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Abstract

Background: Human coronavirus-NL63 (HCoV-NL63) has been isolated from children with respiratory tract infections and its prevalence in Korea has not been reported.

Objectives: This study was designed to investigate the presence and the clinical features of HCoV-NL63 during two winter seasons.

Study design: During April 2004–April 2006, nasopharyngeal specimens from children hospitalized with acute respiratory disease were tested for common respiratory viruses, including RSV, influenza A, influenza B, parainfluenza viruses, and adenovirus by IFA. hMPV infection was excluded by nested RT-PCR using primers for F-gene. To detect HCoV-NL63, previously described nested PCR assays for 1a and 1b were used. PCR products of the 1a gene for HCoV-NL63 were sequenced.

Results: Out of 872 nasopharyngeal aspirate from children aged under 16 years, 14 (1.7%) were positive for HCoV-NL63. Most of the patients had croup (64.2%) or bronchiolitis (21.4%). The peak prevalence was found in November (28.5%). Most were collected between November 2004 and February 2005.

Conclusions: HCoV-NL63 may be one of the causative agents of acute respiratory tract infection, especially croup.

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Keywords: HCoV-NL63; Croup; Acute bronchiolitis; Children

1. Introduction

Human respiratory syncytial virus (RSV), parainfluenza viruses, adenoviruses, influenza viruses, and recently discovered human metapneumovirus (hMPV) are the most commonly isolated viruses in acute respiratory infection in children (Peiris et al., 2003a,b; Selwyn, 1990). However, the etiologic agents are still unknown in a large proportion of respiratory infections. Human coronaviruses are positive sense RNA enveloped viruses (Nokso-Koivisto et al., 2000). HCoV-229E and HCoV-OC43 are major causes of common colds.

In 2002, a novel human corona virus, HCoV-SARS, was identified as a cause of severe acute respiratory syndrome (SARS) (Nokso-Koivisto et al., 2000; Peiris et al., 2003a,b) and a new kind of group 1 human coronavirus-NL63 (HCoV-NL63) was discovered in the Netherlands (van der Hoek et al., 2004). Another group from the Netherlands also identified HCoV-NL in a boy with pneumonia (Fouchier et al., 2004). Although HCoV-NL63 has been associated with upper and lower respiratory tract infections in children, there are no published reports in Korea.

We used nasopharyngeal aspirate specimens from hospitalized children with acute respiratory disease to determine the prevalence and clinical features of HCoV-NL63 during two winter seasons in Korea.

2. Methods

2.1. Patients and specimens

A total of 1005 nasopharyngeal specimens were consecutively collected from children under 16 years of age, who were admitted with acute respiratory disease at SanggyePaik Hospital...
Hospital from April 2004 to April 2006. A total of 827 specimens were tested with IFA (DAKO, Cambridgeshire, UK) for common respiratory viruses; RSV, influenza virus A, influenza B, parainfluenza viruses, and adenoviruses, were sought. The specimens were stored at $-70^\circ\text{C}$ until further tested. Informed consent was obtained at admission from parents. The possibility of hMPV infection was excluded by nested RT-PCR using specific primers to amplify a part of the F-gene as previously described (van den Hoogen et al., 2004). Clinical definitions were: croup was hoarseness of voice, barking cough, and inspiratory stridor due to laryngeal obstruction; tracheobronchitis was cough and rhonchi and no laryngeal obstruction or wheezing; bronchiolitis was expiratory wheezing with or without tachypnea, air trapping, and substernal retractions; pneumonia was rales or evidence of pulmonary consolidation on physical examination or radiograph; bronchial asthma was acute wheezing occurring three or more times in children of any age or occurring one or more times in children aged $\geq 3$ years. The diagnoses of the patients were croup in 29, pneumonia in 290, bronchiolitis in 257, acute bronchitis in 40, upper respiratory tract infection in 21, and bronchial asthma in 190. The median age was 15 months (range = patients ranged from 1 to 192 months in age): children 5 years of age or younger constituted 92.7% (759/827) of the study population. The Ethics Committee of Faculty Medicine, Inje University, Seoul, Korea, approved the study.

