<table>
<thead>
<tr>
<th>Instructions for use</th>
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<tr>
<td>Detecetion of Human Bocaviruses 1 to 4 from Nasopharyngeal Swab Samples Collected from Patients with Respiratory Tract Infections</td>
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</tbody>
</table>

**Title**

Detection of Human Bocaviruses 1 to 4 from Nasopharyngeal Swab Samples Collected from Patients with Respiratory Tract Infections

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Detection of human bocavirus 1-4 from nasopharyngeal swab samples collected from patients with respiratory tract infections

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Human bocavirus (HBoV) 1, 2, 3 and 4 were detected in 132 (15.5%), 5 (0.6%), 3 (0.4%) and 5 (0.6%) of 850 nasopharyngeal swab samples collected from children with respiratory tract infections, respectively. Out of the 145 HBoV1-4-positive samples, 62 (42.8%) were co-detected with other respiratory viruses. (47 words)
Human bocavirus (HBoV, lately denoted HBoV1), belonging to the family *Parvoviridae*, subfamily *Parvovirinae* and genus *Bocavirus*, was first identified by molecular screening of pooled human respiratory tract samples in 2005 (4). HBoV1 has been detected worldwide in 1.6% to 19% of patients with respiratory tract infections (4, 6, 8, 10, 23, 26, 27). Recently, serological and quantitative PCR analyses have provided compelling evidence for HBoV1 being an etiologic agent for respiratory tract infections (RTIs) (3, 8, 13, 21, 24, 30, 34). In 2009–2010, three additional species of human bocaviruses, HBoV2, HBoV3 and HBoV4, were discovered from fecal samples (5, 19, 20). In contrast to HBoV1, HBoV2-4 were predominantly detected in human stool samples and were therefore thought to be involved in enteropathogenesis (5, 18, 20). Only HBoV2 of HBoV2-4 has been detected in nasopharyngeal samples from children so far (16, 31). In this study, we examined the presence of HBoV2-4 as well as HBoV1 in nasopharyngeal swab samples (NPSs) from children with RTIs.

A total of 850 NPSs were collected from 757 children (436 males and 321
females) aged 0 - 136 months (average age, 17.9 months) with RTIs at four
hospitals (KKR Sapporo Medical Center, Hokkaido Social Insurance Hospital,
Sapporo Kosei General Hospital and Nemuro City Hospital) in Hokkaido,
Japan during the period from June 2005 to August 2011 after obtaining in
formed consent from the children’s parents. DNA was extracted from 200 μl of
NPSs by using the method according to Chomczynski’s protocol (11).
Elution volume of the extractions was 90 μl. Pan-bocavirus nested PCR
targeting the VP1 region was used for detection of HBoVs as described by
Kapoor et al. (19). Both sense and antisense strands of the second PCR
products were sequenced directly by using a BigDye terminator cycle
sequencing ready reaction kit (Perkin-Elmer Applied Biosystems, Tokyo,
Japan) with an ABI Prism 310 genetic analyzer (Perkin-Elmer Applied
Biosystems). The nucleotide sequences of the second PCR products were
assembled by using CLUSTAL W software. Phylogenetic trees were
generated by the neighbor-joining method with the MEGA program (22).
Using DNA extracted from fecal specimens collected in Vietnam as templates
of the pan-bocavirus nested PCR, the first-round PCR products of HBoV1
and HBoV2 were cloned into the vector pT7Blue (Novagene, Inc., Madison,
WI, U.S.A.). The sequences were deposited at GenBank (accession numbers JQ734543 and JQ734544). The corresponding regions of HBoV3 (EU918736) and HBoV4 (FJ973561) were synthesized and cloned into pUC57 by Genscript (Piscataway, NJ, U.S.A.). These four vectors were used as positive controls for the nested PCR. DNA solutions containing 5, 25, 125, 625 and 3125 copies of these vectors per µl were prepared to determine the sensitivities of the nested PCR. All of the specimens were also assayed for the presence of thirteen other respiratory viruses: human respiratory syncytial virus (hRSV), human metapneumovirus (hMPV), human rhinovirus (HRV), parainfluenza viruses (PIV 1-3), influenza A and B viruses (FluA and FluB), human enterovirus (HEV), human coronaviruses (HCoV), adenoviruses (AdV), KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV). The PCR and RT-PCR protocols used for detecting these thirteen viruses were the same as those previously described (1, 2, 12, 15, 17, 25, 28, 29, 32).

