HRV detection by using specific RT-PCR and sequencing: Viral RNA was extracted from all HRV-positive samples by using the QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA, USA). We used three sets of

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primers to amplify the VP4/VP2 and 5' UTR regions for detection of HRVs according the methods described before (19–21). Primers 9895F (nucleotide position 534–560 [5'-GGGACCACTACTTTGGGTGTCCGTGT-3']) and 9565R (nucleotide position 1083–1058, according to HRV1b described by Hughes et al. (22) [5'-GCATCIGGYARYTTCCACCACCANCC-3']) were used to amplify the VP4/VP2 region in most of the known HRV species (~550 bp) (19). Primers RVC556F (5'-ACTACTTTGGGTGTCCGTGTTTC-3') and RVC886R (5'-TTTCCRATAGTGATTTGCTTKAGC C-3') were used to identify the VP4/VP2 region of HRV-C (20). Samples that gave negative results for these screenings were subjected to the third PCR by using primers that amplify the 5' UTR region of HRVs: primers P1-1 (5'-CAAGCACTTCTGTWCCCC-3'), P3-1 (5'-ACGGACACCCAAAGTAG-3'), P2-1 (5'-TTAGC5'CACATTCAGGGGC-3'), P2-2 (5'-TTAGCCACATTCAGGAGCC-3'), and P2-3 (5'-TTAGCCGCATTCAGGGG-3'). P1, P2, and P3 primers were located at bases 163–181, 443–463 and 535–551, respectively in the HRV16 genome. PCR fragment A (~900 bp) was used to determine the 5' UTR sequences of all 101 HRV serotypes. It was amplified using pan-HRV PCR forward primer P1-1, which annealed to the conserved region of P1, and serotype-specific reverse primer annealed to the 5' end of VP2 gene. PCR fragment B (~390 bp) was generated with pan-HRV PCR forward primer P1-1 and reverse primer P3-1. PCR fragment C (~300 bp) was generated with forward primer P1-1 and an equimolar mixture of reverse primers P2-1, P2-2, and P2-3. The variable sequences of P1 and P2 were used for the molecular typing assay (21). All PCR products were cloned into pGEM-T Easy vectors (Promega, Madison, WI, USA) and verified by sequencing.

Phylogenetic analysis and calculation of nucleotide p-distances: Sequences of HRV PCR products were analyzed by using nucleotide BLAST (blastn, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the types of HRV strains detected in this study. According to the results of Blastn, if the nucleotide sequence identity of the HRV PCR product of a sample was $\geq 90\%$ of the prototype strain of a certain type, it was assigned to this type, else it was considered untyped. Phylogenetic trees were constructed with the MEGA 5.02 software package (23) by using the neighbor-joining method and applying the maximum composite likelihood model with bootstrap values calculated from 100 replicates. Pairwise nucleotide p-distances were calculated using the program Sequence Distances within the package.

Statistical analyses: The chi-square (χ^2) test was used to assess the association of variables. *P* values (*P*) less than 0.05 were considered statistically significant.

Accession numbers: The nucleotide sequences generated in this study are available at GenBank, under these accession numbers: 5' UTR: KJ749855-KJ749864, KM462658-KM462700, and VP4/VP2: KM462701-KM462817.

RESULTS

HRVs detection in clinical samples: Of all the NPS samples, 295 (11.0%) tested positive for HRVs. Of these, we typed 170 (57.6%) samples: 117 strains were typed by the VP4/VP2 method and 53 by the 5' UTR method. Using the VP4/VP2 method, 52 (44.4%), 12 (10.3%), and 53 (45.3%) strains were typed as HRV-A, HRV-B, and HRV-C, respectively (Fig. 1A, B, C). Of the 53 HRV strains that were found positive only by the 5' UTR method, 28 (52.8%), 2 (3.8%), and 23 (43.4%) strains were typed as HRV-A, HRV-B, and HRV-C, respectively (Fig. 2A, B, C). The most frequently detected strain was HRV-A (47.1% [80/170]), followed by HRV-C (44.7% [76/170]), and HRV-B (8.2% [14/170]) was the least frequent. Of the 295 HRV-positive samples, 156 (52.9%) were from outpatient visitors and 139 (47.1%) from hospitalized patients. The median age of the hospitalized patients was the same for all HRV species, whereas the median age of HRV-B was higher than HRV-A and C species (for outpatients and hospital patients of HRV-B is 14 years old and 5.5 years old, respectively [Table 1]).

