

1 **Prevalence and Clinical Characterization of a Newly Identified**
2 **Human Rhinovirus C Species in Children with Acute Respiratory**
3 **Tract Infection**

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10 None of the authors has a conflict of interest.

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12 This work was partly supported from“973”National Key Basic Research Program of China (Grant No.
13 2007CB310500)

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23 Keywords: Human Rhinovirus C; Children; Acute Respiratory Tract Infection.

24 Running headline: Human rhinovirus C species in China

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45 Human rhinovirus C (HRV-C) is a newly identified genotype of HRV found in
46 patients with respiratory tract infections (RTIs); however, its epidemiological profile
47 and clinical characteristics are not well understood. In this study, Chinese children with
48 RTIs were screened for HRV-C, and their epidemiological and clinical characteristics
49 were analyzed. From December 2006 to November 2007, 406 nasopharyngeal aspirates
50 from children younger than fourteen years of age with RTIs were screened for HRV and
51 other common respiratory viruses by PCR or RT-PCR. Two-hundred twenty-four
52 (55.2%) of the specimens were infected with at least one virus, including 53 patients
53 with HRV (13%). HRV-A, HRV-B, and HRV-C were detected in 22, 12, and 19
54 specimens, respectively. HRV-C was detected mainly from December 2006 to April
55 2007 and from October to November 2007, with peaks in December and April (10/19).
56 Acute upper respiratory infection and bronchopneumonia were observed in 53 and 37%
57 of the cases, respectively. The most common symptoms were cough (82%), runny nose
58 (53%), and fever (37%). Wheezing and bronchiolitis were less common in those
59 infected with HRV-C than in those infected with RSV. Partial sequencing of *VP4/VP2*
60 revealed that the HRV-C strains were 56–62% identical at the amino acid level with
61 HRV-B and HRV-A reference strains, and 80–99% identical with HRV-C reference
62 strains. In conclusion, HRV-C is an important cause of RTIs in children, and highly
63 diversified strains of HRV-C are prevalent in China. HRV-C may produce different
64 epidemiological features, and patients infected with HRV-C may exhibit different
65 clinical features from those infected with RSV or HRV-A/HRV-B.
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67 Viruses are the most frequent cause of acute respiratory infections and are a leading
68 cause of childhood mortality (23). Human rhinoviruses (HRVs) are not only the most
69 common causative agents of mild upper respiratory tract infections (RTIs), but are also
70 associated with more serious diseases such as pneumonia or acute wheezing episodes
71 associated with bronchiolitis and acute asthma in children (11, 15). More than 100
72 HRV serotypes have been identified. Traditionally, HRVs have been divided into two
73 strains, human rhinovirus A (HRV-A) and human rhinovirus B (HRV-B). Considerable
74 variation may exist in the genetic characteristics of different HRV strains, and the
75 viruses seem to circulate without any identifiable pattern (15).

76 Over the past few years, a new HRV variant has been detected in patients with an
77 acute RTI in America, Australia, and Hong Kong that shares 53–57% homology at the
78 amino acid level with HRV-A and HRV-B (13, 14, 17). The chief symptoms of patients
79 infected with this new strain vary by location: patients from Australia and Hong Kong
80 present mainly with bronchiolitis, wheezing, and asthmatic exacerbation, whereas
81 those from America present mainly with flu-like symptoms(13, 14, 17). However, the
82 clinical impact of this new viral strain in children is unclear. In this 1-year prospective
83 study conducted during 2006 and 2007, 406 children younger than fourteen years of age
84 with an RTI were screened for this new viral strain and other respiratory viruses. The
85 presenting symptoms and epidemiological characteristics associated with the strain
86 were also analyzed.

87 **MATERIALS AND METHODS**

88 *Study subjects, specimen collection, and processing.* From December 1, 2006, to

89 November 31, 2007, 406 children fourteen years or younger who presented to the
90 Department of Pediatrics at the First Hospital of Lanzhou University with acute
91 respiratory symptoms were recruited for this study.

