
Eun Hwa Choi,1,3 Hoan Jong Lee,1,2 Sun Jung Kim,3 Byung Wook Eun,1 Nam Hee Kim,3 Jin A Lee,1 Jun Ho Lee,1 Eun Kyung Song,1 So Hee Kim1 Ji Yong Park,1 and Ji Yeon Sung1

1Department of Pediatrics, Seoul National University College of Medicine, and 2Virus Research Center, Clinical Research Institute, Seoul National University Hospital, Seoul, and 3Seoul Medical Science Research Institute, Seoul National University Bundang Hospital, Gyeonggi-do, Korea

Background. This study was performed to evaluate the associations of newly recognized viruses, namely, human metapneumovirus (hMPV), human coronavirus (HCoV)–NL63, and human bocavirus (HBoV) with lower respiratory tract infections (LRTIs) in previously healthy children.

Methods. To determine the prevalences of 11 viruses—respiratory syncytial virus (RSV), adenovirus, rhinovirus, parainfluenza viruses (PIVs) 1 and 3, influenza viruses A and B, hMPV, HCoV, HCoV-NL63, and HBoV—among infants or children with LRTIs, in association with their epidemiologic characteristics, we performed multiplex reverse-transcriptase polymerase chain reaction on nasopharyngeal aspirates obtained from 515 children ≤5 years old with LRTIs during the period 2000–2005.

Results. Viruses were identified in 312 (60.6%) of the 515 patients. RSV was detected in 122 (23.7%), HBoV in 58 (11.3%), adenovirus in 35 (6.8%), PIV-3 in 32 (6.2%), rhinovirus in 30 (5.8%), hMPV in 24 (4.7%), influenza A in 24 (4.7%), PIV-1 in 9 (1.7%), influenza B in 9 (1.7%), and HCoV-NL63 in 8 (1.6%). Coinfections with ≥2 viruses were observed in 36 patients (11.5%). Twenty-two patients (37.9%) infected with HBoV had a coinfection. Bronchiolitis was frequently diagnosed in patients who tested positive for RSV, PIV-3, or rhinovirus, whereas influenza A, PIV-1, and HCoV-NL63 were commonly found in patients with croup. The age distributions of patients with viral infections differed; notably, RSV was responsible for 77% of LRTIs that occurred in infants ≤3 months old. The number of hMPV infections peaked between February and April, whereas the number of HCoV-NL63 infections peaked between April and May.

Conclusions. This study describes the features of LRTIs associated with newly identified viruses in children, compared with those associated with known viruses. Additional investigations are required to define the role of HBoV in LRTI.
we performed virological studies of Korean infants and children with acute LRTIs. Specifically, we evaluated the relative prevalences and the epidemiologic characteristics of rhinovirus, hMPV, conventional HCoV, HCoV-NL63, and HBoV and compared these findings with those of common respiratory viruses, such as RSV, PIV, adenovirus, and influenza virus.

**PATIENTS, MATERIALS, AND METHODS**

**Patients and respiratory specimens.** The study population consisted of children ≤5 years old with acute LRTIs (i.e., bronchiolitis, pneumonia, or croup). All illnesses were diagnosed at the Seoul National University Children’s Hospital (Korea) or the Seoul National University Bundang Hospital (Korea) between September 2000 and August 2005. Samples obtained from children with major risk factors other than recurrent episodes of wheezing were excluded, as those obtained from children with hospital-related infections. Diagnostic definitions were as follows [12, 13]: pneumonia required rales on auscultation or demonstration of an infiltrate by chest X-ray; bronchiolitis was characterized by a cough, tachypnea, retraction, and expiratory wheezes, often accompanied by rales; and croup required a barking cough with stridor. The principal investigator determined the clinical diagnosis on the basis of a review of medical records conducted by 5 of the investigators. The predominant clinical diagnosis was determined when >1 diagnosis was present.

