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Epidemiologic, clinical, and virologic characteristics of human rhinovirus infection among otherwise healthy children and adults

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Background: Human rhinovirus (HRV) is a major cause of influenza-like illness (ILI) in adults and children. Differences in disease severity by HRV species have been described among hospitalized patients with underlying illness. Less is known about the clinical and virologic characteristics of HRV infection among otherwise healthy populations, particularly adults.

Objectives: To characterize molecular epidemiology of HRV and association between HRV species and clinical presentation and viral shedding.

Study design: Observational, prospective, facility-based study of ILI was conducted from February 2010 to April 2012. Collection of nasopharyngeal specimens, patient symptoms, and clinical information occurred on days 0, 3, 7, and 28. Patients recorded symptom severity daily for the first 7 days of illness in a symptom diary. HRV was identified by RT-PCR and genotyped for species determination. Cases who were co-infected with other viral respiratory pathogens were excluded from the analysis. We evaluated the associations between HRV species, clinical severity, and patterns of viral shedding.

Results: Eighty-four HRV cases were identified and their isolates genotyped. Of these, 62 (74%) were >18 years. Fifty-four were HRV-A, 11 HRV-B, and 19 HRV-C. HRV-C infection was more common among children than adults (59% vs. 10%, \( P < 0.001 \)). Among adults, HRV-A was associated with higher severity of upper respiratory symptoms compared to HRV-B (\( P = 0.02 \)), but no such association was found in children. In addition, adults shed HRV-A significantly longer than HRV-C (\( P_{\text{trend}} = 0.01 \)).

Conclusions: Among otherwise healthy adults with HRV infection, we observed species-specific differences in respiratory symptom severity and duration of viral shedding.

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1. Background

Human rhinovirus (HRV)—the most prevalent respiratory virus—causes up to half of common colds [1,2] and imposes a significant economic burden [3]. HRV has also been associated with bronchiolitis [4], pneumonia [5], and exacerbation of breathing difficulties in populations with underlying respiratory conditions,
including asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) [6–8]. Prior to the era of molecular testing, rhinovirus was considered to be a relatively mild pathogen of questionable importance. However, with the ability to more readily identify HRV infection and species—including the newly identified species (HRV-C)—evidence is emerging that severity of HRV may be dependent upon the species and/or serotype.

The three species of HRV (A–C) comprise a large group of genetically diverse viruses with more than 150 serotypes [9,10]. HRV-A and C are associated with more severe clinical manifestations in children [11–13] and adults [14]. In particular, HRV-C is associated with more severe illness in young children, particularly those with asthma [12,15,16] or cystic fibrosis [17]. However, less is known about the epidemiology and clinical characteristics of HRV infection in otherwise healthy populations, especially adults without underlying illness. Moreover, little is known about the persistence of HRV shedding, which may influence duration of symptoms, clinical course, and infectiousness.

### 2. Objectives

The objective of the study was to understand the full spectrum of HRV disease and species-specific differences in symptom severity, clinical course, and viral shedding among patients with HRV infection in a longitudinal study of influenza-like illness (ILI) among otherwise healthy individuals.

### 3. Study design

#### 3.1. Overview of the ARIC study

Established in July 2009, the Acute Respiratory Infection Consortium (ARIC) is a multi-site, multi-disciplinary clinical research network for the study of ILI among otherwise healthy military personnel and beneficiaries. At the core of the ARIC is the natural history study, an observational, longitudinal cohort study to determine the etiology, epidemiology, and clinical characteristics of HRV infections among military personnel and beneficiaries.
of ILI among patients presenting for care at five US-based military treatment facilities (Table 1).