92 Nasopharyngeal aspirates (NPAs) were collected from each patient. The
93 demographic data, clinical findings, and severity of disease for each patient are
94 presented in Table 1. Informed consent was obtained from the parents of the children.
95 The study protocol was approved by the hospital ethics committee.

96 After collection, the NPAs were stored at -80°C until further processing. DNA and
97 RNA were extracted from 0.2 ml of each NPA using a QIAamp Viral DNA Mini Kit and
98 QIAamp Viral RNA Mini Kit (Qiagen, Beijing, China), respectively.
99 Complementary DNA synthesis was carried out with SSIII viral reverse transcriptase
100 and random hexamer primers (Invitrogen), and PCR amplification was performed with
101 rTaq DNA polymerase (Takara, Beijing, China). All laboratory tests were conducted at
102 the National Institute for Viral Disease Control and Prevention, China Center for
103 Disease Control and Prevention.

104 **HRV detection.** The primers used to amplify a 549-bp fragment of HRV (forward
105 [5'-GGG ACC AAC TAC TTT GGG TGT CCG TGT-3'] and reverse [5'-GCA TCI
106 GGY ARY TTC CAC CAC CAN CC-3']) were described previously (19). PCR was
107 performed under the following conditions: 94°C for 8 min followed by 35 cycles of
108 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 8 min.

109 **Detection of other respiratory viruses.** Human metapneumovirus (hMPV), respiratory
110 syncytial virus (RSV), influenza virus (IFVA and IFVB), parainfluenza virus (PIV

111 types 1–3), and human coronavirus (229E, OC43, NL63, and HKU1) were detected by
112 RT-PCR (5, 6, 21, 22), and adenovirus (AdV) and human bocavirus (HBoV) were
113 detected by PCR (1, 12). All positive PCR products were purified using a QIAquick
114 PCR Purification Kit (Qiagen) and sequenced by Invitrogen (Shanghai, China) to
115 confirm their specificity.

116 **Sequence analysis and nucleotide sequence accession numbers.** The nucleotide and
117 deduced amino acid sequences of *VP4/VP2* were compared with reference HRV strains
118 available from GenBank. Phylogenetic analysis was conducted using MEGA version
119 3.1. The 53 partial sequences of *VP4/VP2* have been submitted to GenBank (accession
120 numbers EU822829–EU822880 and EU822882).

121 **Statistical analysis.** The statistical significance of differences between the various
122 groups was tested using the χ^2 test and Fisher's exact test. The duration of patient
123 hospitalization was compared between different virus-infected groups using an
124 independent sample *t*-test. A P-value of <0.05 was considered statistically significant.
125 All analyses were performed using SPSS version 13.0.

126 RESULTS

127 **Patient characteristics.** The patients enrolled in this study were aged between one day
128 and 168 months (29.9 ± 31.5 months, mean \pm SD), with 88% (357/406) under five years
129 of age. The boy to girl ratio was 1.5:1; the outpatient to inpatient ratio was 1:1.7.

130 **Virological findings in children with acute RTI.** Of 406 samples tested, 224 (55.2%)
131 were positive for at least one viral agent. RSV, which was detected in 118 children
132 (29%), was the most common viral agent, followed by HRV (detected in 53 [13%]),

133 AdV (in 52 [13%]), PIV-3 (in 38 [9%]), hMPV (in 29 [7%]), HBoV (in 29 [7%]),
134 HCoV-NL63 (in 15 [4%]), HCoV-HKU1 (in 12 [3%]), influenza A (in 6 [2%]), PIV-1
135 (in 2 [0.5%]), and HCoV-229E (in 1 [0.2%]). PIV-2 and HCoV-OC43 were not detected
136 in any patients. Most virus-associated RTIs occurred during low-temperature months
137 (winter and early spring). For example, 69% (81/118) of all RSV infections were
138 detected during the months of March, April, and November in 2007, and 69% (36/52)
139 of all AdV infections were detected in December of 2006 and January and March of
140 2007. Similarly, PIV, IFV, and hMPV were detected mainly during the months of
141 December and January, and HBoV was detected in December and April; HCoV-NL63
142 and HCoV-HKU1 were detected mainly in autumn and winter (Fig. 1A). Of the 224
143 virus-positive samples, 142 were also positive for additional viruses, resulting in a
144 coinfection rate of 63%. No significant differences in age distribution or sex ratio were
145 found among the different viral groups.