2.2. RT-PCR and sequencing

Viral RNA was extracted from each sample by QIAamp viral mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s protocol. Reverse transcription of 0.5 $\mu$g of each RNA sample was performed in a final volume of 20 $\mu$L containing 5 $\mu$M of random hexadeoxyxynucleotides, 1 mM of each dNTP, 2 units of RNase inhibitor, and 9 units of reverse transcriptase (Bioneer, Daejeon, Korea). After incubation at 42 $^\circ\text{C}$ for 1 h, the samples were heated for 5 min at 94 $^\circ\text{C}$. To detect HCoV-NL63, previously described nested PCR assays for 1a and 1b were used (Arden et al., 2005). All PCR assays were performed using 1 $\mu$L of cDNA and 0.6 $\mu$M of each primer. To validate the amplification process and to exclude the presence of carryover contamination, positive and negative controls were run on each PCR, and positive samples were verified against an independent RNA extraction.

PCR products of the 1a gene were sequenced. Amplicon was purified using QIAqucik (Qiagen GmbH, Hilden, Germany) and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing products were resolved with an ABI 3730 XL autoanalyzer (Applied Biosystems, Foster City, CA, USA). Phylogenic trees were constructed using MEGA version 3.0 (Kumar, Tamura, Nei 2004).

![Fig. 1. Seasonal distribution of HCoV-NL63 positive samples. The number in parenthesis after each month gives the number of the samples tested.](image-url)
Table 1
Clinical characteristics of HCoV NL-63 positive patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (months)</th>
<th>Sex</th>
<th>Date (sample collection)</th>
<th>Clinical manifestations</th>
<th>Duration of symptoms (days)</th>
<th>Underlying disease</th>
<th>Clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>M</td>
<td>May 2004</td>
<td>Cough, sputum</td>
<td>3</td>
<td>None</td>
<td>Croup</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>F</td>
<td>Nov 2004</td>
<td>Cough, sputum</td>
<td>4</td>
<td>None</td>
<td>BA exacerbation</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>M</td>
<td>Nov 2004</td>
<td>Cough, Fever, dyspnea</td>
<td>2</td>
<td>None</td>
<td>Croup</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>F</td>
<td>Dec 2004</td>
<td>Cough</td>
<td>1</td>
<td>None</td>
<td>Croup</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>M</td>
<td>Jan 2005</td>
<td>Cough, dyspnea</td>
<td>2</td>
<td>None</td>
<td>Croup</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>M</td>
<td>Feb 2005</td>
<td>Cough, dyspnea</td>
<td>1</td>
<td>None</td>
<td>Croup</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>M</td>
<td>Feb 2005</td>
<td>Cough, dyspnea</td>
<td>2</td>
<td>None</td>
<td>Croup</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>F</td>
<td>Feb 2005</td>
<td>Cough, Fever, dyspnea</td>
<td>4</td>
<td>None</td>
<td>Croup</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>M</td>
<td>Mar 2005</td>
<td>Cough, fever</td>
<td>3</td>
<td>None</td>
<td>Bronchiolitis</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>M</td>
<td>Nov 2004</td>
<td>Cough, Fever, dyspnea</td>
<td>2</td>
<td>None</td>
<td>Croup</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>M</td>
<td>Nov 2004</td>
<td>Cough, sputum</td>
<td>10</td>
<td>None</td>
<td>Bronchiolitis</td>
</tr>
<tr>
<td>12</td>
<td>33</td>
<td>M</td>
<td>May 2005</td>
<td>Cough, sputum</td>
<td>2</td>
<td>None</td>
<td>Bronchiolitis</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>F</td>
<td>Apr 2006</td>
<td>Cough</td>
<td>2</td>
<td>None</td>
<td>Croup</td>
</tr>
<tr>
<td>14</td>
<td>58</td>
<td>F</td>
<td>Apr 2006</td>
<td>Cough, fever</td>
<td>2</td>
<td>None</td>
<td>Pneumonia</td>
</tr>
</tbody>
</table>

BA, bronchial asthma.