In all, 11.0% (205/1856) of HRVs were detected in patients 0–5 years old, 12.8% (31/243) in patients 6–10 years old, 10.9% (21/193) in patients 11–16 years old, 10.3% (26/253) in patients 17–46 years old, and 8.3% (12/144) in those over 47 years old. Although HRV was detected more frequently in the patients aged 6–10 years, it was not statistically significant ($\chi^2 = 2.28$, $P > 0.5$). In addition, 28.8% (85/295) patients with HRV had other cohabiting respiratory viruses, and the most frequently co-detected virus was bocavirus (24.7%). Other co-detections included the human coronavirus, 20.0%; RSV, 16.5%; parainfluenza virus, 12.9%; enterovirus, 8.2%; adenovirus, 7.1%; human metapneumovirus, 5.9%; influenza virus, 2.4%; and parechovirus, 2.4% (Table 2).

Seasonality of HRV infections: HRV infections were detected every month between 2008 and 2013 with the peaks being in late summer and early fall (July to October [Fig. 3]). Moreover, the monthly distribution

Table 1. Incidences of HRV infections in different age groups

Age group	No. of samples	No. of HRV positive samples(%)	Outpatient			Hospitalized patient		
			HRV-A	HRV-B	HRV-C	HRV-A	HRV-B	HRV-C
0–5	1856	205 (11.0)	21	3	33	26	3	26
6–10	243	31 (12.8)	9	0	4	0	1	3
11–16	193	21 (10.9)	7	1	2	1	0	2
17–46	253	26 (10.3)	8	3	1	5	2	1
≥ 47	144	12 (8.3)	2	1	4	1	0	0
Median age	2	2	8	14	2	1	5.5	1

Fig. 2. Phylogenetic analysis of HRVs based on nucleotide sequence of 5' UTR region. Using the 5' UTR nucleotide sequence (300 nt), neighbor-joining analysis was performed by applying the maximum composite likelihood model with bootstrap values calculated from 100 replicates and the software MEGA version 5.2. Phylogenetic trees based on the nucleotide sequences of HRV-A (A), HRV-B (B), and HRV-C (C). HRV strains detected in Mongolia are designated (◆).

Table 2. Mixed viral infection detected in HRV infected patients

Virus co-detected	No. of HRV positive sample <i>n</i> = 85 (%)	HRV-A <i>n</i> = 26 (%)	HRV-B <i>n</i> = 4 (%)	HRV-C <i>n</i> = 32 (%)
Bocavirus	21 (24.7)	8 (30.8)	1 (25.0)	9 (28.1)
Coronavirus	17 (20.0)	6 (23.1)	3 (75.0)	6 (18.8) ¹⁾
RSV	14 (16.5)	4 (15.4)	0 (0.0)	5 (15.6)
Parainfluenza virus	11 (12.9)	6 (23.1)	0 (0.0)	4 (12.5)
Enterovirus	7 (8.2)	0 (0.0)	0 (0.0)	3 (9.4) ¹⁾
Adenovirus	6 (7.1)	1 (3.8)	0 (0.0)	2 (6.3)
Metapneumovirus	5 (5.9)	0 (0.0)	0 (0.0)	2 (6.3)
Influenza virus	2 (2.4)	0 (0.0)	0 (0.0)	2 (6.3)
Parechovirus	2 (2.4)	1 (3.8)	0 (0.0)	0 (0.0)

¹⁾: One patient had HRV-C, coronavirus HKU1, and enterovirus triple infection.

differed among the three HRV species. HRV-A was detected usually in late summer (July to August) and early fall (September to October) during 2009–2011. Although HRV-B infection constituted a small fraction of the overall HRV infections, we did note a modest increase in HRV-B detection rate between fall and winter. HRV-C was usually detected every month, aside from the increasing detection rate between March and June 2009 and between September and October 2010–2012 (Fig. 4).