3.2. Patient population and procedures

From February 2010 to April 2012, we recruited patients aged 0–65 years who presented within 72 h of ILI symptom onset. ILI was defined by fever (temperature ≥ 100.4 °F) accompanied by one of the following respiratory symptoms: cough, sputum production, shortness of breath, chest pain, and/or sore throat. Both inpatient and outpatient subjects were eligible for participation. Patients with the following co-morbidities were excluded: type 1 or 2 diabetes, immunodeficiency, COPD, cystic fibrosis, severe asthma, chronic neuromuscular disease, chronic cardiac disease, or chronic renal disease.

At enrollment, demographic information and clinical symptoms were collected by interview. A nasopharyngeal specimen was collected using a nylon flocked swab (Copan Diagnostics, Corona, CA). Participants returned at days 3 ± 1, 7 ± 2 and 28 ± 7 for collection of clinical symptoms and a nasopharyngeal swab at each visit.

Written informed consent was obtained at enrollment. The study was approved by the Infectious Disease Institutional Review Board of the Uniformed Services University of the Health Sciences (IDCRP-045).

In the current study, we performed retrospective analysis on a sub-population that participated in a prospective study on ILI. We included HRV-positive cases with sufficient nasal swab specimens for HRV genotyping and excluded cases with co-detection of other respiratory viruses (see details in Section 3.4) from February 2010 to April 2012.

3.3. Clinical characteristics and severity measures

The presence and severity of clinical symptoms were recorded at each study visit either by self-report or, in subjects <4 years, by observation using a standardized four-point scale (0: none; 1: mild; 2: moderate; and 3: severe). Symptom grading was explained by research personnel. In addition, patients or their guardians completed symptom diaries for 7 days since ILI onset using the same four-point severity scale. Patients also reported number of days with reduced activity. Potential lower respiratory tract involvement was inferred by abnormality of chest radiograph exam (if done) and physical exam performed by health attendants, including the presence of crinkles, egophany, decreased breath sounds, inspiratory wheezing, expiratory wheezing, stridor, rhonchi, accessory muscle breath, dullness to percussion, nasal flaring and grunting. Clinical severity was characterized by composite scores (sum score) of symptoms in four categories: upper respiratory (earache, runny nose, sore throat and sneezing), lower respiratory (cough, breathing difficulty, hoarseness and chest pain), systemic illness (muscle ache, fatigue, headache and chills) and total severity (the above 12 symptoms). The measure was modified from severity scores published by Hayden et al. [18] and was only applied to adults because the measurements were originally designed and verified among adult ILI patients.

3.4. Detection of respiratory virus and HRV genotyping

Nasopharyngeal swabs were placed immediately into viral transport media, frozen at either −70 ° or −80 °C, and shipped to the Naval Health Research Center (San Diego, CA). HRV was identified using real-time reverse transcription polymerase chain reaction (rtRT-PCR) as previously reported [19]. The presence of other viral respiratory pathogens (i.e., influenza virus, adenovirus (type A–F), respiratory syncytial virus, coronavirus (HKU1, NL63, OC43, and 229E), parainfluenza virus (type 1–4), human metapneumovirus and bocavirus) was assessed by multiplex assays (xTAG Respiratory Viral Panel, Luminex, Austin, TX or PLEX-ID Viral IC Spectrum, Abbott, Chicago, IL). We excluded cases with co-detection from the analysis to avoid possible interference in clinical severity from non-HRV respiratory viruses.

HRV sequencing was targeted toward nucleotide 165–1079 of the VP4/VP2 coding region of the genomic RNA [20,21], the most commonly studied region of HRV. Genotyping and serotyping was performed by aligning RNA sequences of our samples with standard HRV sequences from the GenBank database and previous reports (Fig. 2). Nucleotide sequences from our study samples are available at GenBank (accession number: KF957884-KF957967 and KF957971). Detailed methods of HRV detection, sequencing and genotyping, in addition to standard sequences used in genotyping analysis, are available in the Appendix.