Nasopharyngeal aspirates were prospectively collected from all subjects. Specimens were obtained either at the time of visiting an emergency department or immediately following hospital admission. Viral RNA was detected in nasopharyngeal aspirates using RT-PCR.

**Viral diagnosis.** Samples of nasopharyngeal aspirates were kept frozen at −70°C. Viral RNA in nasopharyngeal aspirates was extracted using a QIAamp Viral RNA Mini kit (Qiagen), in accordance with the manufacturer’s instructions. cDNA was synthesized using random hexamers and Superscript RT (Invitrogen). Multiplex RT-PCR assays were developed to detect 11 viruses, namely, RSV and PIV-3 (panel 1), hMPV and rhinovirus (panel 2), influenza viruses A and B and PIV-1 (panel 3), coronaviruses OC43 and 229E and HCoV-NL63 (panel 4), and HBoV (panel 5). Adenovirus was detected by culture in HEP-2 monolayers, because this cell culture–based assay has a diagnostic sensitivity similar to that of adenovirus-specific PCR [14].

In brief, 17.5 μL of total RNA was mixed with 1 μL of random hexamers at a concentration of 15 μM and then incubated at 65°C and chilled on ice. A reaction mix of 11.5 μL containing 6 μL of 5x first strand buffer, 3 μL of 100 mM dithiothreitol, 1 μL of deoxyribonucleotide triphosphate mix at a concentration of 10 mM, 1 μL of RNase inhibitor, and 100 units of SuperScript II reverse transcriptase (Invitrogen) was then added. The reaction was incubated at 25°C for 10 min and at 42°C for 60 min, and after a denaturation step at 94°C for 3 min, the cDNA was used as a template for subsequent PCR. The 20-μL reaction mixtures consisted of 1x GeneAmp PCR buffer Gold (Applied Biosystems), 2.0 mM MgCl₂, each deoxyribonucleotide triphosphate at a concentration of 0.2 mM, 20 pmol of primers, and 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). After incubation for 5 min at 95°C, amplification was performed at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The primers used were designed in this study or modified from the published methods [15–19] to amplify the virus-specific genomes (table 1). Amplified products were then separated on agarose gels, and virus-specific PCR products were identified.

The sensitivity of the multiplex RT-PCR for 5 viruses (RSV, PIV-1, PIV-3, hMPV, and influenza viruses A and B) was determined for each of the targets using virus suspensions in cell culture media that were quantified in terms of TCID₅₀ using standard virological techniques [20].

All specimens were tested by the multiplex RT-PCR for the 11 respiratory viruses and by viral culture and immunofluorescent antigen detection for 6 viruses (RSV, PIV-1, PIV-3, adenovirus, and influenza viruses A and B), as described elsewhere [3]. Samples were considered to be positive if the 2 PCR results using separately extracted copies of viral RNA were positive or if a single positive PCR result was confirmed by viral culture or immunofluorescent antigen detection methods. Each PCR included distilled water as a negative control, as well as positive controls for the corresponding copies of viral cDNA in each panel.

**Clinical database.** Respiratory symptoms and signs were recorded on a standardized form during the emergency department visit or while the patient was hospitalized. Medical records were reviewed to determine the clinical manifestations and any underlying conditions of patients. Clinical data were entered into a database by a person who did not have knowledge of virus identities.

**Statistical analysis.** The χ² test or Fisher’s exact test was used to compare samples with respect to percentage of each sex, symptoms, and the clinical diagnoses, and the Mann-Whitney U test was used to compare the mean age at onset of illness between the various groups. Analyses were performed using GraphPad InStat software, version 3.06 (GraphPad Software).

**RESULTS**

**Patient characteristics.** A total of 2198 nasopharyngeal aspirates associated with a diagnosis of LRTI in the previously healthy children (≤5 years old) were collected during the study period. Tested specimens were selected by a random number assignment from the list of archived samples. A total of 515 nonconsecutive specimens (23.4%) were chosen for RT-
Table 1. Primer sequences for multiplex RT-PCR.

