JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2007, p. 3218–3223 Vol. 45, No. 10
0095-1137/07/$08.00+0 doi:10.1128/JCM.02140-06

Seroepidemiology of Human Bocavirus in Hokkaido Prefecture, Japan▽

Rika Endo,[1] Nobuhisa Ishiguro,[1]* Hideaki Kikuta,[1] Shinobu Teramoto,[1] Reza Shirkoohi,[1] Xiaoming Ma,[1] Takashi Ebihara,[2] Hiroaki Ishiko,[3] and Tadashi Ariga[1]

Department of Pediatrics,[1] and Department of Microbiology and Immunology,[2] Hokkaido University Graduate School of Medicine, Sapporo, and Advanced Medicine Research Department, Mitsubishi Chemical Medience Corporation, Tokyo,[3] Japan

Received 17 October 2006/Returned for modification 27 December 2006/Accepted 1 August 2007

A new human virus, provisionally named human bocavirus (HBoV), was discovered by Swedish researchers in 2005. A new immunofluorescence assay using *Trichoplusia ni* insect cells infected with a recombinant baculovirus expressing the VP1 protein of HBoV was developed, and the levels of immunoglobulin G antibody to the VP1 protein of HBoV in serum samples were measured. The overall seroprevalence rate of antibodies against the VP1 protein of HBoV in a Japanese population aged from 0 months to 41 years was 71.1% (145 of 204). The seropositive rate was lowest in the age group of 6 to 8 months and gradually increased with age. All of the children had been exposed to HBoV by the age of 6 years. A rise in titers of antibody against the VP1 protein of HBoV during the convalescent phase was observed for four patients with lower respiratory tract infections, and HBoV DNA was detected in nasopharyngeal swab and serum samples from all four patients. These results suggest that HBoV is a ubiquitous virus acquired early in life and that HBoV might play a role in the course of lower respiratory tract infections.

In September 2005, a new human virus provisionally named human bocavirus (HBoV), belonging to the *Parvoviridae* family, *Parvovirinae* subfamily, and *Bocavirus* genus, was cloned by molecular screening of pooled human respiratory tract samples in Sweden (3). Recently, the same virus has been identified in patients with respiratory tract infections in Australia, Japan, Canada, the United States, France, Germany, Korea, Thailand, the United Kingdom, South Africa, Switzerland, China, Finland, Italy, The Netherlands, and Iran (2, 5–7, 11, 13, 20, 21, 26–30, 33, 35, 40, 42–44, 47). HBoV seems to be a new member of the community-acquired respiratory viruses such as respiratory syncytial virus, adenovirus, influenza virus, parainfluenza virus, and rhinovirus, which cause common respiratory tract infections in the community (3, 5). The purpose of this study was to clarify the seroprevalence of HBoV in Japan. HBoV encodes two nonstructural proteins (NS1 and NP-1) and two capsid proteins (VP1 and VP2) (3). Capsid (VP1 and VP2) proteins of human parvovirus B19 (B19), which belongs to the *Parvoviridae* family, *Parvovirinae* subfamily, and *Erythrovirus* genus, are known to be immunodominant antigens (9, 15, 39), and they have been expressed in numerous prokaryotic and eukaryotic expression systems in order to use them as diagnostic reagents for B19 infection (8, 10, 17, 34). The VP1 proteins of HBoV are therefore likely to evoke an antibody response. In the present study, a new immunofluorescence assay (IFA) using *Trichoplusia ni* (Tn5) insect cells infected with a recombinant baculovirus expressing the VP1 protein of HBoV was developed, and levels of immunoglobulin G (IgG) antibody to the VP1 protein of HBoV in sera were measured.

MATERIALS AND METHODS

Serum samples. A total of 204 serum samples were obtained from patients (aged 0 months to 41 years) who were outpatients or inpatients at six hospitals (see Acknowledgments) in Hokkaido Prefecture, Japan, from 1998 to 2005. All samples were collected after obtaining informed consent from the children's parents or the adults.

Nasopharyngeal swab and serum samples from patients with lower respiratory tract infections. From January 2006 to January 2007, a total of 161 nasopharyngeal swab samples were collected from children (aged 2 months to 6 years and 1 month) with lower respiratory tract infections (LRTI) at four hospitals (see Acknowledgments) in Hokkaido Prefecture, Japan. Serum samples from patients in the acute and/or convalescent phase of LRTI were also obtained. All samples were collected after obtaining informed consent from the children's parents.

Cells. Sf9 insect cells were cultured in SF900 II medium (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum. *Trichoplusia ni* (Tn5) insect cells were cultured in EX-CELL 405 medium (JRH Biosciences, Lenexa, KS).

Expression of HBoV and B19 VP1 proteins in a baculovirus-insect cell system. A baculovirus expression kit (Bac-to-Bac system) was used to prepare VP1 proteins expressed in a baculovirus-insect cell system in accordance with the instructions of the manufacturer (Invitrogen, Carlsbad, CA). The genomic DNA of VP1 protein from HBoV strain JPBS05-52 (GenBank accession no., EF035488) was amplified by PCR with primers HBoV VP1 start (5′-ATC GTC TCG CAT GAG TAA AGA AAG TGG CAA-3′) and HBoV VP1 end (5′-GCC TCG AGT TAC AAT GGG TGC ACA CGG C-3′). The genomic DNA of B19 VP1 protein (a kind gift from Y. Munakata and K. Ishii [41] and T. Ito) was amplified by PCR with primers B19 VP1 start (5′-ATC GTC TCG CAT GAG TAA AGA AAG TGG CAA-3′) and B19 VP1 end (5′-GCC TCG AGT TAC AAT GGG TGC ACA CGG C-3′). (The restriction sites in the primers used for cloning are underlined.) The PCR products were digested with BsmBI and XhoI and cloned into the NcoI and XhoI sites of the baculovirus transfer vector pFASTBAC HTa. The resulting plasmids (pFASTBAC HTa-HBoV VP1 and pFASTBAC HTa-B19 VP1) and pFASTBAC HTa were transformed into *Escherichia coli* DH10 Bac, a host for the baculovirus expression vector (Invitrogen, Carlsbad, CA), for transposition into the bacmid. The recombinant bacmids were isolated and transfected into Sf9 cells with a liposome-mediated transfection kit (Invitrogen, Carlsbad, CA). Recombinant baculoviruses (His-HBoV VP1-Bac, His-B19 VP1-Bac, and His-Bac) were used to infect Tn5 cells for 72 h.