146 **Detection of human rhinovirus C (HRV-C).** Phylogenetic analysis of the coding
147 regions of the viral protein VP4/VP2 revealed that among the 53 patients infected with
148 HRV, 22 (42%) were infected with genotype A, 12 (23%) were infected with genotype
149 B, and 19 (36%) were infected with the new strains, HRV-C. The rates of coinfection
150 for genotypes A, B, and HRV-C with another virus were 36% (8/22), 75% (9/12), and
151 42% (8/19), respectively. RSV was the most common additional respiratory virus
152 detected in the three groups, accounting for 26% (14/53) of all HRV infections. Human
153 coronaviruses, including NL-63 and HKU1, were other common pathogens causing
154 coinfections. In the HRV-C infection group, 4 patients were co-infected with RSV, and

155 1 was co-infected with HMPV, HBoV, NL63, and HKU1.

156 **Epidemiology of HRV.** A HRV was detected each month of the year except June, with
157 the highest detection rates between December 2006 and April 2007 (51%, 27/53) and
158 September to November of 2007 (40%, 21/53). HRV-C was found mainly from
159 December 2006 to April 2007 and October to November of 2007, with peaks in
160 December and April (10/19). HRV-A was detected throughout the study year except in
161 June and July. HRV-B was only detected during the autumn and winter months, with a
162 peak in November (Fig. 1B). The age range of the 53 HRV-infected children was 1–108
163 months (30.4 ± 32.7 months, mean age \pm SD). The male: female ratio was 33:20. About
164 89% (17/19) of the children infected with HRV-C were <5 years of age, and 74% (14/19)
165 were <36 months of age. Although HRV-A was detected in children of all age groups,
166 HRV-B was largely detected in children of less <1 year, 25–36 months, and >5 years of
167 age (Fig. 2).

168 **Phylogenetic analysis of HRV.** Partial sequences (549 bp) of VP4/VP2 from the HRVs
169 obtained from children with RTIs in China were aligned with reported rhinovirus
170 prototype strains including HRV 2 (X02316), HRV 6 (DQ473486), HRV 53
171 (DQ473507), HRV 75 (DQ473510), HRV 92 (AY040238), HRV 97 (AY040242), HRV
172 A (DQ473509), HRV NY-003 (DQ875929), HRV QPM (EF186077), HRV C strain 024
173 (EF582385), HRV C strain 025 (EF582386), HRV C strain 026 (EF582387), HRV
174 C101507 (687515), HRV C103007 (687519), and HRV C103107 (687520),
175 HRV-CO-203(EU743927), HRV-CO-1396(EU743926), HRV PNC89098(EU590103),
176 HRV PNC90620(EU590111), HRV NY-041 (DQ875921), HRV NY-063 (DQ875924).

177 Alignment of the sequences in the present study with the HRV prototype strains
178 resulted in three cluster: HRV-A, HRV-B, and a novel genetic cluster (Fig. 3). The
179 sequences from the strains of the novel cluster were 56–62% identical at the amino acid
180 level with those from HRV-A and HRV-B, while retaining 80–99% identity with
181 HRV-C reference strains. Sequences (Lz127, Lz135, Lz218,) and three sequences
182 (Lz167, Lz185, and Lz333) showed marked similarity to HRVC 024 (EF582385) and
183 HRVC 025 (EF582386) from Hong Kong; sequences (Lz163, Lz239) and (Lz89, Lz383)
184 showed similarity to HRV-CO-203(EU743927) from Colorado and HRV C103107
185 (687520) from Beijing, respectively; the sequences from Lz123 and Lz124 shared
186 strong similarity with HRV NY-003 (DQ875929) and HRV QPM (EF186077),
187 respectively. The percent identity among the strains ranged from 87.9 %to 100 %within
188 group A and from 90.8 %to 100 %within group B.