<table>
<thead>
<tr>
<th>Panel</th>
<th>Virus</th>
<th>Sequences (5′ → 3′)</th>
<th>Target gene</th>
<th>Amplicon size, base pairs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RSV</td>
<td>F: ACT AAG TTA GCA GCA GG</td>
<td>Nucleoprotein</td>
<td>230</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCT GCG AAG ATT CCT TCA AC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PIV-3</td>
<td>F: CTG GGC TTC ATC AGT AGA GA</td>
<td>Matrix protein</td>
<td>370</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGG CAT TGT GTT CAG TGC TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>hMPV</td>
<td>F: CAT GCC CAC TAT AAA AGG TCA G</td>
<td>L gene</td>
<td>171</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Rhinovirus</td>
<td>F: GCA CTT CTG TTT CCC C</td>
<td>5′ UTR</td>
<td>202</td>
<td>Modified from [16]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGC AGC CAC GCA GGC T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PIV-1</td>
<td>F: TGC AGA CGG CAT ATC TCC TCT GGA</td>
<td>HN</td>
<td>307</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Influenza A virus</td>
<td>F: AAG GGC TTT CAC CGA AGA GG</td>
<td>Nonstructural protein</td>
<td>171</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>Influenza B virus</td>
<td>F: GGG ATA TAC GTA ATG TGT TGT</td>
<td>Nonstructural protein</td>
<td>489</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCA CTG CCT GCT GTA CAC TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HCoV 229E and OC43</td>
<td>F: GCG CAA AAT AAT GAA TTA ATG CC</td>
<td>Replicase</td>
<td>550</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>HCoV-NL63</td>
<td>F: GCC AAA GTT CTT GTC CCA GTA TTA AC</td>
<td>Replicase</td>
<td>215</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTC GCA CCA CCA TAT GAA TCC TG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F: GCC GCA GTC AAA AGT CCA GAA TTA AC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>HBoV</td>
<td>F: TAT GCC CAA GGC AAT CTG CCA AG</td>
<td>NS1 gene</td>
<td>291</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCC GGC ACA ACA TGA GAA ACA GA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE. HBoV, human bocavirus; HCoV, human coronavirus; hMPV, human metapneumovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

PCR after excluding unavailable samples and repeated samples collected from the same patient. The monthly proportion of selected samples ranged from 20% to 28% of the total number of archived specimens (figure 1). Of the study population, 306 (59%) were male. The mean age among children was 15.4 months; 20% were infants <4 months old, 28% were 4–12 months old, 25% were 13–24 months old, and 27% were >24 months old. The remaining 1683 samples that were not selected for this study did not differ significantly from selected samples with respect to mean age at disease onset, sex, seasonal distribution, or clinical diagnosis.

Prevalence of respiratory viruses among children with an LRTI. The detection sensitivity of the assay for 5 viruses (RSV, PIV-1, PIV-3, hMPV, and influenza viruses A and B) was greatest for influenza A, with a value of 10^-4 TCID50. Similarly, the assay detected 5 x 10^-3 TCID50 of RSV and PIV-3 and 10^-3 TCID50 of PIV-1, hMPV, and influenza B. Detectable viral RNA concentrations for rhinovirus, coronaviruses OC43 and 229E, HCoV-NL63, and HBoV were 5–10 ng of the total RNA extracted from the cell culture supernatants or clinical specimens. Of the 515 samples tested, 312 (60.6%) were positive for any of the 11 respiratory viruses (table 2). The detection rate of multiplex RT-PCR was increased because of additional viruses that can be detected by this method and because of greater sensitivity, compared with viral culture or antigen detection methods. Of these 312 virus-positive samples, 36 were also positive for additional viruses, resulting in a coinfection rate of 11.5%. Virus frequencies were as follows: RSV was detected in 122 children (23.7%), HBoV in 58 (11.3%), adenovirus in 35 (6.8%), PIV-3 in 32 (6.2%), rhinovirus in 30 (5.8%), hMPV in 24 (4.7%), influenza A in 24 (4.7%), PIV-1 in 9 (1.7%), influenza B in 9 (1.7%), HCoV-NL63 in 8 (1.6%), and conventional HCoV in 1 (replicase 1a gene sequencing analysis revealed this to be HCoV-OC43). Rhinoviruses were found to be associated with acute LRTI at a relatively high frequency. The prevalence of hMPV was similar to that of influenza virus A. In contrast to these, HCoV-NL63 was present at a low prevalence in the study group.

