

Antibodies against Structural and Nonstructural Proteins of Human Bocavirus in Human Sera[▽]

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Immunofluorescence assays (IFAs) for detection of human bocavirus (HBoV) proteins (VP1, VP2, NP-1, and NS1) were developed. The VP1 IFA was the most sensitive for detection of IgG antibody and suitable for screening. IgG antibodies in convalescent-phase sera from HBoV-positive patients were detected by VP1 and VP2 IFAs. Sensitivities of NP-1 and NS1 IFAs were low.

Human bocavirus (HBoV), belonging to the family *Parvoviridae*, subfamily *Parvovirinae*, and genus *Bocavirus*, was cloned by molecular screening of pooled human respiratory tract samples in 2005 (2). Until the identification of HBoV, human parvovirus B19 (subfamily *Parvovirinae*, genus *Erythrovirus*) had been the only known human pathogen in the family *Parvoviridae* (42). HBoV has been detected in patients with respiratory tract infections in many countries by PCR or real-time PCR, the rate of HBoV detection ranging from 1.5 to 19% (1, 3–5, 8, 9, 13, 14, 24, 26–28, 30, 31, 33, 35–39, 41). However, the causative role of HBoV in respiratory tract infection remains unclear. In fact, HBoV is codetected with other respiratory viruses in many cases of lower respiratory tract infection (1, 3, 8, 9, 13, 14, 20, 30, 34, 39, 41).

Real-time PCR for HBoV has been used to analyze the pathophysiology of HBoV infections. High loads of HBoV in nasopharyngeal samples, mainly in the absence of other viral agents, have been found in some studies, suggesting a causative role in acute respiratory tract infections (1, 17). Another study showed that the load of HBoV in nasopharyngeal samples from patients with bronchiolitis was significantly higher than that in patients with the diagnosis of febrile seizures (34).

Seroepidemiological study is also an important tool for diagnosis of and research on HBoV infection. Antibodies against HBoV in serum are raised after HBoV infection, suggesting that HBoV infection evokes a systemic immune response (11, 18, 19, 23). Recently, we developed an immunofluorescence assay (IFA) to measure titers of specific antibodies against HBoV VP1, which is one of the structural proteins of HBoV (11). HBoV encodes two structural proteins (VP1 and VP2) and two nonstructural proteins (NP-1 and NS1) (2). The recombinant structural proteins (VP1 and VP2) of B19 have been used for the detection of IgG and IgM antibodies against B19 (6, 22). More recently, the nonstructural protein (NS1) of

B19 was shown to play a significant role in serodiagnosis of acute infection, thereby supplementing the role played by B19 structural proteins as diagnostic antigens (10, 12, 15). The purpose of this study was to compare the efficiencies of IFAs using individual proteins of HBoV.

A baculovirus expression kit (Bac-to-Bac system) was used to prepare histidine (His)-tagged VP1, VP2, NP-1, and NS1 proteins for expression in a baculovirus-insect cell system in accordance with the instructions of the manufacturer (Invitrogen, Carlsbad, CA). The genomic DNA corresponding to VP1, VP2, NP-1, and NS1 proteins of HBoV from strain JPBS05-52 (GenBank accession numbers EF035488, EU984096, and EU984097) was amplified by PCR with the following primers: HBoV VP1 start (5'-ATC GTC TCG CAT GAG TAA AGA AAG TGG CAA-3'), HBoV VP2 start (5'-ATC GTC TCG CAT GTC TGA CA CTG ACA TTC A-3'), HBoV VP1 and VP2 end (5'-GCC TCG AGT TAC AAT GGG TGC ACA CGG C-3'), HBoV NP-1 start (5'-ATC GTC TCG CAT GAG CTC AGG GAA TAT GAA-3'), HBoV NP-1 end (5'-GCC TCG AGT TAA TTG GAG GCA TCT GCT T-3'), HBoV NS1 start (5'-ATC CAT GGC TTT CAA TCC TCC T-3'), and HBoV NS1 end (5'-CGC TCG AGT TAC TTA CTT GGT G-3') (the restriction sites in the primers used for cloning are underlined). The following procedures for making baculoviruses were described previously (11). *Trichoplusia ni* (Tn5) cells in T25 flasks were infected with recombinant baculoviruses expressing His-tagged VP1, VP2, NP-1, and NS1 proteins of HBoV and with mock baculovirus at a multiplicity of infection of 10 virus particles per cell and were resuspended in 250 μ l of phosphate-buffered saline (PBS). The procedures for Western blot analysis were described previously (11). The results of Western blot analysis are shown in Fig. 1A. The predicted molecular sizes of His-VP1, His-VP2, His-NP-1, and His-NS1 fusion proteins of HBoV were 78.1, 63.4, 29.0, and 75.0 kDa, respectively, which were consistent with the molecular sizes of these proteins determined by Western blotting using anti-six-histidine tag monoclonal antibody.