IFA using the baculovirus-insect cell system and absorption test. Tn5 cells infected with the recombinant baculovirus (His-HBoV VP1-Bac, His-B19 VP1-Bac, and His-Bac) were spotted onto slides. The cell smears were air dried, fixed in acetone for 10 min, and incubated for 30 min at 37°C with serum samples diluted serially, beginning at 1:40. After being incubated, the slides were washed three times in phosphate-buffered saline (PBS) for 10 min each time. They were then incubated for 30 min at 37°C with fluorescein isothiocyanate-conjugated

* Corresponding author. Mailing address: Department of Pediatrics, Hokkaido University Graduate School of Medicine, N-15, W-7, Kita-ku, Sapporo 060-8638, Japan. Phone: 81-11-706-5954. Fax: 81-11-706-7898. E-mail: nishigur@med.hokudai.ac.jp.

▽ Published ahead of print on 15 August 2007.

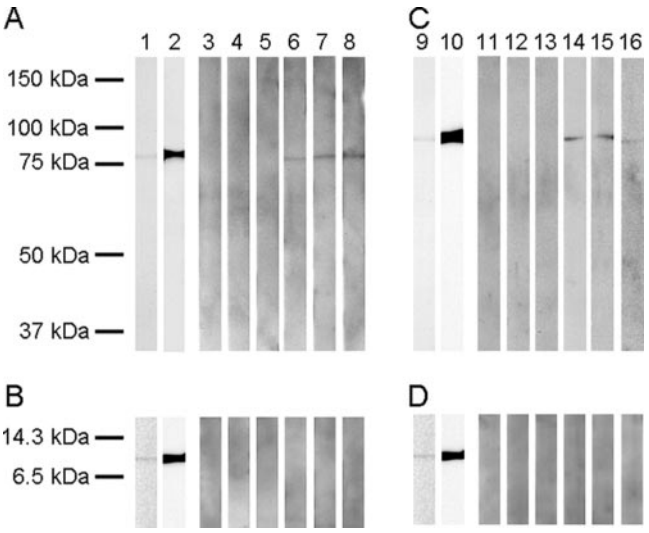

FIG. 1. Western blot analysis of (A) histidine-tagged VP1 protein of HBoV, (C) histidine-tagged VP1 protein of B19, and (B, D) histidine-tagged control protein. The gels were stained with Coomassie brilliant blue (lanes 1 and 9). Anti-six-histidine monoclonal antibody (lanes 2 and 10), HBoV IFA-negative sera (lanes 3, 4 and 5), HBoV IFA-positive sera (>1:1,280) (lanes 6, 7 and 8), B19 IFA-negative sera (lanes 11, 12 and 13), and B19 IFA-positive sera (>1:1,280) (lane 14, 15 and 16) were incubated on nitrocellulose membranes.

rabbit anti-human IgG (Dako, Glostrup, Denmark) at a serum dilution of 1:40. After being incubated, they were washed three times in PBS for 10 min each time, air dried, and mounted with PBS-glycerin (1:1). The stained preparations were then examined at ×400 magnification under a fluorescence microscope (model BX60; Olympus, Tokyo, Japan). Serum samples that reacted with the VP1 proteins of HBoV and B19 at dilutions of more than 1:40 were considered positive for antibodies to HBoV and B19 (HBoV and B19 IFAs). A T25 flask of uninfected Tn5 cells or Tn5 cells infected with recombinant baculoviruses (His-HBoV VP1-Bac, His-B19 VP1-Bac, and His-Bac) was resuspended in 500 μl of PBS. The suspension was sonicated for 10 s on ice with a sonicator (Handy Sonic model UR-20P; Tomy Seiko Co., Tokyo, Japan). For the absorption test, 10 μl of serum was incubated with 25 μl of the sonicated lysate for 6 h on ice and serially diluted with PBS from 1:40 to 1:2,560 and then used for HBoV and B19 IFAs.

