Seroepidemiology of Human Bocavirus Defined Using Recombinant Virus-Like Particles

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Background. Human bocavirus (HBoV) is a newly identified human parvovirus for which seroepidemiology and antigenic properties remain undefined.

Methods. The HBoV VP2 gene, expressed from a baculovirus vector, produced virus-like particles (VLPs), which were used to raise rabbit anti-HBoV antisera and to develop an enzyme-linked immunosorbent assay (ELISA). The VLP-based ELISA was used to screen for HBoV-specific immunoglobulin G antibodies in a convenience sample of 270 serum specimens, mostly from children, obtained at Yale–New Haven Hospital; 208 specimens were also screened for erythrovirus B19–specific antibodies by a B19 VLP–based ELISA.

Results. Immunofluorescence and ELISA showed that human parvoviruses HBoV and B19 are antigenically distinct. By the HBoV VLP–based ELISA, 91.8% and 63.6% of serum specimens from infants in the first and second months of life, respectively, were found to be seropositive, as were 45.4% from 3-month-old infants and 25.0% from 4-month-old infants. The percentages of HBoV-seropositive children increased to 40.7%–60.0% for children 5–47 months of age and to >85% for individuals >48 months old. However, the overall percentage of B19-seropositive individuals was <40.5% for all age groups screened.

Conclusions. HBoV infection is common during childhood, but a minority of children and young adults screened have evidence of B19 infection.

In 2005, Allander et al. [1] described the identification of a previously unknown human parvovirus in individuals with respiratory tract disease. On the basis of phylogenetic analysis, this new agent was determined to be the first known human virus to belong to the genus Bocavirus and was therefore designated “human bocavirus” (HBoV). Previously, parvoviruses that infected humans fell into 2 of the 5 vertebrate-infecting genera, the dependoviruses and the erythroviruses [2]. Of the dependoviruses, 3 adeno-associated virus serotypes (types 2, 3, and 5) circulate in the human population but are not associated with disease, whereas human parvovirus B19, a species within the erythroviruses, is the etiologic agent of erythema infectiosum, a common rash of childhood; aplastic crisis in hemolytic syndromes, such as sickle cell disease; polyarthralgias and arthritis in adults; chronic anemia in immunocompromised hosts; and hydrops fetalis in human fetuses.

To date, >20 polymerase chain reaction (PCR)–based studies have identified HBoV DNA in respiratory secretions. The virus appears to have worldwide distribution [1, 3–12]. Many studies have found that HBoV DNA is present in 1%–8% of respiratory specimens screened [1, 3, 8, 12–15], but others suggest that the rate is >18% [7, 16]. In our previous study [8], HBoV was detected in 5.2% of children with respiratory tract disease who tested negative for respiratory syncytial virus (RSV), influenza A and B virus, parainfluenza viruses 1–3, and adenoviruses and in 0% of control subjects, suggesting a causal role for the virus in respiratory tract disease. When reported, coinfection with HBoV and other common human respiratory viruses is often observed, and the rate of coinfection can be as high as 80% or more [6]. Therefore, the role that HBoV plays in dis-
ease remains undefined. Most HBoV-positive specimens are from children, but this may represent a sampling bias. HBoV DNA has also been detected in blood [6, 16] and in stool [17–19], although the significance of these findings remains unclear [20].

One of the major obstacles to studying the epidemiology and biology of HBoV is the lack of reagents, specifically infectious virus and monospecific anti-HBoV antibodies. Currently, there are no reports of its successful propagation in cell culture or in animal models. Japanese researchers have recently reported the expression of the HBoV VP1 gene and have used this recombinant protein to screen for HBoV-specific antibodies [21]. We used an approach previously used to create other parvoviral virus-like particles (VLPs) involving cloning and expressing one of the viral structural proteins, VP2, in insect cells from recombinant baculovirus vectors. Baculovirus-expressed VP2 (but not VP1) self-assembles to form VLPs that can be purified and used to develop detection assays for immunoglobulins directed against structural determinants in the viral particle. We have used such an ELISA to demonstrate that HBoV and B19 are antigenically distinct and to explore the seroepidemiology of HBoV and B19 in a study population in New Haven, Connecticut.