189 ***Clinical characteristics of HRV-C infection in children.*** The age range of the patients
190 who tested positive for HRV-C was from 4 days to 7 years. Among the 19
191 HRV-C-positive children, 9 were inpatients with a mean hospital stay of 6.6 days. Ten
192 children (53%) had an upper RTI, 7 children (37%) had bronchopneumonia, 1 (5%) had
193 acute laryngitis, and 1 had suppurative tonsillitis. The most common symptom was a
194 cough (n = 16, 82%), followed by a runny nose (n = 10, 53%), crackle (n = 10, 53%),
195 and a fever (n = 7, 37%). Other clinical presentations included wheezing (n = 3, 16%),
196 cyanosis (n = 2, 11%), dyspnea (n = 2, 11%), vomiting (n = 1, 6%), and hoarseness (n =
197 1, 5%). Of 11 children who underwent chest radiography, 6 had abnormal findings and
198 5 were confirmed with pneumonia. Complete blood counts were performed for 16 of

199 the 19 children, and the mean white blood cell (WBC) count was 92,500 cells/ μ L (range
200 45,000–178,000 cells/ μ L). Bacterial cultures of the NPAs were obtained for 6 patients,
201 two of which were *Haemophilus influenzae* (Lz89) and *Streptococcus pneumoniae*
202 (Lz333), and four of which were normal flora (Lz8, Lz127, Lz 167, and Lz269).

203 A few distinct clinical characteristics were noted when different groups of patients
204 were compared (Table 1). HRV-C mono-infection was more common than RSV
205 mono-infection in patients with AURI ($P = 0.007$), whereas patients diagnosed with
206 bronchiolitis were more common in the RSV mono-infection group than in the HRV-C
207 mono-infection group. Although wheezing occurred frequently in those patients with
208 HRV-C co-infection (33%), RSV infection (33%), and HRV-A/HRV-B mono-infection
209 (26%), patients with HRV-C mono-infection did not present with wheezing. Among the
210 patients with HRV-C mono-infection, HRV-C co-infection, and RSV mono-infection, no
211 significant differences were found in terms of sex, proportion of patients aged <3 years,
212 presence of a fever ($\geq 38^{\circ}\text{C}$), cough, cyanosis, dyspnea, and crackle. The number of
213 patients requiring hospitalization was lower in the HRV-C mono-infection group than in
214 the HRV-A or HRV-B mono-infection group ($P = 0.028$); however, the duration of
215 hospitalization was not significantly different between the HRV-C mono-infection group,
216 HRV-A or HRV-B mono-infection group, and RSV group.

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DISCUSSION

218 In the present study, more than half of the children (55%) were infected with at least
219 one viral agent, with HRV being the second most common virus. The detection rate of
220 viruses was lower than that of other studies (9, 18, 20). One reason of that is the low PCR

221 sensitivity with these pair of primers used in this study . Another one is the low clinical
222 visit rate of patients with minimal RTIs symptoms in the general hospital, and they
223 usually get medical care easily from community hospitals. HRV coinfections were
224 more frequent in the present study than in other studies(8, 10, 16). RSV and coronavirus
225 (NL63 and HKU1) were the major coinfection viruses. The reason is partly due to the
226 high detection rate of RSV and the similar seasonal circulation pattern of these viruses.

227 The detection rates for three HRV species, HRV-A, HRV-B, and a novel species,
228 HRV-C, were 5.4, 3.0, and 4.7%, respectively. AURI and bronchopneumonia were the
229 main diagnoses for the HRV-C-positive patients. A previous report indicated that 5% of
230 RTIs among inpatients are caused by HRV-C (7). In our study, 9 of 19 patients were
231 hospitalized, and 11 were found to have HRV-C monoinfection. These results indicate
232 that HRV-C is an important etiological factor in children with an RTI.

