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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.

Acute viral respiratory illnesses are major health problems in infants and children. Traditionally, respiratory syncytial virus (RSV), parainfluenza virus (PIV), influenza virus, and adenovirus have been viewed as being the leading causes of acute viral lower respiratory tract infections (LRTIs) [1–4].

However, in addition to previously known viruses, many respiratory viruses have been recently identified as causative agents of lower respiratory illnesses in children. Rhinovirus, which generally has been considered a cause of mild upper respiratory illnesses in children and adults, is now considered to be a major cause of acute LRTIs and asthma exacerbations [5–7]. However, the extent to which rhinoviruses contribute to LRTIs in otherwise healthy children is unclear. More recently, new viruses, such as human metapneumovirus (hMPV) [8] and human coronavirus (HCoV)–NL63 [9, 10], have been suggested to cause LRTIs in children. Moreover, human bocavirus (HBoV) has been identified in the respiratory tracts by molecular screening [11], but its role as a causative agent of LRTI remains to be proven.

To determine the relative contributions made by these newly recognized respiratory viruses to LRTIs in childhood and to characterize their epidemiologic and clinical features, over a period of 5 years (2000–2005)
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 were 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 low sensitivity 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 samples 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-PCR analysis. 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 low sensitivity and any underlying conditions of patients. Clinical data were entered into a database by a person who did not have knowledge of virus identities.

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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 52°C for 10 min and at 25°C for 30 min. 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 of 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.
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 R: CCT GCG AAG ATT CCT TCA AC</td>
<td>Nucleoprotein</td>
<td>230</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F: CTG GGC TTC ATC AGT AGA GA R: TGG CAT TGT GTT CAG TGC TT</td>
<td>Matrix protein</td>
<td>387</td>
<td>This study</td>
</tr>
<tr>
<td>2</td>
<td>hMPV</td>
<td>F: CAT GCC CAC TAT AAA AGG TCA G R: GTC ACC CCA GTC TTT CTT GA</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 R: GGC AGC CAC GCA GGC T</td>
<td>5′ UTR</td>
<td>202</td>
<td>Modified from [16]</td>
</tr>
<tr>
<td>3</td>
<td>PIV-1</td>
<td>F: TGC AGA CGG CAT ATC TCC TCT GGA R: GGT ATG AGA AAT TAC</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 AG R: CCC ATT CTC ATT ACT GCT TC</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 R: GCA CTG CCT GCT GTA CAC TT</td>
<td>Nonstructural protein</td>
<td>489</td>
<td>[15]</td>
</tr>
<tr>
<td>4</td>
<td>HCoV 229E and OC43</td>
<td>F: GCG CAA AAT AAT GAA TTA ATG CC R: GAC GCA CCA CCA TAT GAA TCC TG</td>
<td>Replicase</td>
<td>550</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>HCoV-NL63</td>
<td>F: GCG CTA TGA GGG TGG TTG TAA C R: GGC GCA GTT AAA AGT CCA GAA TTA AC</td>
<td>Replicase</td>
<td>215</td>
<td>[18]</td>
</tr>
<tr>
<td>5</td>
<td>HBoV</td>
<td>F: TAT GGC CAA GGC AAT CGT CCA AG R: GCC GCC GTA ACA TGA GAA ACA GA</td>
<td>NS1 gene</td>
<td>291</td>
<td>[19]</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} TCID_{50}. Similarly, the assay detected 5 × 10^{-3} TCID_{50} of RSV and 10^{-3} TCID_{50} 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

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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 by our definition, hMPV showed a tendency to cause pneumonia. Croup was frequently associated with influenza A, PIV-1, or HCoV-NL63. Fever was more frequently observed in hMPV-infected children (88.2%) or HCoV-NL63–influenza A, PIV-1, or HCoV-NL63. Fever was more frequently found in patients with mixed viral infections (13 [37.1%] of 35 adenovirus-positive samples) than were the 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 different characteristics of bronchiolitis and pneumonia.
influenza A, virus; RSV, respiratory syncytial virus.

that tested positive for parainfluenza 1, influenza B, or human coronavirus (HCoV)–OC43 are not shown. hMPV, human metapneumovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

Our study also has several limitations. It was based on an analysis of samples selected from a list of cases of acute LRTIs in Korean children. In fact, HCoV-NL63 only accounted for 1.6% of cases, and HCoV-OC 43 was rarely found. However, despite the small number of children identified with HCoV-OC43 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.

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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%. *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. †Comparison of the proportion of children >24 months old; for RSV versus adenovirus, influenza A virus, bocavirus, or hMPV; P < .04 for rhinovirus versus adenovirus or bocavirus; and P < .03 for PIV-3 versus adenovirus or bocavirus, by Fisher’s exact test.

Acknowledgements


Potential conflicts of interest. All authors: no conflicts.

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