METHODS

Construction of HBoV VLPs. Nucleic acids from a respiratory specimen positive for HBoV by PCR were extracted with the QIAamp nucleic acid purification kit (Qiagen), as described elsewhere [8]. The HBoV VP2 gene (GenBank accession number EU429804) was amplified in halves by use of HotStarTaq DNA polymerase (Qiagen), using the HBoV ST2 sequence (GenBank accession number DQ000496) to direct the choice of primer sequences. The left half was amplified using a rightward primer (5'-CCATCGGGCGCGATCCTACATGTCTGACACTGACTGATTCAAG-3') that covered an EcoRI site in the middle of the HBoV VP2 gene, with the addition of a SacI site that allowed the 2 fragments to be joined using a leftward primer (5'-TTGTTGGTTTTGAGTATGCATTCAAG-3') that covered the predicted translational start site of the VP2 gene, and with the addition of a BamHI site upstream of it, and a leftward primer (5'-TTGTTGGTTTTGAGTATGCATTCAAG-3') that covered an EcoRI site in the middle of the HBoV VP2 gene in the HBoV ST2 sequence. The right half of the VP2 gene was generated in a similar way, using a rightward primer (5'-AATGAAAGAGGATACATCACCTCCTCTCTC-3') that overlapped the same EcoRI site and a leftward primer (5'-GCTTGGTACCTAGAGCTCACATTCAACACTTATTTGATG-3') that included the TAA codon at the common C-terminal of the HBoV VPs and that contained a SacI site that allowed the 2 fragments to be cloned sequentially into the pFastBac vector (Invitrogen).

The resulting construct, pFB-HBoV-VP2, was sequenced, and a recombinant baculovirus was generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen), by transformation into competent, bacmid-containing DH10 Escherichia coli cells and transfection of the high-molecular-weight DNA that this generated into Sf9 insect cells (Invitrogen). The resulting baculovirus was expanded and then titered by plaque assay on Sf9 monolayer cultures. A recombinant baculovirus expressing VLPs of human parvovirus B19 was provided by B. Kaufmann (Purdue University) [22] and was expanded and titered the same way.

Expression and purification of VLPs. Sf9 cells were infected at a multiplicity of 5 pfu/cell with either HBoV- or B19-expressing recombinant baculovirus and were harvested 96 h after infection. The cells were resuspended in TE 8.7 buffer (50 mmol/L Tris–HCl and 0.5 mmol/L EDTA [pH 8.7]) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics), frozen and thawed 3 times, and cleared by centrifugation at 15,000 g in a Sorvall SS-34 rotor. Then, 5.8-ml quantities of supernatant were purified by sedimentation to equilibrium in iodixanol gradients made as steps of 1 mL at 45%, 1 mL at 40%, 1.5 mL at 35%, 1.5 mL at 30%, and 1 mL at 25% in TE 8.7 buffer. Gradients were centrifuged in a Beckman SW41 rotor at 150,000 g and 18°C for 18 h. Fractions were collected and analyzed on SDS-PAGE gels, and HBoV VP2-containing samples were examined for VLPs by means of a Philips 410 transmission electron microscope.

ELISA. Purified HBoV and B19 VLPs were used as antigens in ELISAs. Briefly, 96-well plates (Costar EIA/RIA; Corning) were coated with purified VLPs (100 ng/mL in PBS) and incubated overnight at 4°C. After 3 washes (in PBS plus 0.05% Tween 20), the wells were blocked with 5% nonfat milk (in PBS plus 1% Tween 20) for 1 h at 37°C. Thereafter, serial dilutions of serum were added to the wells and incubated at 37°C. After 1 h, serum was removed, and the wells were washed with PBS/Tween buffer. A species-appropriate horseradish peroxidase–conjugated secondary antibody (anti-IgG [H + L] diluted 1:20,000; Jackson ImmunoResearch Laboratories) was added to each well, and plates were incubated for 1 h at 37°C. Bound secondary antibody was measured by optical densitometry after a 10-min incubation with 3,3’,5,5’-tetramethyl benzidine (Pierce). Chromogenic reactions were stopped with 5N H3PO4. Optical densities (ODs) were read at 450 nm (Dynatech MR5000 microplate reader).

