Detection of Viruses Identified Recently in Children With Acute Wheezing

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The etiologic role of recently identified respiratory viruses for acute wheezing in children is not yet clear. The purpose of this study was to investigate the prevalence of recently identified viruses, including human metapneumovirus (hMPV), human bocavirus (hBoV), human coronavirus NL63 (hCoV-NL63), and human coronavirus HKU1 (hCoV-HKU1) in children with acute wheezing. Viral etiology was identified in 231 children hospitalized with acute wheezing, aged from 1 month to 5 years. Viral antigens for common respiratory viruses were detected by IFA or multiplex PCR. RT-PCR was used to detect respiratory rhinoviruses, hCoV-NL63, hCoV-HKU1, and hMPV. PCR assays for hBoV DNA were performed using the primer sets for non-capsid protein (NP1) and nonstructural protein (NS1) genes. Viruses were found in 61.5% (142/231) of the study population and a single virus was detected in 45.5% (105/231) of the study population. Rhinovirus (33.3%), human respiratory syncytial virus (hRSV; 13.8%), and hBoV (13.8%) were the most frequently detected viruses. hMPV and hCoV-NL63 were detected in 7.8% and 1.3% of wheezing children, respectively. HCoV-HKU1 was not detected. In 16.0% of the study population, more than one virus was detected. In children with acute wheezing, rhinovirus, hRSV, and hBoV were most frequently detected. Further studies including healthy control subjects are needed to define the clinical significance of hBoV in acute wheezing in children.

MATERIALS AND METHODS

Study Populations and Sample Collection
A total of 308 nasopharyngeal specimens were consecutively collected from 308 children under 6 years of age.

INTRODUCTION

Viral respiratory infections are the most common cause of acute expiratory wheezing in children. Human respiratory syncytial virus (hRSV) and rhinovirus are known to be important triggers of acute expiratory wheezing in children [Sigurs et al., 2000; Lemanske et al., 2005], and recent studies using rhinovirus specific RT-PCR have indicated that rhinovirus is a major etiologic agent for acute bronchiolitis in infants [Papadopoulos et al., 2002; Jacque et al., 2006]. Recently, several new respiratory viruses have been identified, including human metapneumovirus (hMPV), and the human coronaviruses [hCoV-NL63 and hCoV-HKU1] [van den Hoogen et al., 2001; van der Hoek et al., 2005; Wool et al., 2005]. Of these new viruses, hMPV has been proposed to be an etiologic agent for acute wheezing in young children and adults, although some results have been contradictory [Rawlinson et al., 2003; Jartti et al., 2004; Williams et al., 2005]. Human bocavirus (HBoV) was recently identified as a member of the Parvoviridae family and found to cause respiratory tract infections in people in Sweden [Allander et al., 2005]. Although the clinical significance of hBoV infection is still unclear despite frequent detection in respiratory specimens, hBoV has been detected in children with lower respiratory tract infection or asthma exacerbation [Arden et al., 2006; Arnold et al., 2006; Chung et al., 2006; Ma et al., 2006; Sloots et al., 2006]. The purpose of this study was to investigate the role of recently identified respiratory viruses in acute expiratory wheezing in children.
age, who were hospitalized with acute wheezing at Sanggyepaik Hospital, from February 2006 to November 2006. A total of 231 specimens, which were sufficient for virus diagnostic evaluation, were tested with IFA (DAKO, Cambridgeshire, UK) or multiplex RT-PCR for common respiratory viruses. Acute bronchiolitis was defined as expiratory wheezing with or without tachypnea, air trapping, and substernal retraction in children aged <3 years, and bronchial asthma was diagnosed when acute wheezing occurred three or more times in children of any age or occurred one or more times in children aged >3 years [Mertsola et al., 1991]. All the samples were collected after informed consents were obtained from the parents. The study protocol was approved by the Ethics Committee of the Sanggyepaik Hospital, Inje University. On admission, a nasopharyngeal aspirate sample was collected using a disposable catheter and suction device after application of normal saline 2 ml into the nostril. Nasopharyngeal aspirates were centrifuged and the supernatants were stored at −70°C until tested.

**Detection of Viruses**

Viral antigens for adenovirus, influenza A, influenza B viruses, parainfluenza virus, and hRSV were detected by IFA (DAKO) or multiplex RT-PCR as described previously [Bellau-Pujol et al., 2005]. Viral RNA was extracted from each sample by QIAamp viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol. RT-PCR was used to detect rhinoviruses, hMPV, hCoV-NL63, and hCoV-HKU1 as described previously using primers listed in Table I. Reverse transcription of 0.5 μg of each RNA was performed in a final volume of 20 μl containing 5 μM random hexadeoxynucleotides, 1 mM of each dNTP, two units of RNase inhibitor, and nine units of reverse transcriptase (Bioneer, Daejeon, Korea). All PCR assays were performed using 1 μl of cDNA and 0.6 μM of each primer.