3. Results

From April 2004 to April 2006, a total of 827 nasopharyngeal specimens from children, hospitalized with acute respiratory disease, were tested for common respiratory viruses. IFA diagnosis indicated that 151 (18.2%) specimens were positive for RSV, 9 (1.1%) for parainfluenza virus, 5 (0.6%) for influenza A virus, 2 for adenoviruses, and 1 for influenza B virus. RT-PCR detected 83 (10.0%) samples containing hMPV, and 14 (1.7%) containing HCoV-NL63. HCoV NL-63 was detected during May (1 specimen), November (4 specimens), and December (1 specimen) in 2004; January (1 specimen), February (3 specimens), and May (2 specimens) in 2005; April (2 specimens) in 2006 (Fig. 1). Most of HCoV NL-63 positive specimens were collected between November 2004 and February 2005. None were detected during the winter season of 2005–2006. HCoV-NL63 was detected in one case with RSV and in one case with RSV and hMPV.

The HCoV NL-63 positive patients were 5–58 months old (median, 11.5 months) and the ratio of males to females was

![Fig. 2. Phylogenetic analysis of Korean (KR), Canadian (CAN), and Queensland (Q) 1a gene sequences from human coronavirus NL63 strains presented on a topology tree prepared in MEGA3. Nucleotide alignment of a 425 bp portion of the 1a gene was prepared using BioEdit V7.0. The nucleotide distance matrix was generated using the Kimura two-parameter estimation. Nodal confidence values indicate the results of bootstrap resampling (n = 1000). Two main sequence clusters are apparent and all Korean strains belong to the upper cluster. GenBank accession numbers: KR (DQ093116-DQ093123, DQ351988, DQ453793-DQ453795, DQ534705-DQ534706), CAN (AY675543, AY675550, AY675542, AY675547), Q (AY746455, AY746458, AY746451), NL63 (NC_005831), and NL (AY518894).]
1.8:1. HCoV-NL63 positive patients were diagnosed as croup in 9 (64.2%), bronchiolitis in 3 (21.4%), bronchial asthma exacerbation in 1, and pneumonia in 1 (Table 1). HCoV-NL63 was positive in 31.0% (9/29) of children with croup in this study population. Of the 20 cases of croup that were not associated with HCoV-NL63, RSV was detected in 5 patients, hMPV in 3 patients, and parainfluenza virus in 3 patients. The clinical presentations of patients were cough (100%), fever (35.7%), sputum production (28.5%), and dyspnea (35.7%). No patients had predisposing factors or underlying diseases.

The positive PCR products were confirmed by sequencing and the partial –sequences of the 1a gene were deposited in GenBank (DQ093116-DQ093123, DQ351988, DQ453793-DQ453795, DQ534705-DQ534706). Direct sequencing of the PCR products of the 1a gene revealed that seven isolates had the same sequences and others limited variation of sequence (Fig. 2).