Serum specimens. Two hundred seventy serum specimens were obtained from the Clinical Chemistry Laboratory at Yale–New Haven Hospital from June 2003 through March 2004 and stored at −20°C. The vast majority (>97%) were obtained from individuals ≤20 years old. The numbers of serum specimens collected for each age group are displayed in the figures. Collection of specimens and clinical data were approved by the Yale University Human Investigation Committee. Pooled human serum (Bioreclamation) contained serum from 24 individuals and was used in the development of the ELISA.

HBoV- and B19-specific rabbit antiserum. Protocols for antibody production were approved by the Yale Institutional Animal Care and Use Committee. HBoV-specific antiserum was produced by 2 initial intradermal and subcutaneous inocula-
tions of a rabbit with a mixture of native and denatured HBoV VLPs in complete Freund’s adjuvant. Subsequent inoculations with incomplete Freund’s adjuvant were subcutaneous. B19-specific rabbit antiserum was produced, as reported elsewhere [23], by inoculation of a rabbit with a purified 284-aa B19-VP fusion protein. Monoclonal antibody (MAb) against B19 particles (clone 521–50) was obtained from L. J. Anderson (Division of Viral Diseases, National Center of Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia).

**RESULTS**

**Indirect immunofluorescence assays.** HBoV or B19 recombinant baculovirus–infected Sf9 cells were fixed in 2.5% paraformaldehyde and permeabilized with 0.1% Triton X-100. Primary antiserum (rabbit anti-HBoV VLPs at a dilution of 1:2000 or mouse anti-B19 MAb at a dilution of 1:100) were added to fixed and permeabilized infected Sf9 cells in 10% normal goat serum for 45 min at room temperature. Thereafter, the cells were washed with PBS, and the binding of primary antibody was detected with a secondary antibody (either fluorescein isothiocyanate [FITC]–conjugated anti–rabbit IgG antibody or Texas red–conjugated anti–mouse IgG antibody [both from Invitrogen]), using a Nikon Optiphot epifluorescence microscope.

**Immunoprecipitation of DNA-containing virions from respiratory specimens with antiserum specific for HBoV capsids.** HBoV virions were extracted from 2 throat swab samples positive for HBoV by PCR via freezing and thawing 3 times in 50 mM Tris–HCl (pH 8.7) per 0.5 mmol/L EDTA and were then pooled and diluted in tissue culture medium. Samples (1/60th of the total per immunoprecipitation) were adjusted to 0.085% SDS, 150 mmol/L NaCl, 15 mmol/L Tris–HCl (pH 8.0), 1 mmol/L EDTA, and 1% Triton X-100 (1.2 mL per immunoprecipitation) and processed for immunoprecipitation, antigen elution, and proteolysis using an EZ-ChIP kit (Upstate), essentially as described by the manufacturer. Eluted DNA was then purified using QIAquick PCR purification units, and 1/25th of each eluate was subjected to PCR amplification using primers specific for the HBoV NP1 gene (amplifying a 686-bp fragment between residues 2396 and 3081 in the ST2 sequence) or the VP gene (generating a 907-bp fragment spanning nt 3422–4328 in the ST2 sequence). Products were analyzed by electrophoresis through 1.4% agarose gels and were detected by ethidium bromide staining. The identities of candidate bands were confirmed by sequencing.

**Statistics.** SEs for proportions were calculated using standard statistical analyses. The HBoV- and B19-seropositive groups were compared using the χ² test.