Of the 850 NPSs tested, 145 (17.1%) had confirmed positive results by sequencing. By determining the phylogenetic relationships among various
HBoV strains, the final prevalences of HBoV1, 2, 3 and 4 in the NPSs were 132/850 (15.5%), 5/850 (0.6%), 3/850 (0.4%) and 5/850 (0.6%), respectively. The five HBoV2 strains belong to the HBoV2A clade described by Kapoor et al. (19). Two of the HBoV1-positive samples were collected from two children, from whom HBoV1 had been detected two months before. The other HBoV1-4-positive samples were collected from different children. The amplicon sizes were 525 bp (nucleotides 3353 to 3877 in accession no. NC_007455.1) for HBoV1 and HBoV2 and 528 bp (nucleotides 3320 to 3847 in accession no. NC_012564.1) for HBoV3 and HBoV4. The sequences were deposited at GenBank (accession numbers JQ346532 to JQ346676). According to calculations by amplification of serial limiting dilutions of cloned HBoV1-4 DNA, the copy numbers of 67 (50.8%) of the 132 HBoV1-positive NPS samples were estimated to be more than 5.5 x 10^4 copies / ml, and those of the remaining 65 HBoV1-positive NPS samples were estimated to be between 1.2 x 10^4 and 5.5 x 10^4 copies / ml. The copy numbers of the HBoV2, 3 and 4-positive NPS samples were estimated to be between 2.3 x 10^3 and 1.2 x 10^4 copies / ml. At least 10-fold differences in sensitivities of the pan-bocavirus PCR for HBoV1-4 have already been
HBoV1 was detected solely in 76 (57.6%) of the 132 HBoV1-positive samples, and one or more other viruses were co-detected in 56 (42.6%) of the 132 HBoV1-positive samples. hMPV (20 samples), hRSV (15 samples) and HRV (7 samples) were the most frequently detected viruses. HBoV2 was detected solely in 3 (60.0%) of the 5 HBoV2-positive samples, and another virus was co-detected in 2 (40.0%) of the 5 HBoV2-positive samples. HBoV3 was co-detected in 3 (100.0%) of all of the 3 hRSV-positive samples. HBoV4 was detected solely in 4 (80.0%) of the 5 HBoV4-positive samples, and another virus was co-detected in 1 (20.0%) of the 5 HBoV4-positive samples (Table 1).

The ages of patients with HBoV1-positive samples ranged from 1 month to 6 years and 9 months (average age, 18.2 months), and the ages of patients with HBoV2-4-positive samples ranged from 1 month to 3 years and 6 months (average age, 17.1 months). HBoV1 genomes were detected in every month with peaks in May (18 cases) and June (17 cases). All of the 13 HBoV2-4-positive samples were collected in fall or winter except for one in April (Supplemental table). Fifty (37.9%) of the 132 HBoV1-positive
patients were admitted to hospital for 3 to 11 days, but none of the 13 HBoV2-4-positive patients were admitted to hospital. The clinical diagnoses of the HBoV-positive patients are summarized in Table 2. Thirty-four HBoV single-positive patients (28 with HBoV1, 2 with HBoV2 and 4 with HBoV4) were diagnosed as having bronchitis. Twenty HBoV1 single-positive patients were diagnosed as having wheezy bronchitis. Twenty-one HBoV single-positive patients (20 of HBoV1 and 1 of HBoV2) were diagnosed as having pneumonia. Although HBoV2-4 were thought to be involved in enteropathogenesis, a gastrointestinal symptom (diarrhea) was observed in only one of the four HBoV4-positive patients and was not observed in any of the HBoV2-3-positive patients. It is known that low-load HBoV1 DNA is long-lasting in the mucosa (7), and quantification of HBoV DNA by real-time PCR is therefore necessary to estimate the time of infection to the present symptoms. In this study, detection of HBoV DNA was done by semi-quantitative nested PCR instead of real-time PCR. Science “semi-quantification” according to the first and second PCR-round sensitivities is limited, the seasonal distributions of HBoVs and the associations of clinical diagnoses with the presence of HBoVs described
above might not be accurate.