3.5. Statistical analysis

We first described differences in demographics, geographic location, and potential risk factors by HRV species. Second, we assessed species-specific differences in severity of clinical symptoms (individual and composite), and hospitalization. Symptom data from children were analyzed separately. We performed chi-square tests (exact test, as appropriate) to examine the association between categorical variables. We performed Kruskal–Wallis tests to examine species-specific differences in individual symptom score and composite scores. We then compared symptoms with P value of Kruskal–Wallis tests lower than 0.05 within each HRV species pair (e.g., HRV-A vs. HRV-B) using Wilcoxon rank-sum tests and false discovery rate (FDR) to adjust for multiple comparisons. Last, we compared the pattern of serial HRV detection by performing Cochran–Armitage Trend tests. Detection of HRV at consecutive visits implied ongoing viral shedding during that time period. A two-sided P value lower than 0.05 was considered statistically significant. Analyses were performed using SAS software, Version 9.3 (SAS Institute, Cary, North Carolina).

4. Results

4.1. Detection of HRV species

Between February 2010 and April 2012, a total of 160 HRV-positive cases were identified. Seventy-six cases were not genotyped due to insufficient nasal swab specimens (n = 64) and/or co-detection with other respiratory viral pathogens (n = 12). The remaining 84 HRV-confirmed cases were genotyped, including 62 (74%) adults, 48 (57%) males, 54 (64%) active duty military members. Among the 84 cases, 54 (64%) had HRV-A, 11 (13%) had HRV-B,
and 19 (23%) had HRV-C. HRV-C was detected more frequently among children (13/22, 59%) than adults (6/62, 10%; \( P < 0.01 \), Fig. 1). The distribution of HRV species did not differ by other demographic characteristics (Table 1). The phylogenetic tree revealed significant genetic diversity in the HRV VP4/VP2 regions (Fig. 2). Forty-four serotypes were identified. There was no genetic correlation or cluster of HRV serotypes among hospitalized cases (Fig. 2), children attending day care centers, and/or adults living in dorms.

### 4.2. HRV species and clinical severity

All cases reported or were observed for symptom severity at enrollment. Adult patients with HRV-A and HRV-C had higher composite scores of lower respiratory and total symptoms compared to those with HRV-B, although the differences were not statistically significant (Table 2). No other differences in clinical severity, including individual symptoms, abnormal chest X-ray findings, abnormal physical exams and duration of reduced activity, were observed at enrollment among either adults or children (Table 2). A total of four patients (5%, one child and three adults) were hospitalized: two with HRV-A and two with HRV-C. All four patients survived.

The majority (89%) of adults reported daily symptom scores through study day 7. Daily composite scores peaked on the second day of illness and decreased thereafter, regardless of HRV genotype and symptom category (Fig. 3). Patients with HRV-A and HRV-C tended to report higher composite scores compared to those with HRV-B. Species-specific differences were detected on day 3...
5. Discussion

Our study of the epidemiology and clinical characteristics of HRV infection among healthy adults and children without underlying medical conditions differs from previous reports that described infection among patients with asthma, COPD, or cystic fibrosis. We excluded cases of HRV in whom other respiratory viral pathogens of illness (upper respiratory symptoms, \(P = 0.02\); total symptoms, \(P = 0.03\), Fig. 3). Pair-wise comparison among three HRV genotypes on day 3 showed that patients with HRV-A had higher scores than HRV-B (median score of HRV-A vs. HRV-B: 7 vs. 4.5, \(P = 0.02\) for upper respiratory; 20 vs. 11.5, \(P = 0.04\) for total symptom scores with FDR adjustment), while no significant differences were found between those with HRV-B and HRV-C, presumably due to small numbers.

### 4.3. HRV species and persistent viral shedding

Fifty (60%) of the 84 patients completing all four study visits were included in the analysis of viral shedding. Eleven (22%) were positive for HRV on day 0 only, 25 (30%) on days 0 and 3, 12 (24%) on days 0, 3, and 7. Only two (4%) were positive for HRV at all four visits (Table 3).