233 In this study, The HRV-C sequences are genetically distinct, sharing only 56–62% of
234 their amino acids with HRV-A and HRV-B, while retaining 77.3–100% identity with
235 each other. And these sequences showed 69–96% amino acid identity with known
236 strains of HRV-QPM. In accordance with previous data, HRV-C showed a high degree
237 of sequence variation between individual strains. Further analysis is needed to confirm
238 this finding.

239 Previous studies indicated that HRV was detected throughout the year, with peaks in
240 spring and autumn in temperate regions (15). Different rhinoviral species occurred in
241 different seasons. The three HRV species in our study were detected predominately in
242 winter and spring. The most frequently detected species was HRV-A, which occurred in

243 all months except June and July. HRV-B circulated during summer, autumn, and winter.
244 It was reported that all HRV-B species in Queensland were from specimens collected
245 during the winter months, whereas the new HRV-C was found mostly during spring (3).
246 In our study, HRV-C infections peaked in early winter (December) and late spring
247 (April). It was previously reported that HRV-QPM infections peaked in winter but were
248 also detected in spring and summer (17). In Hong Kong, all HRV-C infections were
249 found between October and February, with peaks in autumn and winter (14). Thus,
250 HRV subgroups co-circulate throughout the year, and the predominant species vary
251 with location and year.

252 HRV is the second most frequently recognized agent associated with pneumonia and
253 bronchiolitis in infants and young children, and infection with HRV frequently
254 exacerbates pre-existing airway diseases such as asthma (15). In this study, HRVs were
255 the second most frequently detected respiratory virus, and the HRV-infected children
256 suffered from a wide range of upper and lower respiratory tract symptoms. The most
257 common diagnoses among the children infected with HRV-C were upper RTI and
258 bronchopneumonia, indicating that HRV-C contributes substantially to RTIs, which
259 frequently require hospitalization. The number of cases requiring hospitalization in the
260 HRV-C monoinfection group was smaller than that in the HRV-A or HRV-B
261 monoinfection group and AURI occurred more often in the HRV-C monoinfection
262 group than in the RSV infection group. Although the clinical presentations of HRV-C
263 infection were similar to those of RSV, bronchiolitis, wheezing, cyanosis, dyspnea, and
264 heart failure, which are common in cases of HRV-C infection, were not detected as

265 frequently as in RSV infection. These observations support the view that HRV-C is
266 associated with relatively mild upper respiratory infections, rather than being typical
267 for lower respiratory tract disease.

268 The overall severity of disease, median duration of hospitalization, and frequency
269 of major symptoms between the HRV-C mono-infection and HRV-C coinfection groups
270 were similar. Moreover, no difference in disease severity was observed between the
271 HRV-A/HRV-B and HRV-C groups. A study from Hong Kong showed that febrile
272 wheezing and asthma were the most common presentations of HRV-C infection (76%)
273 (14), and children infected with HRV-QPM most commonly present with bronchiolitis
274 and wheezing (17). In the present study, the prevalence of wheezing and asthma was not
275 as high as in reported Hong Kong or Queensland. A possible explanation for this result
276 is that the specimens used in the current study were obtained from both inpatients and
277 outpatients, and the number of patients with underlying chronic diseases was lower
278 than in the samples from Hong Kong or Queensland.

279 In conclusion, our data indicate that the novel genotype HRV-C exhibits significant
280 genetic variation and a distinct epidemiological profile from HRV-A and HRV-B. It can
281 also cause upper and lower respiratory diseases in children and is frequently associated
282 with AURI and bronchopneumonia. Wheezing and bronchiolitis in the HRV-C group
283 were less common than in those with RSV. Additional studies are needed to elucidate
284 the relationship between HRV-C and wheezing, and to define the epidemiological
285 profile and genetic characteristics of the newly recognized species of HRV.