DNA was extracted from the nasopharyngeal aspirates using the QIAamp DNA Blood Mini Kit (Qiagen GmbH). Two PCR assays using different primer sets were performed per sample to detect the NP1 gene or the NS1 gene of hBoV as described previously (Table I). The PCR product was size separated on a 2% agarose gel with ethidium bromide and visualized with ultraviolet light (UV) light.

**Statistical Methods**

The Chi-square test was applied to compare results for children of different age and for specific virus groups. Statistical analyses were performed using MedCalc for Windows (MedCalc Software, Mariakerke, Belgium).

**RESULTS**

Nasopharyngeal specimens taken from 231 children hospitalized with acute expiratory wheezing in the period of February 2006 to November 2006 were included in the study. The median age of the 231 children was 8.5 months (range 1 month to 5 years), and the gender ratio (M/F) was 1.6:1 (144:87). The age distribution of the study population was: 101 children were aged <12 months (43.7%); 94 children were aged 1–<3 years (40.7%); and 36 children were aged 3–5 years (15.6%). 125 children were diagnosed with bronchiolitis, and 106 were diagnosed with acute asthma exacerbation.

Viruses were found in 142 patients (61.5% of the study population) and, of these, single viral agent was detected in 105 patients (45.5% of the total study population; Table II). Rhinovirus (77 patients, 33.3%), hRSV (32 patients, 13.8%), and hBoV (26 patients, 11.3%) were the most commonly detected agents. hMPV was found in 18 (7.8%) of the study population, and parainfluenza virus was detected in 17 patients (7.4%). Human coronaviruses were detected in eight patients (3.4%); hCoV-OC43 in three, hCoV-NL63 in three, and hCoV-229E in two. Human adenovirus, influenza virus A, and influenza virus B were detected in three patients, in two, and in two, respectively. However, hCoV-HKU1 was not detected in the study population. The seasonal distribution of viruses associated with acute wheezing is shown in Figure 1. More than one virus was found in 37 patients (16.0% of the study population). hBoV and hRSV were co-detected in three patients (1.3%), hBoV and rhinovirus in seven patients (3.0%), hRSV and rhinovirus in nine patients (3.9%; Table II). Human adenovirus was co-detected with other virus; hMPV in 1 patient, rhinovirus in 1, and parainfluenza in 1.

Viruses were found in 34 patients (33.7%) of the infants group (patients aged <12 months), 55 patients (58.5%) of the group aged 1–<3 years, and 17 patients (47.2%) of the group aged 3–5 years. Rhinovirus was most frequently detected in all age groups. The rate of hRSV infection was significantly higher in infants group than in any other age group (P<0.05), but the incidences of other viruses among age groups were not different significantly (Table III).

**DISCUSSION**

In this study, respiratory viruses were detected in 61.5% (142 patients) of children hospitalized with acute expiratory wheezing; this proportion of patients is relatively lower than those of previous studies, which detected viruses in 73–88% of patients [Freymuth et al., 1999; Andreoletti et al., 2000; Rawlinson et al., 2003; Thumerelle et al., 2003; Jartti et al., 2004]. This difference may be due to several factors such as lower sensitivity of antigen detection methods and no test for enterovirus. These results show that the most frequently detected viruses in children with wheezing were rhinovirus and hRSV, which is consistent with previous reports [Heymann et al., 2004; Jartti et al., 2004]. RT-PCR has been used to show that rhinoviruses are the most frequently detected viruses in school-aged children with wheezing and that they are also found in
TABLE I. Primers Used in PCR