Prolonged detection of HRV was more common among adults than children. Only four adults (11%) had detection of HRV solely at enrollment, in contrast to 7 (59%) children; thirteen adults (13/38, 34%) had evidence of HRV shedding on or after day 7, while only one child (1/12, 8%) had evidence of HRV shedding on or after day 7 (\(P = 0.01\), by visit category, Table 3). When examined by species, HRV-C was only detectable at day 0 and 3, while HRV-A and HRV-B was detected on day 7 and later (HRV-A vs. HRV-C, \(P = 0.01\) with FDR adjustment). We did not find different shedding patterns between children with HRV-A and HRV-C—the majority of them were tested positive only on enrollment or on enrollment and day 3.

### Table 2

| Disease | Children (age <18 years old) (n = 22) | Adults (age ≥ 18 years old) (n = 62) | \(P^a\) |
|---------|-------------------------------------|-------------------------------------|--------|
| HRV-A(n = 9) | HRV-C(n = 13) | Med N(%) | HRV-A(n = 45) | HRV-B(n = 11) | HRV-C(n = 6) | Med N(%) |
| Upper respiratory symptoms | | | | | | |
| Sore throat | 0.4 (44.4) | 1.9 (69.2) | 0.59 | 1.34 (75.6) | 1.7 (63.6) | 1.5 (46.7) | 0.85 |
| Cough | 0.3 (37.5) | 0.1 (10.0) | 0.22 | 2.41 (91.1) | 2.9 (81.8) | 3.6 (100) | 0.08 |
| Runny nose | 0.1 (11.1) | 0.1 (10.0) | 0.37 | 1.24 (53.3) | 0.3 (27.3) | 1.4 (66.7) | 0.17 |
| Lower respiratory symptoms | | | | | | |
| Shortness of breath | 0.1 (11.1) | 0.2 (15.4) | 0.74 | 1.3 (66.7) | 0.4 (36.4) | 0.2 (33.3) | 0.12 |
| Gastroenteritis symptoms | | | | | | |
| Decreased appetite | 2.7 (77.8) | 2.12 (92.3) | 0.19 | 2.3 (66.7) | 2.7 (63.6) | 2.6 (100) | 0.47 |
| Vomiting | 0.1 (11.1) | 0.3 (23.1) | 0.46 | 0.9 (20.0) | 0.3 (27.3) | 0.0 (0.0) | 0.43 |
| Diarrhea | 0.1 (11.1) | 0.1 (7.7) | 0.84 | 0.1 (24.4) | 0.5 (45.5) | 0.2 (33.3) | 0.57 |
| Abdominal pain | 0.1 (11.1) | 0.0 (0.0) | 0.26 | 0.13 (28.9) | 0.5 (45.5) | 1.5 (46.7) | 0.06 |
| Systemic symptoms | | | | | | |
| Headache | 0.2 (22.2) | 0.0 (0.0) | 0.10 | 2.36 (80.0) | 2.9 (81.8) | 2.5 (100) | 0.33 |
| Red eyes | 0.4 (44.4) | 0.6 (46.2) | 0.82 | 0.21 (46.7) | 1.6 (54.5) | 1.5 (46.7) | 0.51 |
| Chills | 0.0 (0.0) | 0.1 (7.7) | 0.41 | 1.37 (82.2) | 1.9 (81.8) | 2.4 (66.7) | 0.68 |
| Nausea | 0.0 (0.0) | 0.1 (7.7) | 0.37 | 0.21 (46.7) | 1.7 (63.6) | 1.4 (66.7) | 0.53 |
| Joint pain | 0.0 (0.0) | 0.0 (0.0) | NA | 2.34 (75.6) | 2.10 (90.9) | 2.5 (83.3) | 0.09 |
| Muscle aches | 0.0 (0.0) | 0.0 (0.0) | NA | 2.35 (77.6) | 2.9 (81.8) | 2.4 (66.7) | 0.75 |
| Fatigue | 2.6 (66.7) | 2.9 (69.2) | 1.00 | 2.43 (95.6) | 2.10 (90.9) | 2.5 (83.3) | 0.83 |
| Dizziness | 0.0 (0.0) | 0.0 (0.0) | NA | 0.21 (46.7) | 0.5 (45.5) | 1.4 (66.7) | 0.61 |
| Lower respiratory symptoms | | | | | | |
| Presence of self-reported LRT symptoms | 1 (11.1) | 2 (14.3) | 1.00 | 33 (73.3) | 6 (54.5) | 3 (50.0) | 0.32 |
| Abnormal chest X-ray or PE finding | 1 (16.7) | 4 (30.8) | 0.48 | 5 (15.6) | 0 (0.0) | 0 (0.0) | 0.74 |
| Did not have PE | 3 | 1 | 13 | 2 | 2 | |
| Fever (>100.4 °F) | 8 (88.9) | 13 (92.9) | 1.00 | 11 (24.4) | 2 (18.2) | 2 (33.3) | 0.79 |
| Hospitalization | 0.0 (0.0) | 1 (7.1) | 0.41 | 2 (4.4) | 0 (0.0) | 1 (16.7) | 0.30 |
| Days of reduced activity | 2 (2–3) | 2 (2–2) | 0.69 | 2 (1–3) | 2 (1–2) | 2 (1–3) | 0.56 |