SDS-polyacrylamide gel electrophoresis and Western blot analysis. Histidine-tagged proteins were purified from insect cell extracts with Ni-NTA agarose (QIAGEN, Santa Clarita, CA) by following the procedures supplied by the manufacturer. Briefly, the cells were lysed with lysis buffer (50 mM potassium phosphate–300 mM NaCl buffer [pH 8.0]) containing 1% NP-40 and 20 mM imidazole. After centrifugation, the supernatant was applied to a column filled with Ni-NTA agarose, and the column was then washed with the same lysis buffer. The protein was then eluted with elution buffer (50 mM potassium phosphate–300 mM NaCl buffer [pH 8.0]) containing 1% NP-40 and 250 mM imidazole. Twenty nanograms of purified protein was subjected to sodium dodecyl sulfate (SDS)–8% polyacrylamide gel electrophoresis under reducing conditions, and the recombinant proteins were detected by Coomassie brilliant blue staining. The separated proteins were electrotransferred onto a nitrocellulose membrane. After being blocked with 1% bovine serum albumin, serum (at a dilution of 1:400) or anti-six-histidine monoclonal antibody (at a dilution of 1:1,000; BioDynamics Laboratory Inc., Tokyo, Japan) was allowed to bind to the filter and then to react with horseradish peroxidase-conjugated goat anti-IgG (at a dilution of 1:1,000) or goat anti-mouse Ig polyclonal antibodies (at a dilution of 1:1,000; Biosource International, Camarillo, CA), and the proteins were detected by a chemiluminescence assay method (ECL Plus Western blotting detection reagent; Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Detection of the HBoV genome in nasopharyngeal swab samples by PCR and sequencing. DNA and RNA were extracted from nasopharyngeal swab samples by using Chomczynski's protocol (12). The PCR primers and conditions used for detection of HBoV have been published previously (3). Both sense and antisense strands of the 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).

Detection of other viruses in nasopharyngeal swab samples. Reverse transcription-PCR was carried out for the detection of human enteroviruses, human rhinoviruses, human respiratory syncytial virus, human parainfluenza virus types 1, 2, 3, and 4, influenza viruses A and B, human coronavirus, and human metapneumovirus, and PCR was carried out for the detection of adenovirus (1, 19, 23, 32, 37, 38, 45).

Detection of HBoV in serum samples by real-time PCR. Nucleic acids were extracted from 200 μl of each serum sample, using a QIAamp blood mini kit (QIAGEN, Santa Clarita, CA), and DNA was eluted in 50 μl of reaction mixture. The primers and probe for real-time PCR were selected from the NP-1 gene of

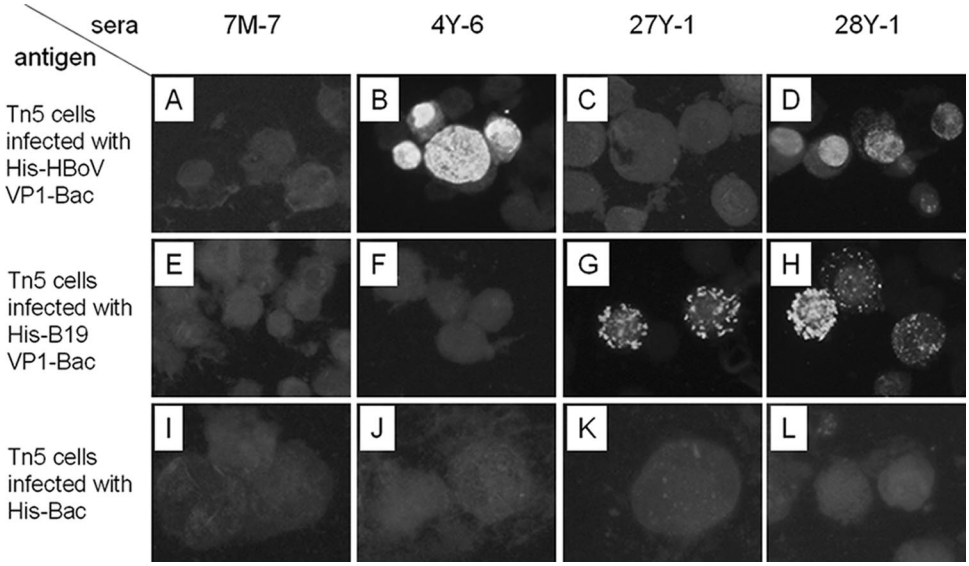

FIG. 2. Detection of VP1 proteins of HBoV and B19 by IFA. Tn5 cells infected with His-HBoV VP1-Bac (A, B, C, and D), His-B19 VP1-Bac (E, F, G and H), or His-Bac viruses (I, J, K, and L) were stained, using serum samples 7M-7 (A, E, and I), 4Y-6 (B, F, and J), 27Y-1 (C, G, and K), and 28Y-1 (D, H, and L) at dilutions of 1:40.

TABLE 1. Results of HBoV and B19 IFAs of individual serum samples

Sample	No. of samples with indicated B19 IFA result		Total no. of samples
	B19-negative	B19-positive	
HBoV-negative	45	14	59
HBoV-positive	95	50	145
Total	140	64	204

HBoV. The 50-μl amplification reaction mixture contained 5 μl of sample DNA, 25 μl of TaqMan gene expression master mix (Perkin-Elmer Applied Biosystems, Tokyo, Japan), 900 nmol/liter of each primer (forward, 5′-GAG GAA GAG ACA CTG GCA GAC-3′; reverse, 5′-AAG ACG ATA GGT GGC TGA TTG G-3′), and 250 nmol/liter of a probe (FAM-CAT CAC AGG AGC AGG AGC CGC AGC-TAMRA). Amplification was performed using an ABI Prism 7000 sequence detection system (Perkin-Elmer Applied Biosystems, Tokyo, Japan) with the following instrument settings: 50°C for 2 min, 95°C for 10 min, and then 40 cycles at 95°C for 15 s and 60°C for 1 min. The plasmid pHBoV_NS1real, containing the PCR product of the qualitative PCR in the vector pT7Blue (Novagene, Inc., Madison, WI), was used as a positive control and for the standard curve. The results were analyzed using ABI Prism 7000 SDS software. Viral loads were calculated from the threshold cycle values of individual samples with respect to the standard curve. The minimum viral load that would allow reproducible quantification was 10 copies per reaction, corresponding to 500 copies/ml for the serum samples.