Seasonal distribution. The monthly distributions of detected viruses over the 5-year study period are shown in figure 1. Monthly variations in percentage contributions to the diagnosed viral LRTIs ranged from 0% to 88%. The number of RSV infections increased during late fall and peaked between November and January. PIV-3 was prevalent from April to June, and rhinovirus was detected year-round, with a peak occurring during late summer and fall. The prevalence of hMPV increased during late winter and peaked between February and April (68% of total isolates). hMPV was not detected during the 2003–2004 epidemic period, although comparable numbers of samples were tested. HCoV-NL63 was identified, with peaks occurring between April and May (44% of total isolates).

Clinical characteristics. The clinical features of children...
who were tested for these viruses are summarized in table 3. The age distributions of infants and children associated with each virus differed. LRTIs caused by RSV were predominant among younger infants (mean age, 9 months), compared with LRTIs associated with adenovirus, HBoV, hMPV, or influenza A (P<.01 for each comparison). In particular, a greater proportion of RSV infections occurred in infants ≤3 months old, compared with other virus-associated LRTIs (P<.04 for all comparisons between RSV and 6 viruses [adenovirus, HBoV, PIV-3, influenza virus A, hMPV, and HCoV-NL63]) (figure 2). In addition, rhinovirus accounted for a larger proportion of LRTIs in young infants ≤3 months than adenovirus or HBoV (P<.03). In contrast, adenovirus, hMPV, and influenza A were more frequently detected in children >24 months old.

Figure 1. Monthly occurrence of acute lower respiratory tract infections associated with 11 respiratory viruses isolated from children over the 5-year period 2000–2005.
The clinical diagnoses of the 265 patients with LRTIs associated with 8 common viruses are summarized in table 3. Bronchiolitis or pneumonia was frequently observed in patients with positive results for RSV, HBoV, PIV-3, hMPV, or rhinovirus. Of these, RSV and rhinovirus were more likely to cause bronchiolitis, whereas wheezing occurred more frequently in patients infected with RSV than in patients with an LRTI associated with other viruses, the above findings indicate that HBoV was associated with high rates of mixed infections. Viruses that were frequently found to be coinfecting with HBoV were as follows, in descending order: adenovirus (7 cases), hMPV (5 cases), RSV (5 cases), and PIV-3 (3 cases). HBoV was identified throughout each year during the 5-year study period, with a peak (57% of total isolates) occurring from May to July, and the mean age of HBoV-infected children was 22.3 months. The clinical diagnoses of 36 HBoV-associated LRTIs were bronchiolitis for 9 patients (25%), pneumonia for 20 patients (55.6%), croup for 3 patients (8.3%), and asthma exacerbation for 4 patients (11.1%).

**DISCUSSION**

In this study, we evaluated the overall prevalences of 11 respiratory viruses identified by multiplex RT-PCR in a cohort of previously healthy children with acute LRTIs. In addition, we compared the relative contributions and clinical features between the acute LRTIs associated with the recently identified viruses (rhinovirus, hMPV, HCoV-NL63, and HBoV) and those caused by the previously known viruses (RSV, parainfluenza viruses, adenovirus, and influenza viruses) during 5 consecutive years, 2000–2005.