| Virus       | Primer                  | Sequence                        | References                          |
|-------------|-------------------------|---------------------------------|-------------------------------------|
| hBoV        | NP-1 188F               | 5'-GACCTCTGTAAGTACTTATTAC-3'     | Sloots et al. [2006]                 |
|             | 542R                    | 5'-CTCTGTTGACTGTAATACA-G'       | Sloots et al. [2006]                 |
|             | NS1 hBoV1.2             | 5'-CTGATCGATCTCCATGTTTAC-3'     |                                     |
|             | hBoV02.2                | 5'-CTGACGCTGATATCTTATC-3'       |                                     |
| hMPV        | F-gene (forward)        | 5'-GCAAGCTGACTGATCTTCTGAAAC-3'  | Van den Hoogen et al. [2004]        |
|             | (Reverse)               | 5'-GCAAATCTGCTCCATTGACAACAC-3'  |                                     |
|             | (Forward)               | 5'-ACATGCAACTCATCGGACAAATACAA-3' |                                     |
|             | (Reverse)               | 5'-ACATGCTGGTACCTCCAATTTTG-3'   |                                     |
| Rhinovirus  | OL26                    | 5'-ACATTTGCGGATATCTTAC-3'       | Gama et al. [2004]                  |
|             | OL27                    | 5'-CGGACACCCAAAGTAG-3'          | Gama et al. [2004]                  |
|             | JWA 1-b                 | 5'-CATCCGGGCGCCGAGA-3'          | Johnston et al. [1993]              |
| hCoV-NL63   | 1b gene (forward)       | 5'-GTTGATGCTATATGGAAATTTG-3'    | Arden et al. [2003]                 |
|             | (Reverse)               | 5'-CTCAGGTGATATAATCTTCC-3'      |                                     |
|             | 1a gene (forward)       | 5'-TTGGTAAAAAGAAGAATAC-3'       |                                     |
|             | (Reverse)               | 5'-TCAATGCTATAACAGTGTCAT-3'     |                                     |
| hCoV-HUK1   | LPW1465 (forward)       | 5'-GTTGAACTGTCGATATTTTCA-3'     | Woo et al. [2005]                   |
|             | LPW1822 (reverse)       | 5'-CTATGATAATCATATGAAATTTG-3'   |                                     |
|             | LPW1467 (forward)       | 5'-GGGTATGCTATTATGAAATTTG-3'    |                                     |
|             | LPW1285 (reverse)       | 5'-CTACTAATGAAATTTGCA-3'        |                                     |
| hCoV-OC43   | MF1 (forward)           | 5'-GCCCTATGTCGATATTTTCA-3'      | Bell-Pujol et al. [2005]            |
|             | MF3 (reverse, 1st)      | 5'-GCTAATGACTGTCGATATTTTCA-3'   |                                     |
|             | MF2i (reverse, 2nd)     | 5'-CTCCAAAATTCGCTTCC-3'         | Bell-Pujol et al. [2005]            |
| hCoV-229E   | MD3 (reverse)           | 5'-CCGTAACACTCGTTAATTTTCA-3'    |                                     |
|             | MD1 (forward, 1st)      | 5'-TGCCCATATTAAATTTTCA-3'       |                                     |
|             | MD2i (forward, 2nd)     | 5'-CCGTAACACTCGTTAATTTTCA-3'    |                                     |

50% of the adults with acute wheezing [Rawlinson et al., 2003; Thumerelle et al., 2003; Heymann et al., 2004; Jartti et al., 2004]. In the present study, influenza A viruses were detected infrequently in the study population, which may be due to the low rate of influenza virus infection during the study period. In previous reports, co-detection of viruses was reported for 18–20% of children hospitalized with asthma, and hRSV and rhinovirus or hRSV and enterovirus were the most frequently detected virus combinations [Jartti et al., 2004; Nagayama et al., 2006]. In this study, of the 137 children in whom viruses were detected, 33 children were infected with more than one virus. Rhinovirus and hRSV, rhinovirus and hBoV, and hRSV and hMPV were the most common co-infections.

In the present study, the prevalence of viral infection was greater in males than females (M/F, 1.6:1), which is similar to those of previous studies suggesting male susceptibility to viral infection due to gender-specific immune response [Johnston et al., 2005; Vabret et al., 2006]. In previous studies [Thumerelle et al., 2003; Weissbrich et al., 2006], most cases of asthma hospitalization occurred in September, and viral respiratory infections, especially rhinovirus, were considered to be associated with asthma exacerbation. In this study, peak rate of rhinovirus detection in wheezing children was in September, but hBoV and hMPV was in peak in May. It is an interesting finding that hBoV was detected most frequently during spring season, because it was found throughout the year in previous study [Chung et al., 2006]. This finding is different from those of previous studies, which show year-round detection of hBoV and mostly in winter months [Arden et al., 2006; Arnold et al., 2006; Weissbrich et al., 2006]. Additional year-round studies over a longer period are needed to know the exact seasonality of hBoV infection.

TABLE II. Detection of Viruses in 231 Children With Acute Wheezing

| Detection of virus         | Number of positive (%) |
|---------------------------|------------------------|
| Single detection          | 105 (45.4)             |
| Rhinovirus                | 56 (24.2)              |
| hRSV                      | 17 (7.4)               |
| hBoV                      | 26 (11.3)              |
| hMPV                      | 13 (5.6)               |
| Others                    | 9 (3.9)                |
| Mixed detection           | 37 (16.0)              |
| hRSV + rhinovirus         | 7 (3.0)                |
| Rhinovirus + hBoV         | 7 (3.0)                |
| hRSV + hMPV               | 4 (1.7)                |
| hRSV + hBoV               | 3 (1.3)                |
| Others                    | 14 (6.1)               |

hRSV, human respiratory syncytial virus; hBoV, human bocavirus; hMPV, human metapneumovirus.