RESULTS

Expression of HBoV and B19 VP1 proteins. The predicted molecular sizes of the histidine-tagged VP1 fusion proteins of HBoV and B19 were 78.1 and 89.0 kDa, respectively, which were consistent with the molecular sizes of these proteins as determined by SDS-polyacrylamide gel electrophoresis (78 and 89 kDa, respectively) (Fig. 1A, lane 1, and C, lane 9). These fusion proteins reacted with anti-six-histidine monoclonal antibody by Western blotting (Fig. 1A, lane 2, and C, lane 10).

Detection of antibodies by Western blot analysis. Human sera reacted with the histidine-tagged VP1 fusion proteins of HBoV and B19 by Western blotting. Representative results are shown in Fig. 1. Antibodies against the histidine-tagged VP1 fusion protein of HBoV were detected in sera from three patients whose HBoV IFA titers were >1:1,280 (Fig. 1A, lanes 6, 7, and 8) but not in sera from three patients whose HBoV IFA titers were <1:40 (Fig. 1A, lanes 3, 4, and 5). The sera did not react with the histidine-tagged control protein (Fig. 1B, lanes 3 to 8). Six (66.6%) of nine serum samples with HBoV IFA titers of >1:1,280 reacted with the histidine-tagged VP1 fusion protein of HBoV by Western blotting (data not shown). Only one (11.1%) of nine serum samples with HBoV IFA titers of 1:40 to ~1:640 reacted with the histidine-tagged VP1 fusion protein of HBoV by Western blotting (data not shown). Antibodies against the histidine-tagged VP1 fusion protein of HBoV were not detected in 12 serum samples with HBoV IFA titers of <1:40 (data not shown).

Antibodies against the histidine-tagged VP1 fusion protein of B19 were also detected in sera from three patients whose B19 IFA titers were >1:1,280 (Fig. 2C, lanes 14, 15, and 16) but not in sera from three patients whose B19 IFA titers were <1:40 (Fig. 1C, lanes 11, 12, and 13). The sera did not react with the histidine-tagged control protein (Fig. 1D, lanes 11 to 16). Five (55.5%) of nine serum samples with HBoV and B19 IFA titers of >1:1,280 reacted with the histidine-tagged VP1 fusion protein of B19 by Western blotting (data not shown). Only one (11.1%) of nine serum samples with B19 IFA titers of 1:40 to ~1:640 reacted with the histidine-tagged VP1 fusion protein of B19 by Western blotting (data not shown). Antibodies against the histidine-tagged VP1 fusion protein of B19 were not detected in 12 serum samples with B19 IFA titers of <1:40 (data not shown).

Detection of antibodies against HBoV and B19 by IFAs. Specific antibodies against HBoV and B19 in 204 human serum samples were measured by IFAs. Representative results are shown in Fig. 2. More than 80% of the cells expressed the VP1 proteins of HBoV and B19 (data not shown). Specific antibodies against both HBoV and B19 were detected in serum sample 28Y-1 (Fig. 2D and H). A specific antibody against HBoV was detected, but no antibody against B19 was detected in serum sample 4Y-6 (Fig. 2B and F). A specific antibody against B19 was detected, but no antibody against HBoV was detected in serum sample 27Y-1 (Fig. 2C and G). Human sera did not react with Tn5 cells infected with recombinant His-Bac (Fig. 2I, J, K, and L). The results of HBoV and B19 IFAs in individual serum samples are summarized in Table 1. The cutoff for a positive test was a serum dilution of 1:40, because nonspecific signals were observed at a serum dilution of 1:20 for some sera (data not shown). Fifty serum samples were positive for both HBoV and B19 IFAs, 95 serum samples were

TABLE 2. Age distribution of patients with antibodies against HboV

Age group	No. of samples with antibody against HBoV at indicated dilution:							No. of positive samples/total no. of samples	% Positive
	<1:40	1:40	1:80	1:160	1:320	1:640	>1:1,280		
<3 mo	2	4	6	6	2	1		19/21	90.5
3–5 mo	6	2	1		1			4/10	40.0
6–8 mo	17					1		1/18	5.6
9–11 mo	12	1	1	1	2		1	6/18	33.3
1 yr	15	1	4	5			1	11/26	42.3
2–3 yr	3	2	1	4	4	2	2	15/18	83.3
4–5 yr	2	1		8	4	2	2	17/19	89.5
6–9 yr		5	2	4	2	6	1	20/20	100
10–19 yr		4		9	5	1	1	20/20	100
>20 yr	2	3	8	8	8	4	1	32/34	94.1
Total	59	23	23	45	28	17	9	145/204	71.1

TABLE 3. Age distribution of patients with antibodies against B19

Age group	No. of samples with antibody against B19 at indicated dilution:							No. of positive samples/total no. of samples	% Positive
	<1:40	1:40	1:80	1:160	1:320	1:640	>1:1,280		
<3 mo	14					1	6	7/21	33.3
3–5 mo	3	3	1	1	2			7/10	70.0
6–8 mo	13	2	2	1				5/18	27.8
9–11 mo	17	1						1/18	5.6
1 yr	26							0/26	0.0
2–3 yr	15			1	1	1		3/18	16.7
4–5 yr	16					1	2	3/19	15.8
6–9 yr	10						10	10/20	50.0
10–19 yr	7				1	4	8	13/20	65.0
>20 yr	19	1	1		1	2	10	15/34	44.1
Total	140	7	4	3	5	9	36	64/204	31.4

positive for HBoV IFA and negative for B19 IFA, 14 serum samples were positive for B19 IFA and negative for HBoV IFA, and 45 serum samples were negative for both HBoV and B19 IFAs.