\(^a\) P-value of chi-square test comparing score of HRV-B with HRV-A/C in adults was 0.02.

\(^b\) P-value of Wilcoxon rank-sum test comparing symptom scores and days of reduced activity between children with HRV-A and HRV-C.

\(^c\) P-value of Kruskal-Wallis test comparing symptom scores and days of reduced activity across three HRV species in adults.

\(^d\) One child with HRV-A and three with HRV-C did not respond to this question.

\(^e\) Med represents median severity score of the designated symptom. Number and proportion of patients reporting the designated symptom and hospitalization in each category.

\(^f\) Average of three respiratory symptoms (sore throat, cough and shortness of breath) and systemic symptoms (chills, muscle ache, headache, fatigue, and dizziness) with their standard deviations in parentheses.

\(^g\) P-value of Fisher’s exact test comparing the proportion of patients with the outcome across HRV species.
Fig. 3. Median and interquartile range of composite symptom scores among adults, by HRV genotypes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Red solid line: HRV-A; blue dashed line: HRV-B; green dotted line: HRV-C.
**P ≤ 0.05, *P > 0.05 & P ≤ 0.1 for comparison across three HRV genotypes.

were detected. The prospective, longitudinal design of the study allowed us to evaluate both clinical and virologic (i.e., shedding) characteristics of HRV infection in these patients and to minimize bias of patient-recalled symptoms using a standardized severity score.

The prevalence of HRV species differed by age. HRV-C infection was more common in children than adults, while the majority of adults were infected with HRV-A. Other groups have described high prevalence of HRV-C in children, regardless of clinical status [11,12,22]. However, information on the frequency of HRV-C detection in adults with respiratory illness is less consistent, ranging from 4.8% to 30% [7,14,23–25], regardless of patients’ comorbid status. It is unclear why such variation existed in adult population. While our data, along with others’, suggest age differences in the prevalence of HRV species, there are as yet no data explaining reasons of these differences. It may be that a longer history of exposure and disease in adults versus children may account for the diversity of clinical presentation and the patterns of viral shedding [26–28].

We found adult patients with HRV-A had higher composite severity scores compared to those with HRV-B in the early (i.e., day 3 of illness) stages of illness. Similar trends were also found among HRV-C adult patients, although the finding was not statistically significant. In addition, all four hospitalized cases had either HRV-A or HRV-C. The findings were in accordance with the literature—HRV-A and/or HRV-C are associated with more severe manifestations, including severe upper respiratory illness and pneumonia [14,29]. While symptoms related to HRV infection are generally mild among patients without underlying health conditions, such species-specific variation implies differences in viral pathogenesis and/or host immunity. We were unable to confirm such differences using clinical data collected at enrollment. Differences in the timing of presentation and/or enrollment may have contributed to the result. Patients were enrolled within 72 h after illness, but this relied upon their initial presentation to the facility. Even within such a short period, subtle differences in the severity of clinical symptoms may have been masked.