Phylogenetic analysis and typing of HRV infections: Based on the nucleotide sequences, HRV-A, B, and C were provisionally assigned to have 27, 6, and 26 types, respectively, in the VP4/VP2 phylogenetic trees (Fig. 1A, B, C). The number of HRV-A strains in the phylogenetic tree based on the nucleotide sequences was as follows: HRV 46, five strains; HRV12 and 78, four

Fig. 3. Distribution of positive samples for HRV per month (averages for 4 years).

Fig. 4. Distribution of positive samples for HRV species per month.

strains; HRV1, 8/95, 11, 19, 20–22, 29, 36, 80, 82, 89, and 96, two strains; and HRV7, 10, 13, 24, 49, 53, 58, 63, 67, 68, and 71, one strain. The number of HRV-B strains in the phylogenetic tree based on the nucleotide sequences was as follows: HRV79 and 86, three strains; HRV6 and 52, two strains; and HRV4 and 83, one strain. The number of HRV-C strains in the phylogenetic tree based on the nucleotide sequences was as follows: NAT045 (HRV-C2), five strains; NAT069 (HRV-C36), four strains; C025 (HRV-C5), 06-20 (HRV-C15), 7316563 (HRV-C19), Resp5613/07 (HRV-C32), and 06-230 (HRV-C43), three strains; NAT001 (HRV-C1), C024 (HRV-C4), C026 (HRV-C6), NAT083 (HRV-C12), RV471 (HRV-C21), RV-1123 (HRV-C25), RV177 (HRV-C26), N22 (HRV-C27), and tu304 (HRV-C38), two strains; and QPM (HRV-C3), g2-4 (HRV-C16), 06-582 (HRV-C17), N46 (HRV-C28), 06-739b (HRV-C35), NAT059 (HRV-C37), g2-23 (HRV-C41), g2-28 (HRV-C42), and PNG7293-3193 (HRV-C48), one strain.

Based on the nucleotide sequences, HRV-A, B, and C were provisionally assigned to 20, 2, and 5 types, respec-

tively, in the 5' UTR phylogenetic trees (Fig. 2A, B, C). The number of HRV-A strains in the phylogenetic tree based on the nucleotide sequences was as follows: HRV 12, four strains; HRV80, three strains; HRV1a, 47, and 80, two strains; and HRV1b, 7, 21, 22, 31, 34, 36, 46, 52, 65, 66, 71, 75, 77, and 91, one strain, respectively. Two HRV-B strains were provisionally assigned to HRV 52 and 91 based on the nucleotide sequences. The number of HRV-C strains in the phylogenetic tree based on the nucleotide sequences was as follows: W37, nine strains and NAT045 (HRV-C2), C024 (HRV-C4), C025 (HRV-C5), and W38, four strains.

Interspecies p-distances of HRVs: We calculated the interspecies of HRVs by the distribution of p-distances. Among the strains detected in our study and the reference strains in the VP4/VP2 region, the p-distances (mean $\pm$ SD) for HRV-A, HRV-B, and HRV-C were 0.254 ± 0.048 , 0.248 ± 0.047 , and 0.356 ± 0.087 , respectively (Fig. 5A, B, C). Among our strains and the reference strains in the 5' UTR region, the p-distances (mean $\pm$ SD) for HRV-A, HRV-B, and HRV-C were 0.211 ± 0.084 , 0.188 ± 0.069 , and 0.344 ± 0.134 , re-

Fig. 5. Distribution of nucleotide p-distances for HRV-A (A), HRV-B (B), and HRV-C (C) based on the nucleotide sequences of the VP4/VP2 region.

Fig. 6. Distribution of nucleotide p-distances for HRV-A (A), HRV-B (B), and HRV-C (C) based on the nucleotide sequences of the 5' UTR region.

spectively (Fig. 6A, B, C).