Seroprevalence rate of anti-HBoV antibodies. The overall seroprevalence rate of anti-HBoV antibodies in the Japanese population in Hokkaido Prefecture for patients aged from 0 months to 41 years was 71.1% (145 of 204). The age-related seroprevalence rates of anti-HBoV antibodies were 90.5% (19 of 21) in the age group under 3 months, 40.0% (4 of 10) in the 3- to 5-month-old group, 5.6% (1 of 18) in the 6- to 8-month-old group, 33.3% (6 of 18) in the 9- to 11-month-old group, 42.3% (11 of 26) in the 1-year-old group, 83.3% (15 of 18) in the 2- to 3-year-old group, 89.5% (17 of 19) in the 4- to 5-year-old group, 100% (20 of 20) in both the 6- to 9- and 10- to 19-year-old groups, and 94.1% (32 of 34) in the over 20-year-old group. Antibody titers of HBoV varied from 1:40 to >1:1,280 (Table 2).

Seroprevalence rate of anti-B19 antibodies. The overall seroprevalence rate of anti-B19 antibodies in the Japanese population in Hokkaido Prefecture for patients aged from 0 months to 41 years was 31.4% (64 of 204). The age-related seroprevalence rates for anti-B19 antibodies were 33.3% (7 of 21) in the age group under 3 months, 70.0% (7 of 10) in the 3- to 5-month-old group, 27.8% (5 of 18) in the 6- to 8-month-old group, 5.6% (1 of 18) in the 9- to 11-month-old group, 0% (0 of 26) in the 1-year-old group, 16.7% (3 of 18) in the 2- to 3-year-old group, 15.8% (3 of 19) in the 4- to 5-year-old group, 50.0% (10 of 20) in the 6- to 9-year-old group, 65.0% (13 of 20) in the 10- to 19-year-old group, and 44.1% (15 of 34) in the over 20-year-old group. Antibody titers of B19 varied from 1:40 to >1:1,280 (Table 3).

Absorption test. To test the specificity of antibodies against HBoV and B19, the specific antibody titers of the serum samples were measured following absorption of sera (positive for both HBoV and B19 as determined by IFA) by cell sonicates of recombinant baculovirus (His-HBoV VP1-Bac, His-B19 VP1-Bac, and His-Bac)-infected or uninfected Tn5 cells. For the absorption test, four serum samples were selected on the basis of the results of HBoV and B19 IFAs. For the samples selected, (i) the titers against both HBoV and B19 were more than 1:320 and (ii) the differences in HBoV and B19 IFA titers in the serum sample were within fourfold. The results are shown in Table 4. The antibody against HBoV was absorbed by the cell sonicate of His-HBoV VP1-Bac-infected Tn5 cells but not by the cell sonicate of His-B19 VP1-Bac-infected Tn5 cells. The antibody against B19 was absorbed by the cell sonicate of His-B19 VP1-Bac-infected Tn5 cells but not by the cell sonicate of His-HBoV VP1-Bac-infected Tn5 cells. Therefore, specific antibodies to the VP1 proteins of HBoV and B19 were detected by the HBoV and B19 IFAs, respectively.

Detection of anti-HBoV antibodies and HBoV DNA in acute and convalescent-phase sera from patients with HBoV infection. Eight (5.0%) of the 161 nasopharyngeal swab samples from children with LRTI were positive for HBoV DNA. Direct sequencing of the PCR products of these samples showed that their sequences were identical to the sequences of published HBoV (data not shown). Both acute- and convalescent-phase sera were obtained from four patients whose nasopharyngeal swab samples were positive for HBoV as determined by PCR. The titers of specific antibodies against HBoV and B19 were measured in acute- and convalescent-phase sera of those four patients. The titers of the antibody against HBoV increased from <1:40 (acute phase) to 1:640 to ~1:2,560 (convalescent phase) in four patients (Table 5), but the antibody against B19 was not detected in either the acute- or convalescent-phase sera of four

TABLE 4. Titers of antibodies against HBoV and B19 after absorption by Tn5 cells

Serum sample and antibody	Antibody titers after absorption by uninfected Tn5 cells	Antibody titers after absorption by Tn5 cells infected with indicated strain:		
		His-Bac	His-HBoV VP1-Bac	His-B19 VP1-Bac
3Y-6				
Anti-HBoV	1:320	1:320	<1:40	1:320
Anti-B19	1:640	1:640	1:640	<1:40
10Y-1				
Anti-HBoV	1:1,280	1:1,280	<1:40	1:1,280
Anti-B19	1:1,280	1:1,280	1:1,280	<1:40
26Y-2				
Anti-HBoV	1:640	1:640	<1:40	1:640
Anti-B19	1:640	1:640	1:640	<1:40
31Y-2				
Anti-HBoV	1:2,560	1:2,560	<1:40	1:2,560
Anti-B19	1:640	1:640	1:640	<1:40

TABLE 5. Titers of antibodies against HBoV and HBoV load in sera from four patients with acute HBoV infections

Patient	Age of patient	Sex of patient	No. of days after onset for acute-phase serum	Indicated result for acute-phase serum:		No. of days after onset for convalescent-phase serum	Indicated result for convalescent-phase serum:	
				Titers of anti-HBoV IgG antibody	HBoV copy number (copies/ml)		Titers of anti-HBoV IgG antibody	HBoV copy number (copies/ml)
1	11 mo	Male	1	<1:40	7.32×10^4	27	1:1,280	$<5 \times 10^2$
2	1 yr 1 mo	Male	5	<1:40	3.05×10^5	119	1:640	$<5 \times 10^2$
3	9 mo	Male	3	<1:40	2.17×10^5	28	1:1,280	1.86×10^4
4	1 yr 4 mo	Male	5	<1:40	1.48×10^6	32	1:2,560	$<5 \times 10^2$

patients (data not shown). HBoV DNA was detected in four (100%) of the four acute-phase serum samples and one (25%) of the four convalescent-phase samples. Viral loads in sera ranged from $<5 \times 10^2$ to $\sim 1.86 \times 10^4$/ml (convalescent phase) to 7.32×10^4 to $\sim 1.48 \times 10^6$/ml (acute phase) (Table 5).