We did not find that HRV-C or HRV-A was more severe in children, unlike previous reports [12,13,24,30,31]. Since we restricted enrollment to children without underlying conditions, it is possible that HRV-C is not more severe than other HRV species in this group. This finding adds context to the literature regarding HRV-C in children, suggesting that the observed higher severity of some HRV infections may be confined to specific risk groups.

Persistent viral shedding may indicate different levels of virulence, host immune response and infectiousness [32,33]. In sharp contradistinction to influenza infection where children shed virus longer than adults [34–36], our data suggest that adults shed HRV longer than children. In addition, we observed adults to shed HRV-A longer than HRV-C. Longer shedding of HRV-A among adults may be correlated to more severe clinical presentation with such species. Further studies are needed to understand interaction of host response and viral virulence. To the best of our knowledge,
Table 3

Patterns of viral shedding among HRV cases who completed 4 study visits, by species and age group.

| Age group | HRV detection at consecutive visits | HRV-A | HRV-B | HRV-C | Total |
|-----------|------------------------------------|-------|-------|-------|-------|
| 0–17 years of age (n=12) | Day 0 | 3 | 2 | 0 | 5 |
| | Day 0 and 3 | 2 | 0 | 0 | 2 |
| | Day 0, 3, and 7 | 1 | 0 | 0 | 1 |
| | Day 0, 3, 7, and 28 | 0 | 0 | 0 | 0 |
| | Total | 5 | 4 | 0 | 12 |
| 18–65 years of age (n=38) | Day 0 | 7 | 16 | 9 | 32 |
| | Day 0 and 3 | 4 | 3 | 1 | 8 |
| | Day 0, 3, and 7 | 4 | 2 | 0 | 6 |
| | Day 0, 3, 7, and 28 | 1 | 0 | 0 | 1 |
| | Total | 7 | 21 | 12 | 38 |

Note: numbers of HRV detection in group 3 (Day 0, 3, and 7) and group 4 (Day 0, 3, 7, and 28) were combined in the Cochran–Armitage Trend test given the small number.

- $p$-value of Cochran–Armitage Trend test comparing HRV shedding pattern at 4 study visits between HRV-A and HRV-C among children.
- $p$-value of Cochran–Armitage Trend test comparing HRV shedding pattern at 4 study visits between designated HRV species among adults with FDR adjustment.
- $p$-value of Cochran–Armitage Trend test comparing HRV shedding pattern (regardless of HRV species) at 4 study visits between children and adults.

This is the first study to describe species-specific and age-specific differences in the patterns of viral shedding during natural HRV infection.

Our study has several limitations. First, the small sample size, especially among children (n=23), may have precluded us from identifying an association between HRV species and clinical outcomes. Second, the study population was comprised of individuals who presented to healthcare facilities with ILL. This may not reflect the epidemiology of HRV infection among individuals in the community who do not seek medical care. In addition, the majority of HRV cases in this study were from a single site. This may limit the generalizability of our results to different geographical regions. On the other hand, our study population also had a relatively small proportion of hospitalized patients and may not be fully representative of severe cases of HRV infection. Third, symptom diaries were not completed by all patients. It is possible that some participants suspended use of their diaries when their symptoms subsided. To address this, we performed a sensitivity analysis using data only from participants who completed the diary per protocol (n=42). The analysis confirmed the results that adult patients with HRV-A had higher composite severity scores than those with HRV-B (data not shown). Finally, shedding of virus was measured by detection of RNA, not using cell culture. The fastidious nature of HRV has led to the use of PCR as the main tool for detection, so we feel this is acceptable, though it is only inferential of infectious particles. In addition, the duration of shedding was measured by consecutive detection of HRV in nasal swabs while genotyping was performed for only the baseline specimen. It is possible that the HRV detected at subsequent visits could have been new infections.