IFAs using Tn5 cells infected with recombinant baculoviruses expressing VP1, VP2, NP-1, and NS1 proteins of HBoV were developed as described previously (11, 16). A total of 205 serum samples were randomly obtained from outpatients or

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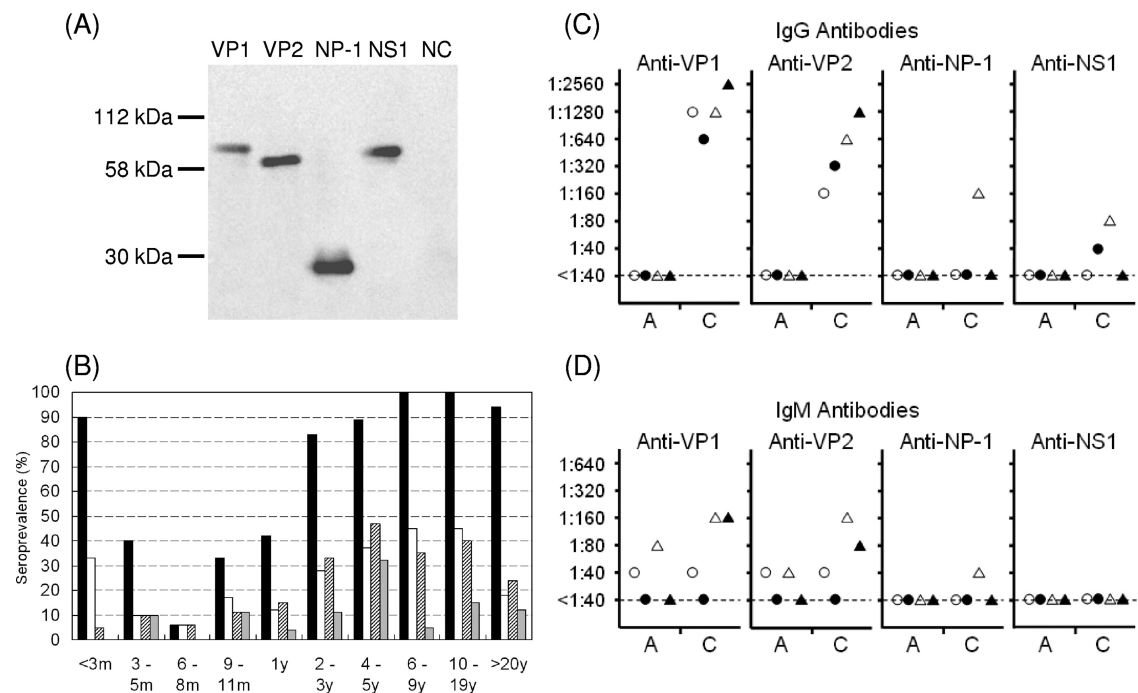


FIG. 1. (A) Western blot analysis of His-VP1 (VP1), His-VP2 (VP2), His-NP-1 (NP-1), and His-NS1 (NS1) proteins of HBoV expressed by a baculovirus system, compared with negative-control protein (NC). Anti-six-histidine tag monoclonal antibody was incubated on nitrocellulose membranes. (B) Age distribution of seropositive rates for HBoV VP1 IgG (black columns), VP2 IgG (white columns), NP-1 IgG (striped columns), and NS1 IgG (gray columns). m, months; y, years. (C and D) Detection of the IgG and IgM antibodies against VP1, VP2, NP-1, and NS1 proteins of HBoV in acute-phase (A) and convalescent-phase (C) serum samples from four patients with acute HBoV infection by IFAs. White circles, black circles, white triangles, and black triangles correspond to patient numbers 1, 2, 3, and 4, respectively, in reference 11.