Detection of other viruses in nasopharyngeal swab samples. Four HBoV-positive nasopharyngeal swab samples from four patients from whom acute- and convalescent-phase sera were obtained were tested for twelve other viruses described in Materials and Methods. Human rhinovirus was detected in two nasopharyngeal swab samples (Table 5, patients 1 and 3), and human rhinovirus and adenovirus were detected in one sample (Table 5, patients 4).

DISCUSSION

HBoV DNA has recently been detected frequently in children with upper and lower respiratory tract infections (2, 3, 5–7, 11, 13, 20, 21, 26–30, 33, 35, 40, 42–44, 47). A method for detecting the antibody against HBoV in human serum has not been available until now. In the present study, an IFA using Tn5 cells infected with a recombinant baculovirus expressing the VP1 protein of HBoV was developed, and levels of IgG antibody to the VP1 protein of HBoV in sera were successfully measured for the first time (Fig. 2 and Table 2). The antigenicity of HBoV VP1 protein was also demonstrated by Western blot analysis (Fig. 1). It was shown that the seropositive rate of HBoV was lowest (5.6%) in the 6- to-8 month age group and increased with advancing age and that all of the children had been exposed to HBoV by the age of 6 years. Twenty-three (74%) of the 31 infants under 6 months of age in this study had antibodies to HBoV, which might be maternally derived (Table 2). These results are consistent with the fact that HBoV DNA has usually been detected in samples from patients between 6 months and 3 years of age (5–7, 11, 13, 20, 27, 28, 42, 43, 47). Elevation of the titers of HBoV-specific antibody in convalescent-phase sera was observed in four patients with LRTI, and HBoV DNA was detected in nasopharyngeal swab and serum samples from all four patients (Table 5). These four cases were thought to be primary infections by HBoV because of undetectable IgG antibodies against HBoV in acute-phase sera (Table 5). Detection of HBoV DNA in acute-phase sera suggested that HBoV can cause not only a respiratory tract infection but also a systemic infection, like other parvoviruses, and can evoke a humoral immune response (16, 18, 31).

Human parvovirus B19 is a human pathogen that causes a wide spectrum of clinical manifestations (9). B19 VP1 has only 18% amino acid identity to HBoV VP1 (3, 41). To eliminate the possibility that an IFA positive for HBoV was the result of detection of the antibody against B19, Tn5 cells infected with a recombinant baculovirus expressing B19 VP1 protein were prepared and used for an IFA and absorption test. The overall seroprevalence rate and the age group-specific rate of anti-B19 antibody titers are shown in Table 3. These rates are consistent with previously reported data (4, 14, 22, 25, 46), indicating that the VP1 protein of B19 was properly prepared. The existence of HBoV IFA-positive and B19 IFA-negative or HBoV IFA-negative and B19 IFA-positive serum samples indicates that the anti-HBoV and anti-B19 sera did not cross-react with each other (Table 1). The results of absorption tests also demonstrate that the specific antibody to the VP1 protein of HBoV, but not the antibody to that of B19, was detected by the HBoV IFA (Table 4).

Relatively high proportions of coinfection with HBoV and other respiratory viruses (for example, respiratory syncytial virus, adenovirus, human metapneumovirus, human rhinovirus, human parainfluenza viruses types 1 to 4, and influenza viruses types A and B) have recently been reported (2, 5, 11, 13, 20, 21, 24, 30, 36, 44, 47). Although two respiratory viruses (human rhinovirus and adenovirus) were detected in three of four HBoV-positive nasopharyngeal swab samples in this study, these results might be biased due to the limited number of nasopharyngeal swab samples. In addition, the serum and nasopharyngeal swab samples used in this study were collected in only one part of Japan. Further studies are therefore necessary to determine whether HBoV is truly one of the causative agents of LRTI, whether HBoV is widespread throughout Japan, and whether HBoV plays a role in modifying the clinical course of infections in children who are coinfected with other respiratory viruses.

In the present study, we developed an IFA using the VP1 protein of HBoV. The IFA using HBoV VP1 protein provides a useful tool to analyze the spectrum of HBoV infections, the immune responses against HBoV, and the pathogenesis of HBoV infections.

ACKNOWLEDGMENTS

This research was supported in part by a Grant-in-Aid for Scientific Research (C), 2005 (17591065), from the Ministry of Education, Science, Sports and Culture of Japan.

Sera and nasopharyngeal swab samples were kindly provided by Yutaka Takahashi of KKR Sapporo Medical Center; Hiroyuki Sawada and Tsuguyo Nakayama of Hokkaido Social Insurance Hospital; Yachiyo Ohta, Yasutsugu Koga, Takashi Iwai, and Koji Okuhara of Tenshi Hospital; Mutsuko Konno of Sapporo Kosei General Hospital; Nobuyoshi Ishikawa and Akihiro Iguchi of Kitami Red Cross Hospital;

and Kunio Ozutusmi of Nemuro City Hospital. We thank Stewart Chisholm for proofreading the manuscript.