In conclusion, this study lends new insight into the role of HRV with respect to species-specific patterns of clinical symptom patterns and viral shedding in otherwise healthy children and adults with ILL. Our findings may inform development of strategies for prevention of HRV disease and transmission. Moreover, studies are needed to understand host-pathogen interactions across HRV species in different age groups, which will ultimately lead to improved clinical management of severe HRV infection.

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Competing interests

None to report.

Ethical approval

The study was approved by the Infectious Disease Institutional Review Board of the Uniformed Services University of the Health Sciences (IDCRP-045).

Key points

HRV species A and C were associated with more severe respiratory and systemic symptoms in adults, but not in children. Adults with HRV-A had prolonged duration of viral shedding.
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Appendix.

Details regarding HRV detection, genotyping and phylogenetic analysis

HRV detection

Human rhinovirus was screened from clinical samples using real-time reverse transcription-PCR (RT-PCR). Rhinovirus primers and probe from [37]: F: 5’ CPX GCC ZGC CTG GC 3’; R: 5’ GAA ACA CGG ACA CCC AAA GTA 3’; P: 5’ FAM-TCC GCC CCC TGA ATG YGG C-BHQ1 3’ target the viral 5’ noncoding region. This was part of a multiplex assay, created in house to generate a research use only test. Amplification was performed with an ABI 7500 with reverse transcription at 50°C for 30 min; followed by 40 cycles of amplification at 95°C for 15 s and 55°C for 30 s. Reactions were carried out in 20-μl reaction volumes with 2.5-μl Nuclease-free water, 3.0-μl Primer/probe mix, 2.0-μl PATH-ID Real Time Multiplex Enzyme, 12.5-μl PATH-ID Real Time Multiplex 2X Mix. Primer mix uses a 0.8-μM concentration of rhinovirus primers and a 0.2-μM concentration of rhinovirus probe.

HRV genotyping and phylogenetic analysis

DNA Extraction and PCR. We extracted and amplified viral RNA from 140μl of the viral transport media using a viral RNA kit (QIAamp, Qiagen®). This was performed in 22-μl of reaction mixture consisting of 2.2 μl of nuclease-free water, 3 μl of MgSO4, (5.0 X), 15 μl of 2X reaction mix (Super Script III One-Step RT-PCR System) with Platinum (Taq High Fidelity kit), 0.6 μl 20-μM concentrations of the sense and antisense primers, 0.6 μl Enzyme Mix and 8 μl of template. HRV and coxsackievirus detection was performed by semi-nested reverse transcription-PCR (RT-PCR) targeting nucleotides 165–1079 of the genomic RNA. We used P1-1 HRV (CAAGCACTTCTGTYWCCCC) and 9565, R HRV (GCATCNGG-YARYTTCCACCCCAACC) [38–40]. The amplification was carried out in a thermocycler 7700 (Applied Biosystems). Cycling conditions included a reverse transcription step at 50°C for 30 min and 95°C for 15 min followed by 45 PCR cycles: 94°C for 30 s; 55°C for 30 s; 72°C for 90 s; and final incubation for 72°C for 10 min. The amplified products of 915 bp were analyzed by electrophoresis on an agarose 2% gel. PCR products were purified with CentriSeps Columns (Princeton Separations, Inc.). Purified products were directly used for sequencing of viral nucleic acids from clinical specimens.