inpatients (aged 0 months to 41 years) at six hospitals (see Acknowledgments) in Hokkaido Prefecture of Japan from 1998 to 2005. All samples were collected after obtaining informed consent from the patients or the parents of child patients. Titers of specific antibodies against VP1, VP2, NP-1, and NS1 proteins of HBoV in the 204 human serum samples were measured by IFAs. The serum samples were diluted 1:40 with PBS and applied individually onto slides carrying Tn5 cells infected with recombinant baculoviruses expressing VP1, VP2, NP-1, and NS1 proteins of HBoV and mock baculovirus. Serum samples that had clear fluorescence signals on slides carrying Tn5 cells infected with recombinant baculoviruses and had no fluorescence signals on slides carrying cells infected with mock baculovirus were judged to be IFA positive. The IFA-positive serum samples were then serially diluted twofold in PBS from 1:40 to 1:10,240, and titers were determined by IFAs in the same manner. The results of IFAs were checked by two reviewers (R.S. and R.E.) who were not blinded to the results from the other reviewer. If a serum sample was considered to be positive by both reviewers, the serum sample was judged to be IFA positive, and if a serum sample was considered to be negative by one or both reviewers, the serum sample was judged to be IFA negative. One serum sample that had fluorescence signals on the slide with Tn5 cells infected with mock baculovirus at a 1:40 dilution was excluded from this study. The results for the remaining 204 serum samples are summarized in Table 1. The highest seroprevalence was observed in the IFA for HBoV VP1-specific IgG (146 of 204 samples [71.6%] were positive), with the next highest levels

obtained by the IFAs for HBoV VP2-specific IgG (51 of 204 samples [25.0%] were positive), HBoV NP-1-specific IgG (46 of 204 [22.5%] were positive), and HBoV NS1-specific IgG (20 of 204 [9.8%] were positive). IgG antibodies against VP2, NP-1, and NS1 proteins of HBoV were not detected in serum samples negative for HBoV VP1-specific IgG (data not shown). When the HBoV VP1-specific IgG IFA was used as a standard, the sensitivities of HBoV VP2-specific IgG, HBoV NP-1-specific IgG, and HBoV NS1-specific IgG IFAs were 35, 32, and 14%, respectively (data not shown). The IFA titers of HBoV VP1-specific IgG ranged between 40 and 5,120, with a median titer of 160 and a geometric mean titer of 374 (Table 1). The differences between IFA titers of IgG antibodies against HBoV VP1 and those of IgG antibodies against the

TABLE 1. Seroprevalence of IgG antibodies against HBoV proteins detected by IFAs using a baculovirus expression system

IFA antigen	No. of serum samples positive/no. tested (%)	IFA titer ^a		
		Range	Median	Geometric mean titer
HBoV VP1	146/204 (71.6)	40–5,120	160	374
HBoV VP2	51/204 (25.0)	40–640	80	186
HBoV NP-1	46/204 (22.5)	40–160	40	107
HBoV NS1	20/204 (9.8)	40–80	40	83

^a Differences in titers between antibodies to different proteins (VP1 and VP2 antibodies, VP1 and NP-1 antibodies, VP1 and NS1 antibodies, and VP2 and NS1 antibodies) were statistically significant ($P < 0.01$).