REFERENCES

1. **Aguilar, J. C., M. P. Pérez-Breña, M. L. Garcia, N. Cruz, D. D. Erdman, and J. E. Echevarria.** 2000. Detection and identification of human parainfluenza viruses 1, 2, 3, and 4 in clinical samples of pediatric patients by multiplex reverse transcription-PCR. J. Clin. Microbiol. **38:**1191–1195.
2. **Allander, T., T. Jartti, S. Gupta, H. G. Niesters, P. Lehtinen, R. Osterback, T. Vuorinen, M. Waris, A. Bjerkner, A. Tiveljung-Lindell, B. G. van den Hoogen, T. Hyypia, and O. Ruuskanen.** 2007. Human bocavirus and acute wheezing in children. Clin. Infect. Dis. **44:**904–910.
3. **Allander, T., M. T. Tammi, M. Eriksson, A. Bjerkner, A. Tiveljung-Lindell, and B. Andersson.** 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc. Natl. Acad. Sci. USA **102:**12891–12896.
4. **Anderson, L. J., C. Tsou, R. A. Parker, T. L. Chorba, H. Wulff, P. Tattersall, and P. P. Mortimer.** 1986. Detection of antibodies and antigens of human parvovirus B19 by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **24:**522–526.
5. **Arden, K. E., P. McErlean, M. D. Nissen, T. P. Sloots, and I. M. Mackay.** 2006. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J. Med. Virol. **78:**1232–1240.
6. **Arnold, J. C., K. K. Singh, S. A. Spector, and M. H. Sawyer.** 2006. Human bocavirus: prevalence and clinical spectrum at a children's hospital. Clin. Infect. Dis. **43:**283–288.
7. **Bastien, N., K. Brandt, K. Dust, D. Ward, and Y. Li.** 2006. Human bocavirus infection, Canada. Emerg. Infect. Dis. **12:**848–850.
8. **Beersma, M. F., E. C. Claas, T. Sopaheluakan, and A. C. Kroes.** 2005. Parvovirus B19 viral loads in relation to VP1 and VP2 antibody responses in diagnostic blood samples. J. Clin. Virol. **34:**71–75.
9. **Broliden, K., T. Tolfvenstam, and O. Norbeck.** 2006. Clinical aspects of parvovirus B19 infection. J. Intern. Med. **260:**285–304.
10. **Brown, C. S., M. J. van Bussel, A. L. Wassenaar, A. M. van Elsacker-Niele, H. T. Weiland, and M. M. Salimans.** 1990. An immunofluorescence assay for the detection of parvovirus B19 IgG and IgM antibodies based on recombinant viral antigen. J. Virol. Methods **29:**53–62.
11. **Choi, E. H., H. J. Lee, S. J. Kim, B. W. Eun, N. H. Kim, J. A. Lee, J. H. Lee, E. K. Song, S. H. Kim, J. Y. Park, and J. Y. Sung.** 2006. The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000–2005. Clin. Infect. Dis. **43:**585–592.
12. **Chomczynski, P.** 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques **15:**532–534, 536–537.
13. **Chung, J. Y., T. H. Han, C. K. Kim, and S. W. Kim.** 2006. Bocavirus infection in hospitalized children, South Korea. Emerg. Infect. Dis. **12:**1254–1256.
14. **Cohen, B. J., and M. M. Buckley.** 1988. The prevalence of antibody to human parvovirus B19 in England and Wales. J. Med. Microbiol. **25:**151–153.
15. **Corcoran, A., and S. Doyle.** 2004. Advances in the biology, diagnosis and host-pathogen interactions of parvovirus B19. J. Med. Microbiol. **53:**459–475.
16. **Cossart, Y. E., A. M. Field, B. Cant, and D. Widdows.** 1975. Parvovirus-like particles in human sera. Lancet **i:**72–73.
17. **Doyle, S., S. Kerr, G. O'Keeffe, D. O'Carroll, P. Daly, and C. Kilty.** 2000. Detection of parvovirus B19 IgM by antibody capture enzyme immunoassay: receiver operating characteristic analysis. J. Virol. Methods **90:**143–152.
18. **Durham, P. J., A. Lax, and R. H. Johnson.** 1985. Pathological and virological studies of experimental parvoviral enteritis in calves. Res. Vet. Sci. **38:**209–219.
19. **Ebihara, T., R. Endo, H. Kikuta, N. Ishiguro, H. Ishiko, M. Hara, Y. Takahashi, and K. Kobayashi.** 2004. Human metapneumovirus infection in Japanese children. J. Clin. Microbiol. **42:**126–132.
20. **Foulongne, V., Y. Olejnik, V. Perez, S. Elaerts, M. Rodiere, and M. Segondy.** 2006. Human bocavirus in French children. Emerg. Infect. Dis. **12:**1251–1253.
21. **Fry, A. M., X. Lu, M. Chittaganpitch, T. Peret, J. Fischer, S. F. Dowell, L. J. Anderson, D. Erdman, and S. J. Olsen.** 2007. Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J. Infect. Dis. **195:**1038–1045.
22. **Heegaard, E. D., B. L. Petersen, C. J. Heilmann, and A. Hornsleth.** 2002. Prevalence of parvovirus B19 and parvovirus V9 DNA and antibodies in paired bone marrow and serum samples from healthy individuals. J. Clin. Microbiol. **40:**933–936.
23. **Ishiko, H., Y. Shimada, M. Yonaha, O. Hashimoto, A. Hayashi, K. Sakae, and N. Takeda.** 2002. Molecular diagnosis of human enteroviruses by phylogeny-based classification by use of the VP4 sequence. J. Infect. Dis. **185:**744–754.