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In previous studies, coronaviruses were detected in 0–13% of patients with asthma exacerbation [Freymuth et al., 1999; Papadopoulos et al., 2002; Thumerelle et al., 2003; Jartti et al., 2004; Nagayama et al., 2006]. Recently identified viruses, such as hCoV-NL63 or hCoV-HKU1, are known to be associated with croup or lower respiratory tract infections in children, but these infections are not thought to be highly prevalent.
HMPV has previously been reported to have a role in exacerbation of asthma in children [Rawlinson et al., 2003; Bosis et al., 2005; Williams et al., 2005] and the median ages of children with hMPV or rhinovirus infections are known to be greater than those of children infected with hRSV [Bosis et al., 2005]. In this study, hMPV was detected in 7.8% of the total study population, which is in agreement with those of a recent study showing similar prevalence of hMPV (8.9%) in hospitalized children with wheezing, which indicates that they play a minor role in provoking acute wheezing in children. HCoV-HKU1 was not found in children with acute wheezing, which is different from recent studies showing high prevalence of hCoV-HKU1 in lower respiratory tract infections. This difference of hCoV-HKU1 prevalence may be due to several factors such as size of study population, low sensitivity of primers used, geographical variance, and cyclic pattern of hCoV-HKU1 infections.

In a Korean study, the detection rate of hBoV in children with acute lower respiratory tract infection was 7.5% [Chung et al., 2006]. In this study, hBoV infections are frequently detected in children with acute wheezing, which suggest that hBoV may have an important role in acute wheezing. However, others reported that rhinovirus was the only virus significantly associated with exacerbations of asthma and hBoV was not detected in the study population [Khesursiani et al. 2007]. Although it is possible that the detection of hBoV in nasopharyngeal specimens from children with acute wheezing can be an incidental finding, hBoV was not detected in healthy control children in a recent study [Kesebir et al., 2006]. Further studies are needed to clarify the clinical role of hBoV-only infections and infections with hBoV and other respiratory viruses. In particular, these should include the prevalence of hBoV infection in healthy control subjects, assessment of disease severity by other clinical variables, and determination of immunologic effects.

HMPV has previously been reported to have a role in exacerbation of asthma in children [Rawlinson et al., 2003; Bosis et al., 2005; Williams et al., 2005] and the median ages of children with hMPV or rhinovirus infections are known to be greater than those of children infected with hRSV [Bosis et al., 2005]. In infants, hRSV, influenza virus, and hMPV are known to be associated with wheezing [Chiu et al., 2002; Heymann et al., 2004; Williams et al., 2005]. Viral infections are still important triggering factors in asthmatic children aged >2 years, and rhinoviruses are most frequently involved [Johnston et al., 1995; Rawlinson et al., 2003].

One limitation of this study is that outpatients with wheezing were excluded, but it has been previously reported that rates of respiratory virus infection are higher in hospitalized children than in children not requiring hospitalization [Rakes et al., 1999; Thumerelle et al., 2003; Heymann et al., 2005]. Other limitations of this study are not performing PCR for enteroviruses which were frequently detected in young children with asthma in previous studies [Rawlinson et al., 2003; Jartti et al., 2004], and diagnostic tests for Chlamydia pneumoniae and Mycoplasma pneumoniae, both of which are known to be associated with the exacerbation of wheezing. However, it has previously been reported that M. pneumoniae infections are not often detected in patients with exacerbated asthma (1–5% of patients are infected) and C. pneumoniae infections are more commonly associated with prolonged asthmatic symptoms than with acute exacerbation [Cunningham et al., 1998; Freymuth et al., 1999; Thumerelle et al., 2003]. There are some other limitations including low specimen numbers which could account for no detection of hCoV-HKU1, the absence of specimens in some months, and mixed use of antigen detection methods and PCR testing for common respiratory viruses, which could have a different sensitivity or specificity.

In conclusion, rhinovirus, hRSV, and hBoV were the most frequently identified respiratory viruses in children hospitalized with acute wheezing. However, further studies including healthy control subjects and in other geographic areas are needed to clarify the possible association of hBoV with asthma exacerbation in children.

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In conclusion, rhinovirus, hRSV, and hBoV were the most frequently identified respiratory viruses in children hospitalized with acute wheezing. However, further studies including healthy control subjects and in other geographic areas are needed to clarify the possible association of hBoV with asthma exacerbation in children.

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