Since both hMPV and HCoV-NL63 were recognized as being the etiologic agents of LRTIs [8, 9, 21–23], comparative studies on the relative prevalences and clinical characteristics of respiratory tract infections caused by newly identified viruses in children have been hampered, primarily because of the different sensitivities of the diagnostic tests employed [24–26]. In addition, the majority of previous studies have used molecular methods to investigate the roles of hMPV and HCoV-NL63 in acute respiratory diseases by using subjects that differ with respect to the number of respiratory specimens tested or results obtained by viral culture or by antigen detection methods [22, 27]. Moreover, simultaneous infections by other viruses are often documented; thus, contributions made by individual viruses to respiratory diseases may be over- or underestimated.

In this study, we used multiplex RT-PCR, which has been widely used to identify recently recognized viruses, such as hMPV, HCoV-NL63, and HBoV, to determine the prevalences of these viruses and to reassess the prevalences of older viruses, such as coronavirus and rhinovirus, among children with LRTIs. Eleven tested viruses were found in 312 (60.6%) of the 515 patients.

The clinical characteristics of patients infected with different viruses were relatively distinct. RSV, rhinovirus, and PIV-3 were frequently observed in the patients with bronchiolitis. In contrast, influenza virus, PIV-1, and HCoV-NL63 were found to be major viral agents of croup. Although previous studies have shown that hMPV is more likely to be associated with bronchiolitis or wheezy bronchitis than pneumonia or croup [27, 28], we found that hMPV was frequently associated with viral pneumonia in this cohort. This observation may reflect dif-

<table>
<thead>
<tr>
<th>Virus identified</th>
<th>Antigen detection</th>
<th>Viral culture</th>
<th>Multiplex RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory syncytial virus</td>
<td>91 (17.7)</td>
<td>97 (18.8)</td>
<td>122 (23.7)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>18 (3.5)</td>
<td>35 (6.8)</td>
<td>35 (6.8)</td>
</tr>
<tr>
<td>Parainfluenza virus 1</td>
<td>3 (0.6)</td>
<td>5 (1.0)</td>
<td>9 (1.7)</td>
</tr>
<tr>
<td>Parainfluenza virus 3</td>
<td>19 (3.7)</td>
<td>24 (4.7)</td>
<td>32 (6.2)</td>
</tr>
<tr>
<td>Influenza virus A</td>
<td>11 (2.1)</td>
<td>19 (3.7)</td>
<td>24 (4.7)</td>
</tr>
<tr>
<td>Influenza virus B</td>
<td>4 (0.8)</td>
<td>6 (1.2)</td>
<td>9 (1.7)</td>
</tr>
<tr>
<td>Human bocavirus</td>
<td>ND</td>
<td>ND</td>
<td>58 (11.3)</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>ND</td>
<td>ND</td>
<td>30 (5.8)</td>
</tr>
<tr>
<td>Human metapneumovirus</td>
<td>ND</td>
<td>ND</td>
<td>24 (4.7)</td>
</tr>
<tr>
<td>Coronavirus NL63</td>
<td>ND</td>
<td>ND</td>
<td>8 (1.6)</td>
</tr>
<tr>
<td>Coronavirus OC43</td>
<td>ND</td>
<td>ND</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>Total</td>
<td>146 (28.4)</td>
<td>185 (36.2)</td>
<td>312 (60.6)</td>
</tr>
</tbody>
</table>

NOTE. ND, not determined.

a The results of PCR for adenovirus are the same as the results of viral culture.

b Indicates the total no. (%) of positive specimens of the total no. of specimens tested.

c A total of 352 viruses was identified from 312 specimens by multiplex RT-PCR assay. More than 1 virus was detected in 36 specimens (11.5%).
influenza A, virus; RSV, respiratory syncytial virus.