Table 1. Comparison of the clinical characteristics between groups of children with acute respiratory infection

Characteristic	Group1	Group2	Group3	Group4	P-value		
	HRVC monoinfection n=11	HRVC coinfection n=8	HRVA/HRVB monoinfection n=19	RSV monoinfection n=73	Group1 vs Group2	Group1 vs Group3	Group1 vs Group4
Male sex	7(64%)	5(63%)	12(63%)	44(60%)	1	1	1
Age≤3 years	6(55%)	7(88%)	12(63%)	58(80%)	0.121	0.712	0.374
No. of hospitalization	5(46%)	4(50%)	17(90%)	46(63%)	0.328	0.028	1
Median duration of hospitalization(day)	5(2-26)	3(3-7)	7(2-17)	10(2-36)	0.377	0.946	0.752
Clinical diagnosis							
AURI	5(46%)	5(63%)	3(16%)	7(10%) ^a	0.007	0.104	1
Acute laryngitis	1(9%)	0	1(5%)	1(1%)	0.246	1	
Suppurative tonsillitis	1(9%)	0	0	0			
Bronchitis	0	0	3(15%)	3			
Bronchiolitis	0	0	2(10%)	20			
Bronchopneumonia	4(36%)	3(38%)	9(47%)	42(58%)	0.212	0.708	1
Clinical manifestations							
runny nose	4(36%)	6(75%)	2(11%)	37(52%)	1	1	1
Fever > 38°C	3(27%)	4(50%)	5(26%)	34(47%)	0.332	1	0.642
Cough	9(82%)	7(88%)	14(74%)	64(88%)	0.632	1	1
Wheezing	0	3(38%)	5(26%)	24(33%)			
cyanosis	1(9%)	1(13%)	3(15%)	8(11%)	1	1	1
dyspnea	1(9%)	1(13%)	2(10%)	15(20%)	0.682	1	1
Crackles	5(46%)	5(63%)	10(53%)	45(62%)	0.34	1	1
heart failure	0	0	0	11			

NOTE. AURI: acute upper respiratory tract infection, RSV: respiratory syncytial virus, Group 1: Human Rhinovirus C mono-infection, Group 2: Human Rhinovirus C coinfected with other viruses, Group 3: Human Rhinovirus A or B mono-infection, Group 4: respiratory syncytial virus mono-infection.

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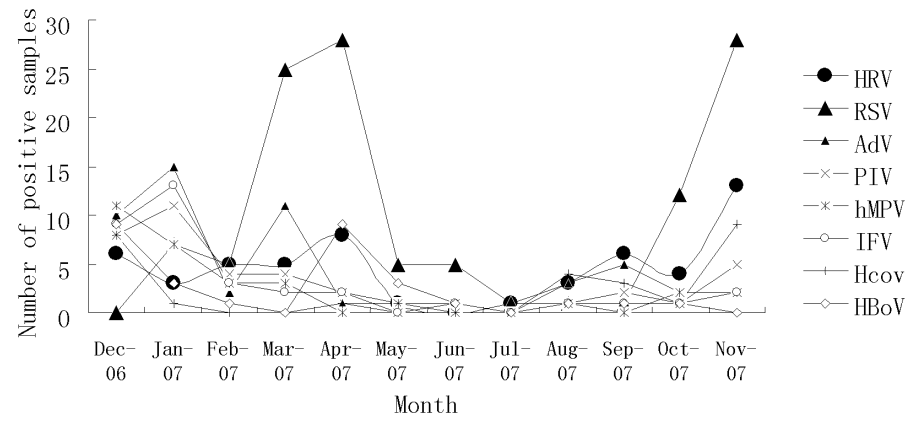
FIGURE LEGENDS

Figure 1. Seasonal distribution of human rhinovirus and other common viruses (A) and human rhinovirus A, B and C during study period (B).

Figure 2. Distribution of various genotypes of human rhinovirus-positive specimens by patient age.

Figure 3. Phylogenetic analysis of the deduced amino acid sequences of VP4/VP2 gene (420bp) of the 53 human Rhinoviruses with reference strains. Phylogenetic trees were constructed by the neighbor-joining method by using MEGA 3.1 and bootstrap values were determined by 1000 replicates. Viral sequences in marks were reference strains, representative human Rhinoviruses sequences and other Rhinoviruses from GenBank are indicated by isolate names. GenBank accession numbers of each strain are given in parentheses.

(A)



(B)