DISCUSSION

In this study, HRVs especially HRV-A and HRV-C were found to be important causes of hospitalization due to respiratory illness in Mongolia between 2008 and 2013. HRVs were detected in 11.0% of the tested NPS samples. Majority of the HRV-positive patients were infected by HRV-A (47.1%), followed by HRV-C (44.7%). HRV-B infections accounted for only 8.2% of the cases. Our findings are similar to the results found in RTI studies conducted in some Asian countries (24–27). In studies conducted in other countries, HRV-A and HRV-C were found to be predominant as well since they were detected in 50–73% of the tested samples, whereas HRV-B was the least common species (5,14,28). In this study, we have detected 37 types of HRV-A, 6 types of HRV-B, and 28 types of HRV-C strains in Mongolia, and the more prevalent types were HRV-A12, A46, A78, and A80; HRV-B6, B35, B52 and B86; and HRV-C NAT045, W37, C025 and C024.

In contrast to our finding, in Beijing, China, the most prevalent HRV serotypes were HRV-A90, A95, A54, and A18, and HRV-B48, B3, B37, and B27 in acute respiratory infection patients and HRV-A12, A36, A4, A22, and HRV-B42 in patients with pneumonia (24). In our study, the calculated p-distances for HRV-C strains were (VP4/VP2, 0.356 ± 0.087 ; 5' UTR, 0.344 ± 0.134) greater than those for HRV-A strains (VP4/VP2, 0.254 ± 0.048 ; 5' UTR, 0.211 ± 0.084). Recent studies suggested (7, 29, 30) that the correct type assignment of HRV-C strains is possible based on VP4/VP2 coding regions showing 10% divergence from the reference strains. An another report (31) suggested that divergence of the VP4/VP2 coding regions may not differ significantly among HRV-A, B, and C strains, although the divergence of HRV-C may be greater than that of HRV-A and -B.

However, species assignment (A, B, or C) is sometimes difficult due to putative recombination events between HRV-A and HRV-C strains (32–34). As has been noticed previously, some HRV-A sequences (e.g., HRV-A51, A65, A71, A12, A45, and A78 types) co-localize with a subset of HRV-C sequences which were consequently designated as “HRV-Ca” clade due to their 5' UTR sequences resembling those of HRV-A. Strains with species A-like 5' UTR sequence have been named HRV-Ca, and the remainder is assigned under HRV-C (33). No significant differences were found between the clinical outcomes or epidemiology of HRV-Ca and HRV-Cc isolates (35).

In our results, we found that, the HRV species was frequently co-detected with the bocavirus. Similarly, a study from Hong Kong (36) co-detected HRV-C species and the bocavirus. In a recent study in Thailand (27), HRV species were often co-detected with human metapneumovirus in as high as 40% cases. However, in recent reports from China and Italy (24,37), HRV was most frequently co-detected with RSV in children with pneumonia. HRVs are known as a cause epidemics in early fall and late spring (1). In our study, HRV infections were detected every month throughout the 4-years period with peaks late summer and early fall. In addition,

HRV-A was detected usually in late summer and early fall, HRV-B in fall and winter, and HRV-C during late spring, fall, and winter. In some studies (25,36) that included clinical specimens collected throughout the year, HRV appeared to show seasonal patterns of infection. In Hong Kong, HRV infections occurred throughout the year, although a higher incidence has been observed during fall and winter. In a study in Beijing, HRV-A infection was not detected during May and June, however, HRV-C was detected during May and June, not between July and September, and HRV-B was detected frequently between July and November (24). A recent study from USA (38) reported that HRV infections increased threefold during peak prevalence months of March, September, and October, and a clear seasonal pattern in the severity of HRV illness was seen. We did not find this kind of pattern in Mongolia. In our study, HRV-A and HRV-C were more frequently detected in younger children, while HRV-B was detected mostly in older children. A study in Thailand (39) detected HRV-A more frequently in younger children and adults, and HRV-C in children aged 1 to 4 years.

In summary, we analyzed HRV infections throughout the year in Mongolia during 2008–2013 and observed higher incidences during late summer and early fall. In addition, the median age of HRV-infected hospitalized patients was found to be less than that of HRV-infected outpatients (Table 1).

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Conflict of interest None to declare.

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