24. **Kaplan, N. M., W. Dove, A. F. Abu-Zeid, H. E. Shamoon, S. A. Abd-Eldayem, and C. A. Hart.** 2006. Human bocavirus infection among children, Jordan. Emerg. Infect. Dis. **12:**1418–1420.
25. **Kelly, H. A., D. Siebert, R. Hammond, J. Leydon, P. Kiely, and W. Maskill.** 2000. The age-specific prevalence of human parvovirus immunity in Victoria, Australia compared with other parts of the world. Epidemiol. Infect. **124:**449–457.
26. **Lin, F., A. Zeng, N. Yang, H. Lin, E. Yang, S. Wang, D. Pintel, and J. Qiu.** 2007. Quantification of human bocavirus in lower respiratory tract infections in China. Infect. Agent Cancer **2:**3.
27. **Lu, X., M. Chittaganpitch, S. J. Olsen, I. M. Mackay, T. P. Sloots, A. M. Fry, and D. D. Erdman.** 2006. Real-time PCR assays for detection of bocavirus in human specimens. J. Clin. Microbiol. **44:**3231–3235.
28. **Ma, X., R. Endo, N. Ishiguro, T. Ebihara, H. Ishiko, T. Ariga, and H. Kikuta.** 2006. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J. Clin. Microbiol. **44:**1132–1134.
29. **Maggi, F., E. Andreoli, M. Pifferi, S. Meschi, J. Rocchi, and M. Bendinelli.** 2007. Human bocavirus in Italian patients with respiratory diseases. J. Clin. Virol. **38:**321–325.
30. **Manning, A., V. Russell, K. Eastick, G. H. Leadbetter, N. Hallam, K. Templeton, and P. Simmonds.** 2006. Epidemiological profile and clinical associations of human bocavirus and other human parvoviruses. J. Infect. Dis. **194:**1283–1290.
31. **Meunier, P. C., B. J. Cooper, M. J. Appel, and D. O. Slauson.** 1985. Pathogenesis of canine parvovirus enteritis: the importance of viremia. Vet. Pathol. **22:**60–71.
32. **Miura-Ochiai, R., Y. Shimada, T. Konno, S. Yamazaki, K. Aoki, S. Ohno, E. Suzuki, and H. Ishiko.** 2007. Quantitative detection and rapid identification of human adenoviruses. J. Clin. Microbiol. **45:**958–967.
33. **Monteny, M., H. G. Niesters, H. A. Moll, and M. Y. Berger.** 2007. Human bocavirus in febrile children, The Netherlands. Emerg. Infect. Dis. **13:**180–182.
34. **Morinet, F., L. D'Auriol, J. D. Tratschin, and F. Galibert.** 1989. Expression of the human parvovirus B19 protein fused to protein A in *Escherichia coli*: recognition by IgM and IgG antibodies in human sera. J. Gen. Virol. **70**: 3091–3097.
35. **Naghipour, M., L. E. Cuevas, T. Bakhshinejad, W. Dove, and C. A. Hart.** 2007. Human bocavirus in Iranian children with acute respiratory infections. J. Med. Virol. **79:**539–543.
36. **Neske, F., K. Blessing, F. Tollmann, J. Schubert, A. Rethwilm, H. W. Kreth, and B. Weissbrich.** 2007. Real-time PCR for human bocavirus infections and phylogenetic analysis. J. Clin. Microbiol.
37. **Offringa, D. P., V. Tyson-Medlock, Z. Ye, and R. A. Levandowski.** 2000. A comprehensive systematic approach to identification of influenza A virus genotype using RT-PCR and RFLP. J. Virol. Methods **88:**15–24.
38. **Osiowy, C.** 1998. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J. Clin. Microbiol. **36:**3149–3154.
39. **Rayment, F. B., E. Crosdale, D. J. Morris, J. R. Pattison, P. Talbot, and J. J. Clare.** 1990. The production of human parvovirus capsid proteins in *Escherichia coli* and their potential as diagnostic antigens. J. Gen. Virol. **71:**2665–2672.
40. **Regamey, N., U. Frey, C. Deffernez, P. Latzin, and L. Kaiser.** 2007. Isolation of human bocavirus from Swiss infants with respiratory infections. Pediatr. Infect. Dis. J. **26:**177–179.
41. **Saito, T., Y. Munakata, Y. Fu, H. Fujii, T. Kodera, E. Miyagawa, K. Ishii, and T. Sasaki.** 2003. Evaluation of anti-parvovirus B19 activity in sera by assay using quantitative polymerase chain reaction. J. Virol. Methods **107:**81–87.
42. **Simon, A., P. Groneck, B. Kupfer, R. Kaiser, G. Plum, R. L. Tillmann, A. Müller, and O. Schildgen.** 2007. Detection of bocavirus DNA in nasopharyngeal aspirates of a child with bronchiolitis. J. Infect. **54:**e125–e127.
43. **Sloots, T. P., P. McErlean, D. J. Speicher, K. E. Arden, M. D. Nissen, and I. M. Mackay.** 2006. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J. Clin. Virol. **35:**99–102.
44. **Smuts, H., and D. Hardie.** 2006. Human bocavirus in hospitalized children, South Africa. Emerg. Infect. Dis. **12:**1457–1458.
45. **Stephensen, C. B., D. B. Casebolt, and N. N. Gangopadhyay.** 1999. Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. Virus Res. **60:**181–189.
46. **Tsujimura, M., K. Matsushita, H. Shiraki, H. Sato, K. Okochi, and Y. Maeda.** 1995. Human parvovirus B19 infection in blood donors. Vox Sang. **69:**206–212.
47. **Weissbrich, B., F. Neske, J. Schubert, F. Tollmann, K. Blath, K. Blessing, and H. W. Kreth.** 2006. Frequent detection of bocavirus DNA in German children with respiratory tract infections. BMC Infect. Dis. **6:**109.