**RESULTS**

**Self-assembly of recombinant HBoV VP2 into VLPs.** In the absence of any other source of particulate HBoV antigen for the development of an ELISA, we produced VLPs by expressing viral VP2 polypeptides in insect cells from a recombinant baculovirus. For this strategy to work, it was essential to express VP2, not VP1, because parvoviral VP1 polypeptides expressed alone will not self-assemble to form capsids [24, 25]. Figure 1A shows an alignment of the spliced transcript encoding the VP1 protein of the mouse parvovirus minute virus of mice (MVM) with the proposed VP2 open reading frame of the best-characterized bacavirus, bovine parvovirus (BPV) 1 (GenBank accession number DQ335247), and the analogous translation product of HBoV. This provides convincing evidence against the prior assignment of the BPV VP2 start codon, because the catalytic domains of the viral phospholipase A2s align very well between the MVM VP1–specific region and the N-terminal peptide previously proposed for BPV1.

This alignment strongly supports the contention, recently proposed by Qiu et al. [26], that the most likely translational start codon for the bacoviral VP2 is that originally predicted to start BPV1 VP3. On the basis of this alignment, we sequentially cloned PCR products covering the entire VP2 coding sequence into a pFastBac plasmid and used this to generate a recombinant baculovirus. Extracts of Sf9 cells infected with either the HBoV VLP– or B19 VLP–expressing baculoviruses were sedimented through iodixanol gradients previously optimized for the purification of empty capsids of MVM, and assembled 60mer VLPs were collected from the position of the original 40%:35% iodixanol interface. When input samples and fractions from across the HBoV gradient were analyzed by SDS-PAGE, only trace amounts of unassembled VP2 protein were observed in the upper fractions. As shown in figure 1B, however, high yields of particles (~100 µg/gradient) containing the predicted 64-kDa HBoV band sedimented to equilibrium in fractions 5 and 6 at approximately the same position as recombinant baculovirus–derived B19 VLPs analyzed on parallel iodixanol gradients. As expected, the 64-kDa HBoV protein in these particles was completely resistant to trypsin digestion, indicating that it formed part of a tight macromolecular structure (data not shown). Finally, to show that, when examined by electron microscopy, these peak fractions contained the ~28-nm-diameter particles typical of parvoviral empty capsids.

**Immunoprecipitation of HBoV particles from respiratory specimens by HBoV VLP–specific antiserum.** Purified HBoV VLPs were used to raise an HBoV-specific rabbit antiserum, which was tested for its ability to immunoprecipitate DNA-containing HBoV virions from PCR-positive throat swab samples. Antigens eluted from these immunoprecipitates were assessed for their ability to generate sequence–confirmed HBoV amplicons by use of primers from both the viral NP1 and VP genes. As seen in figure 2, serum specimens obtained 2 and 4 months after the first VLP injection effectively immunoprecipitated DNA-containing virions, whereas prebleed serum obtained immediately before the first injection did not. Similarly, rabbit antiserum directed against the B19 capsid failed to precip-
imate viral particles, as did a second nonimmune control rabbit antiserum. Thus, recombinant VLPs share antigens that are exposed on authentic HBoV virions and can be used to elicit an immune response to those virions.

Antigenic distinctness of human parvoviruses HBoV and B19. To explore further the extent to which HBoV and B19 are antigenically distinct, 2 approaches were taken. First, virus-specific antisera were used in immunofluorescence assays to stain insect cells infected with 1 of the 2 recombinant baculoviruses. Infected cells were first exposed to a mixture of mouse antibodies directed against B19 and rabbit antibodies directed against HBoV, allowing differential binding to be assessed using Texas red–labeled anti–mouse IgG and FITC-labeled anti–rabbit IgG. As shown in figure 3, B19-specific antibodies were spes-
Specific for recombinant B19 baculovirus–infected cells, whereas HBoV-specific antibodies bound to recombinant HBoV baculovirus–infected cells but B19-specific antibodies did not. Second, purified HBoV and B19 VLPs were used as antigens in an ELISA to compare the binding of virus-specific rabbit antisera. As demonstrated in figure 4, rabbit antisera raised against either HBoV or B19 VLPs did not cross-react with the heterologous virus. These data indicate that the 2 viruses are serologically distinct.