Clinical characteristic

- Analysis of samples selected from a list of cases of acute LRTI
- HCoV-NL63 is an important etiologic agent of croup during the fall and winter seasons [3, 34]. These findings suggest that or PIV-1, which are the 2 most common causes of croup in proportion of HCoV-NL63–positive croup cases was found to frequently associated with croup (3 of 6 cases). Moreover, the identified with HCoV-NL63 infection, when it was found, it was found. However, despite the small number of children identified with HCoV-NL63 infection, when it was found, it was frequently associated with croup (3 of 6 cases). Moreover, the proportion of HCoV-NL63–positive croup cases was found to be similar to the proportion of cases caused by influenza virus or PIV-1, which are the 2 most common causes of croup in the fall and winter seasons [3, 34]. These findings suggest that HCoV-NL63 is an important etiologic agent of croup during the spring in Korea.

Our study also has several limitations. It was based on an analysis of samples selected from a list of cases of acute LRTI in a hospital database. Therefore, we could not determine the population-based prevalence. Viruses associated with low prevalence and mild symptoms may not be fully described, either. However, comparable numbers of the archived samples were selected in each study year; thus, we believe that the data obtained in the present study may represent the overall activities of viruses responsible for acute LRTIs and their epidemiologic characteristics. During the 5-year observation period, hMPV infection appeared to be rare in the 1-year period of 2003–2004. This finding suggests that hMPV-associated LRTIs are subject to annual variations, which contrasts with the relatively stable annual incidence of RSV infections [35, 36]. These results may be the consequence of spurious observations affected by the selection bias of this study. However, annual variability has also been demonstrated in other studies of periods of only 2 or 3 years and of archived samples during a longer period [26, 37, 38]. Additional prospective studies are needed to characterize the epidemiologic features of hMPV infection.

HBoV, a potential causative agent of LRTI, demonstrated seasonal periodicity during each study year, with a peak prevalence occurring from May to July. The number of HBoV infections peaked slightly later than the number of PIV-3 infections, and during its peak months, the total prevalence of the other 10 viral agents excluding HBoV was 34.7% (50 cases among 144 nasopharyngeal aspirates), which is lower than the
Figure 2. Age distribution of children with lower respiratory tract infections associated with viral agents. The percentage of patients infected with individual viruses is shown for each age group. The sum of the proportion of persons infected with each virus in each of 4 age groups is 100%. a The proportion of infants <3 months old; P < .04 for all comparisons between respiratory syncytial virus (RSV) and 6 viruses (adenovirus, bocavirus, parainfluenza virus [PIV]-3, influenza virus A, human metapneumovirus [hMPV], or human coronavirus [HCoV]-NL63; P < .03 for rhinovirus versus adenovirus or bocavirus, by Fisher’s exact test. b Comparison of the proportion of children 1–24 months old; for RSV versus adenovirus, influenza A virus, bocavirus, or hMPV; for rhinovirus versus adenovirus or bocavirus; and for PIV-3 versus adenovirus or bocavirus, by Fisher’s exact test.

Overall detection rate. The detection rate of 11 viruses during the same period increased from 34.7% to 59%, similar to that of the total detection rate of 60.6%. These findings suggest that HBoV is a major viral agent of respiratory episodes during late spring to early summer. However, the issue of a causal relationship remains unresolved. It is noteworthy that the detection of HBoV DNA was associated with a higher rate of coinfection than other viral agents. As demonstrated above, the prevalence of coinfection of HBoV was similar to that of adenovirus, which is frequently observed as a copathogen because of its long shedding period. In 2 previous studies, HBoV has also been found with other agents with prevalences of 17.5% and 55.6% [11, 19]. Thus, HBoV infection might occur incidentally to respiratory infections caused by other viral agents. At the present time, despite the periodicity shown by HBoV, its role as a true pathogen remains uncertain. Additional studies are required to determine its asymptomatic prevalence in the population, pathogenicity, and viral shedding characteristics.

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Potential conflicts of interest. All authors: no conflicts.

References