In our study, we identified HBoV2, HBoV3 and HBoV4, as well as HBoV1, in NPSs collected from patients with RTIs. The detection rate of HBoV1 (15.5%) in NPSs was within the range of previously reported rates (1.6-19%) (4, 6, 8, 10, 23, 26, 27). In contrast, the detection rates of HBoV2 (0.6%), HBoV3 (0.4%) and HBoV4 (0.6%) in NPSs were much lower than that of HBoV1 (15.5%). The hospital admission rates of HBoV2-4-positive patients (0%) were lower than that of HBoV1-positive patients (37.9%). These facts suggest that the roles of HBoV2-4 in RTIs might be limited in comparison with the role of HBoV1. Differences in the duration of persistence of HBoVs in the nasopharynx might also have caused the lower prevalences of HBoV2-4 than that of HBoV1. Previously reported detection rates of HBoV2 in NPSs were 4.3% in China (31) and 2.3% in South Korea (16), which were higher than the rate in our study. HBoV2 was not detected among 6,524 respiratory samples in the United Kingdom and Thailand (9). The majority of respiratory samples in that study (6,138 of 6,524) were not obtained from individual persons but from pooled stock, which might have
reduced the sensitivity of their PCR assay. These different results can be explained partially by the difference in PCR primers or by the regional difference. On the other hand, HBoV2 (3 samples) and HBoV4 (4 samples) were detected without co-detection of other respiratory viruses in a few NPSs, suggesting that HBoV2 and 4 might play some roles in RTIs in children. To clarify the clinical impact of HBoV2-4 in RTIs, quantitative PCR study in various age groups and various clinical groups including healthy controls is needed. Adenoviruses 40 and 41 cause gastroenteritis, but respiratory symptoms are not frequent (21% of cases) (33). The unique physicochemical properties of adenovirus 41 partially explain its enteric tropism (14). Structural studies of HBoV1-4 might be also helpful to clarify the roles of HBoVs in RTI and gastroenteritis.

ACKNOWLEDGEMENTS

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All of the necessary ethics approval for this study was obtained from the Institutional Review Board of Hokkaido University Hospital for Clinical Research. We thank Kunio Ozutusmi of Nemuro City Hospital for providing nasopharyngeal swab samples. We thank Stewart Chisholm for proofreading the manuscript.

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301 Importance of enteric adenoviruses 40 and 41 in acute gastroenteritis


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305 and co-infection with other respiratory viruses in children with acute

FIGURE LEGEND:

Figure 1. Phylogenetic analysis of partial VP1 genes of human bocaviruses amplified by nested PCR. Trees were constructed by the neighbor-joining method with the MEGA program; bootstrap values $\geq 70\%$ are shown.
<table>
<thead>
<tr>
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<th>No. (%) of detection</th>
<th>Single detection</th>
<th>Codetection with one or more viruses</th>
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<tr>
<td></td>
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<tr>
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<td>83</td>
<td>17</td>
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*Among the 132 HBoV1-positive samples, 10 were detected simultaneously with two or more viruses (3 with hMPV and KIPyV, 3 with hRSV and HRV, 1 with WUPyV and HRV, 1 with hMPV and PIV3, 1 with KIPyV and WUPyV, and 1 with hMPV, KIPyV and WUPyV).
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