**Detection of HBoV-specific antibodies in human serum by HBoV VLP–based ELISA.** Initial screening revealed that serum specimens from many individuals, as well as pooled human serum, contained antibodies that bound to HBoV VLPs, whereas some serum specimens, such as specimen 82, showed no detectable reactivity (figure 5A). Furthermore, some serum specimens, such as that from individual 10, contained antibodies that bound to HBoV VLPs but not to B19 VLPs (figure 5B), whereas the serum from individual 41 contained antibodies that bound to B19 VLPs but not to HBoV VLPs (figure 5C). Figure 5D shows a scattergram of OD values obtained at a 1:80 dilution for HBoV from our group of 270 serum specimens. At this serum dilution, these OD_{450} ELISA values could be operationally separated into 2 groups by means of a cutoff of 0.150, which could be used to classify samples as seropositive or seronegative. (A similar analysis was performed for B19 [data not shown], and the same cutoff value of 0.150 was determined.) On the basis of this presumption, a serum specimen negative for HBoV was defined as having an OD_{450}/H_{32895} < 0.1665 (at dilutions of 1:80, 1:160, and 1:320), with 0.1665 being the mean OD_{450} value plus 3 SDs for serum samples with an OD_{450} < 0.150. Likewise, a serum specimen negative for B19 was defined as having an OD_{450}/H_{32895} < 0.150 (at dilutions of 1:80, 1:160, and 1:320), with 0.150 being the mean OD_{450} value plus 3 SDs for serum samples with an OD_{450} < 0.150.

**HBoV infection during early childhood.** Overall, 270 serum specimens were screened for HBoV-specific antibody, and 195 (72.2%) tested positive (figure 6A). Although ≈90% of in-
Infants ≤2 months old were seropositive for HBoV, this percentage dropped to 25.0% in the 4-month-old age group. This decline likely represents waning maternally acquired antibody. However, the percentage who were seropositive rapidly rebounded, so that, for children 5–47 months old, the percentage of seropositive individuals ranged from 40.7% to 60.0%. Thus, HBoV infection is common during very early childhood, with infection in the majority of children appearing to follow rapidly after the waning of maternal antibody protection. This incidence is relatively stable until the fourth and fifth years of life, when the seroprevalence of HBoV antibody rises sharply to 85% in the ages 48 months. In light of the scattergram (figure 5D), it appears that an OD450 of 0.25 may be a reasonable breakpoint to distinguish between seropositive and seronegative specimens (although this value is not based on statistical analyses). Of the 17 seropositive specimens that would be reclassified as seronegative on the basis of this cutoff value, 13 were from children <12 months old. The general trend in the percentage of seropositive individuals <12 months old did not change with this classification.

Volume constraints allowed only a subset of 208 serum specimens to be screened for both HBoV- and B19-specific antibody, of which 153 (73.6%) tested positive for HBoV-specific antibody and 60 (28.8%) tested positive for B19-specific antibody. The seroepidemiological data for B19 were quite different from those for HBoV (figure 6B). For infants <5 months old, <45% had evidence of B19-specific antibody, suggesting that the seroprevalence of B19 in the mothers of these children was also <45%. The seroprevalence of HBoV and B19 were compared in a subpopulation in which maternally acquired antibody presumably was no longer present—specifically, in individuals ≥12 months of age. The difference between the percentages of seropositive individuals ≥12 months old for HBoV (88/110 [80.0%]) and B19 (32/110 [29.1%]) was statistically significant (P < .0001).

**DISCUSSION**

HBoV has yet to be propagated in cell culture or in experimental animals, and therefore the production of VLPs represents an ideal method for antigen production for seroepidemiological investigations. This is of particular importance for retrospective IgG studies, because it has been shown that antibodies to linear capsid determinants are transitory after infection with human parvovirus B19 and that only those to structural epitopes persist [27]. Furthermore, the VLP-based assay is quantitative and does not rely on subjective interpretations of an indirect immunofluorescence screen [21].