4. Discussion

This is the first report of HCoV-NL63 infections in Korean children hospitalized with acute respiratory disease. HCoV-NL63 circulated in Korean children during 2004–2006. Coronavirus are divided into three different groups: group 1 (HCoV-229E and HCoV-NL63); group 2a (HCoV-OC43 and HCoV-HKU1); group 2b (SARS-CoV); group 3, but human pathogens are only found in groups 1 and 2. Coronavirus HCoV-NL63 infections have been reported in the Netherlands, Australia, Japan, Canada, USA, France, and Hong Kong (Arden et al., 2005; Bastien et al., 2005; Chiu et al., 2005; Ebihara et al., 2005; Esper et al., 2005; Vabret et al., 2005; van den Hoogen et al., 2004), which suggest that this newly discovered human coronavirus has a worldwide distribution. Although, we did not include a healthy control group, HCoV-NL63 presence in the nasal aspirates of children with acute respiratory disease, in the absence of an alternate etiology, suggests that it may have a role in the illness. However, the causal association HCoV-NL63 cannot be established without including a healthy control group. The incidence of HCoV-NL63 in patients with respiratory disease of unknown etiology is reported to be 1.2–9.3% (Esper et al., 2005; Moes et al., 2005; Suzuki et al., 2005; Vabret et al., 2005). In this study, the positive rate of HCoV-NL63 (1.7%) was comparable to that of Australia and Japan (Arden et al., 2005; Suzuki et al., 2005). The differences in HCoV-NL63 positivity rates among several studies may be due to the characteristics of the study populations and the collection time of respiratory specimens. In a Japanese study, HCoV-NL63 was detected in 25.0% of hospitalized children with acute bronchiolitis suggesting that it plays an etiological role in bronchiolitis (Ebihara et al., 2005). In this study, HCoV-NL63 was associated with croup in most of the positive children (9/14, 64.1%), which is consistent with a recent report showing strong association between HCoV-NL63 infection and croup (Moes et al., 2005). However, others have reported that mild and non-specific symptoms are frequent in HCoV-NL63 positive patients and that detection occurred occasionally in healthy children (Boivin et al., 2005; Moes et al., 2005; Suzuki et al., 2005). In this study, HCoV-NL63 infections occurred in previously well patients which is consistent with what was found in previous studies (Arden et al., 2005; Chiu et al., 2005; Ebihara et al., 2005), although others have reported that many children with HCoV-NL63 infection had an underlying disease such as prematurity or cardiac disease (Esper et al., 2005; Moes et al., 2005).

To elucidate, exactly, the clinical spectrum and significance of HCoV-NL63 infection, further population-based studies are needed. In this study, most of the HCoV-NL63 positive patients (85.7%, 12/14) were under 24 months old, and 42.8% (6/14) were under 12 months old. These results are similar to a recent report, which showed that HCoV-NL63 causes lower respiratory tract symptoms in early life and that it is one of the important etiologic agents of acute respiratory infection in young children (Kaiser et al., 2005).

Two out of 14 sequences of HCoV-NL63 detected in this study were identical to that of HCoV-NL63 (NC-005831) and 5 sequences were identical to that of HCoV-NL (AY518894). These results indicate that the 1a gene of HCoV-NL63 is a highly conserved region which aids in its detection by RT-PCR. We do not think that our results are false positives because cross contamination controls were negative and all positive RT-PCR results were confirmed by a second run. Although some reported high frequency (75%, 9/12) of mixed infection (Boivin et al., 2005), we detected co-infections with HCoV-NL63 and another respiratory virus in only two cases.

The prevalence of HCoV-NL63 varies according to the geographical region, seasonality, and year. In the Netherlands, Canada, Japan and Belgium, HCoV-NL63 was mostly detected during winter (Bastien et al., 2005; Foucier et al., 2004; Moes et al., 2005; van der Hoeck et al., 2005), but some have been detected in spring (Chiu et al., 2005; Esper et al., 2005). In this study HCoV-NL63 was detected in spring and winter of 2004, and spring of 2006. Although a comparable number of specimens were included, we could not detect HCoV-NL63 in the respiratory specimens during winter 2005–2006. It is known that outbreaks of other coronaviruses including HCoV-229E and HCoV-OC43 occur every second year (Monto and Lim, 1974). Our results show that outbreaks of HCoV-NL63 infections do not occur every year: further studies are needed to confirm the pattern of periodicity of outbreaks in Korea. Phylogenetic analysis showed that different strains are cocirculating in Korea, which is similar to findings in other countries (Arden et al., 2005; Bastien et al., 2005; Moes et al., 2005; Vabret et al., 2005).

In conclusion, we confirmed the presence of HCoV-NL63 infection in Korean children hospitalized with acute respiratory tract disease. Although the prevalence of HCoV-NL63 is not high, it is one of the important etiologic agents of respiratory tract infections (especially croup) in hospitalized children.
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References