Sequencing and phylogenetic analyses. The VP4/VP2 coding region of all HRV positive samples obtained were sequenced and included in the following phylogenetic analyses. This region was selected for sequencing because VP4/VP2 is the most commonly studied region of HRV. For direct sequencing of viral nucleic acids from clinical specimens, gene fragments were amplified and sequenced with the use of a Big Dye terminator cycle sequencing kit (version 3.1, Applied Biosystems) and internal primers Generic F HRV (AGCCTGGTGCGKCC) and NCR2HRV (ACTACTTGCGTTCGCTGTTC) on a Genetic Analyzer system (version 3130XL, Applied Biosystems). Nucleotide sequences of PCR products (n = 441 nt) were analyzed by sequencing using Sequencher and BioEdit (version 7.0.0, Ibis Pharmaceuticals) and then aligned with the CLUSTAL X version 2.0.1 software to compare with HRV sequences from the GenBank database. Phylogenetic trees were constructed by the neighbor-joining method in MEGA software (version 5). The statistical significance of the tree topology was tested by bootstrapping (1000 replicates). Pairwise distances between and within the genotypes at the nucleotide level were calculated with Kimura 2 parameters and with Poisson correction at the amino acid level with MEGA software. Nucleotide sequences from our study samples are available at GenBank (accession numbers: KF957884-KF957967 and KF957971).

Accession number of standard HRV sequences used in the study

| Reference sequence | GenBank accession # | Reference |
|--------------------|---------------------|-----------|
| HRRV_a              | AB548895, Japan_2005.916 | [43] |
| HRRV_b              | AB548895, Japan_2005.916 | [43] |
| HRRV_c              | HM044210, Malaysia_2009.807 | [43] |
| HRRV_d              | IAR00041, HRV59, Argentina_2011.208 | [43] |
| HRRV_e              | FL15972, HRV59, Argentina_2010.877 | [43] |
| HRRV_f              | HQ123442, Thailand_2006.690 | [43] |
| HRRV_g              | HQ123442 | [43] |
| HRRV_h              | AB548898, Japan_2006.916 | [43] |
| HRRV_i              | HQ444754, Hongkong_2008.916 | [43] |
| HRRV_j              | HQ444754 | [43] |
| HRRV_k              | ICC00135, HRV49, Colombia_2010.96 | [43] |
| HRRV_l              | FL15982, HRV49, Peru_2010.793 | [43] |
| HRRV_m              | FL15982, HRV49, Peru_2010.793 | [43] |
| HRRV_n              | HQ444754, Hongkong_2007.916 | [43] |
| HRRV_o              | HQ444754 | [43] |
| HRRV_p              | ICC00016, HRV48, Colombia_2010.96 | [43] |
| HRRV_q              | AB548896, Japan_2007.916 | [43] |
| HRRV_r              | FL15979, HRV48, Paraguay_2010.71 | [43] |
| HRRV_s              | AB548892, Japan_2007.916 | [43] |
| HRRV_t              | HQ190927, Cuba_2010.585 | [43] |
| HRRV_u              | FL15983, HRV12, Peru_2010.877 | [43] |
| HRRV_v              | HQ190927, Cuba_2010.585 | [43] |
| HRRV_w              | HQ123441, Thailand_2006.521 | [43] |
| HRRV_x              | HQ123441 | [43] |
| HRRV_y              | IAR00030, HRV46, Argentina_2011.208 | [43] |
| HRRV_z              | JF781504, USA_2007.904.000 | [43] |
| HRRV_{a}             | JF781504 | [43] |
| HRRV_{b}             | FL15951, HRV51, Venezuela_2010.71 | [43] |
| HRRV_{c}             | JF781504 | [43] |
| HRRV_{d}             | IPE00570, HRV1a, Peru_2011.71 | [43] |
| HRRV_{e}             | IAR00036, HRV103, Nicaragua_2011.041 | [43] |
| HRRV_{f}             | GQ415052, USA_2008.585 | [43] |
| HRRV_{g}             | GQ415052 | [43] |
| HRRV_{h}             | GQ415051, USA_2000.658 | [43] |
| HRRV_{i}             | AB548900 | [43] |
| HRRV_{j}             | AB548902 | [43] |
| HRRV_{k}             | AB548907 | [43] |
| HRRV_{l}             | AB548907 | [43] |

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