In light of our VLP studies, human parvoviruses HBoV and B19 appear to be antigenically distinct. This is not an unanticipated result, because the viruses are not closely related phylogenetically [1]. Nonetheless, we demonstrated that rabbit antibodies specific for either of the 2 viruses did not bind to VLPs of the heterologous virus. Furthermore, we identified several human serum specimens that contained antibodies to either HBoV or B19 but not to both viruses. These data suggest that immunity that develops as a result of infection with either HBoV or B19 will not protect against infection with the heterologous virus.

Both the HBoV VLPs and the HBoV VLP–specific antisera should prove to be powerful tools for studying the biology of HBoV, allowing us, for example, to identify the range of cell types that are permissive for HBoV infection. Furthermore, the HBoV VLP--specific antibody should be of use as a diagnostic reagent, perhaps allowing for the screening of cells in respiratory secretions by means of an indirect immunofluorescence assay.

![Figure 4. Antigenic distinctness of the human parvoviruses human bocavirus (HBoV) and B19, as demonstrated by ELISA.](https://academic.oup.com/jid/article-abstract/198/1/41/841612/46)
although the sensitivity of this antibody in such an assay remains to be determined. Last, HBoV VLPs may well be an ideal vaccine candidate, if a vaccine is deemed necessary. B19 VLPs have been shown to induce neutralizing antibodies in human volunteers [28]. Recent demonstration of the efficacy of a human papillomavirus virus vaccine provides evidence of the potential utility of VLPs for preventing infection and disease [29].

HBoV infection appears to be extremely common during early childhood. The decline in the proportion of infants <4 months old who are seropositive for HBoV likely represents waning maternally acquired antibody, as observed with RSV and human metapneumovirus, and, like infections caused by these viruses, HBoV infection appears to be very common during the first year of life [30, 31]. This decline in the percentage of seropositivity appears to overlap with the acquisition of HBoV infection during infancy and early childhood. This profile of the seroprevalence is similar to that reported in Japan [21]. The rapid acquisition of HBoV infection in this group after the loss of maternal antibody is reminiscent of the epidemiology of MVM in endemically infected laboratory populations [32]; in such populations, rather than spreading by host-to-host transmission, the virus is acquired from the environment, where it persists.
because of the rugged nature of the parvoviral virion. Further studies, particularly of infection rates in second and subsequent children, will be needed to explore this possibility. There is little, if any, information in the literature concerning the incidence of B19-specific antibodies during the first year of life, but previous studies and our data show that the incidence during the second year of life is 2–3-fold lower than the seroprevalence we report here for HBoV-specific IgG [33–35].

In our study group, the percentage of HBoV-seropositive individuals increased to >85% in children ≥4 years old. This likely represents exposure of children to the virus in day care or preschool environments and parallels the primary period of acqui-
sition for B19 infection [33–35]. Our serum specimens were collected from a clinical chemistry laboratory at a large hospital, so the results may or may not reflect the seroprevalence of HBoV in the general population. The serum specimens were obtained from children with acute or chronic disease as well as from otherwise healthy children who may have had blood sampled for screening purposes or for preoperative evaluation before elective surgery or other procedures. However, it is clear that the sero-epidemiology of HBoV is different from that of B19 in the population screened. Furthermore, our B19 data are consistent with findings previously reported for the general population [33–35], strongly suggesting that the HBoV data may also be representa-
tive of the population at large.

In conclusion, we believe this to be the first report comparing the antigenicity and seroepidemiology of HBoV and B19. Using both human and experimental serum, we demonstrated that the parvoviruses HBoV and B19 are antigenically distinct. We showed that HBoV infection is common during early childhood. The tools developed in this study will help us to better understand the biology and epidemiology of this newly recognized human pathogen.

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