other three proteins tested (HBoV VP2, NP-1, and NS1) were statistically significant ($P < 0.01$ by the Kruskal-Wallis test) (Table 1). The age-related seropositivity rate for IgG antibodies against HBoV VP1 was higher than those for IgG antibodies against the other three proteins (HBoV VP2, NP-1, and NS1), except in the 6- to 8-month-old group (Fig. 1B). It has been shown previously that both HBoV capsid (VP1 and VP2) proteins are derived from overlapping reading frames and that VP1 and VP2 proteins are identical except for an additional 129 amino acids on the amino terminus of the VP1 protein (2). These findings suggest that the unique portion of VP1 containing 129 amino acids may be immunodominant. Further studies are necessary to determine the epitopes recognized by human sera. Recently, it has been reported that the unique region of VP1 from B19 is immunodominant in comparison with the VP2 protein from B19 and elicits a long-lasting immune response because specific IgG antibodies were present in blood donors of all age groups (32, 43). The HBoV VP1-specific IgG IFA showed the highest seroprevalence, and HBoV VP1 protein is therefore the most suitable antigen for IFAs to study the seroepidemiology of HBoV infection. Four groups have previously described an enzyme-linked immunosorbent assay (ELISA) system using recombinant VP2-based virus-like particles (VLP) (7, 18, 23, 25). Further studies are necessary to determine which assay (IFA versus VLP-based ELISA) and which antigen (recombinant VP1 versus recombinant VP2-based VLP) are most sensitive for the detection of HBoV-specific antibodies.

Both acute-phase and convalescent-phase serum samples were obtained from four patients with lower respiratory tract infections whose nasopharyngeal swab samples were PCR positive for HBoV. (Other viruses were codetected in three of the four patients. Data for these patients were reported previously [11].) The levels of IgG and IgM antibodies to each protein of HBoV in serum samples were measured. HBoV VP1- and VP2-specific IgG antibodies were detected in all four convalescent-phase serum samples but not in any of the acute-phase serum samples (Fig. 1C). Considering the lower rate of positive results from the HBoV VP2-specific IgG IFA than from the VP1-specific IgG IFA for 204 serum samples (Table 1), the IgG antibodies against the unique region of VP1 may persist long after infection and the levels of those against the VP2 region may decline rapidly after infection. Similar phenomena were observed in B19 infection (29). Briefly, both anti-linear VP1 epitope IgG and anti-conformational VP2 IgG antibodies were detected after very recent B19 infection and persisted for several months or longer in the majority of subjects (29). IgG antibodies against the VP2 linear epitopes were generally present during active infection or after very recent infection, and during the convalescent phase, they were detected in only about 20% of subjects with signs of past B19 infection (29). In our previous study, it was found that six (66.6%) of nine serum samples with titers of $>1,280$ as determined by the HBoV VP1 IFA reacted with the His-VP1 fusion protein of HBoV in Western blot analyses (11). Therefore, HBoV VP1-specific IgG antibodies in convalescent-phase serum samples were suspected to react partially with a linear epitope of HBoV VP1 protein. Further study is necessary to characterize linear and conformational epitopes of VP1 and VP2 proteins of HBoV. HBoV VP1- and VP2-specific IgM antibodies were detected in

only two of the four acute-phase serum samples, and the titers of IgM antibodies were low (1:40 to 1:80) (Fig. 1D), suggesting that the HBoV VP1- and VP2-specific IgM IFAs may not be suitable for early diagnosis of HBoV infection. These observations demonstrate that the recombinant VP1 and VP2 proteins of HBoV produced by a baculovirus system are useful as a source of antigens for serological tests and that seroconversion with IgG antibodies to the VP1 and VP2 proteins of HBoV is a reliable marker of HBoV infection.

IgG antibodies against nonstructural proteins of HBoV were detected in convalescent-phase serum samples: anti-NP-1 IgG antibody was detected in one case, and anti-NS1 antibodies were detected in two cases (Fig. 1C). IgM antibody against HBoV NP-1 was detected in one convalescent-phase serum sample, and IgM antibody against HBoV NS1 was not detected in any acute- or convalescent-phase serum samples (Fig. 1D). It is known that IgG antibodies against nonstructural protein (NS1) are present in patients with persistent B19 infection (40) and chronic arthritis after B19 infection (21). If a chronic HBoV infection status is found in the future, nonstructural proteins of HBoV may have potential value as diagnostic antigens for HBoV infection.

In conclusion, IFAs for the detection of seroconversion with IgG antibodies against structural (VP1 and VP2) proteins of HBoV are useful for diagnosis of acute HBoV infection. An IFA for the detection of IgG antibody against HBoV VP1 protein is a suitable test for seroepidemiological studies.

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