Evidence of Prior Exposure to Human Bocavirus as Determined by a Retrospective Serological Study of 404 Serum Samples from Adults in the United States

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Recently, molecular screening for pathogenic agents has identified a partial genome of a novel parvovirus, called human bocavirus (HBoV). The presence of this newly described parvovirus correlated with upper and lower respiratory tract infections in children. Lower respiratory tract infections are a leading cause of hospital admission in children, and the etiological agent has not been identified in up to 39% of these cases. Using baculovirus expression vectors (BEVs) and an insect cell system, we produced virus-like particles (VLPs) of HBoV. The engineered BEVs express the HBoV capsid proteins stoichiometrically from a single open reading frame. Three capsid proteins assemble into the VLP rather than two proteins predicted from the HBoV genome sequence. The denatured capsid proteins VP1, VP2, and VP3 resolve on silver-stained sodium dodecyl sulfate-polyacrylamide gels as three bands with apparent molecular masses of 72 kDa, 68 kDa, and 62 kDa, respectively. VP2 apparently initiates at a GCT codon (alanine) 273 nucleotides downstream from the VP1 start site. Three capsid proteins assemble into the VLP rather than two proteins predicted from the HBoV genome sequence. The denatured capsid proteins VP1, VP2, and VP3 resolve on silver-stained sodium dodecyl sulfate-polyacrylamide gels as three bands with apparent molecular masses of 72 kDa, 68 kDa, and 62 kDa, respectively. VP2 apparently initiates at a GCT codon (alanine) 273 nucleotides downstream from the VP1 start site and 114 nucleotides upstream from the VP3 initiation site. We characterized the stable capsids using physical, biochemical, and serological techniques. We found that the density of the VLP is 1.32 g/cm³ and is consistent with an icosahedral symmetry with approximately a 25-nm diameter. Rabbit antiserum against the capsid of HBoV, which did not cross-react with adeno-associated virus type 2, was used to develop enzyme-linked immunosorbent assays (ELISAs) for anti-HBoV antibodies in human serum. Using ELISA, we tested 404 human serum samples and established a range of antibody titers in a large U.S. adult population sample.

Among the family Paroviridae, the genus Parovirinae has many pathogenic species such as feline panleukopenia virus (38, 46), canine parvovirus (39), and Aleutian disease virus of mink (7). However, the only human-pathogenic parvovirus is (38, 46), canine parvovirus (39), and Aleutian disease virus of Many pathogenic species such as feline panleukopenia virus /H11001

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Recently, a second potentially pathogenic human parvovirus called human bocavirus (HBoV) was isolated and assigned the species name named human bocavirus (HBoV) (2). Although HBoV DNA was detected in clinical isolates of children with lower respiratory tract infections (4, 5, 12, 30), it is unclear whether HBoV was the etiological agent or contributed to the pathogenicity of the respiratory infection (34, 45). Until recently, the presence of HBoV in respiratory secretions relied on PCR (13, 16, 32, 35, 37, 40). Using PCR, HBoV DNA has been detected worldwide with 5 to 10% prevalence among children with upper or lower respiratory tract infections (6, 22, 24, 27, 33, 36, 41, 51). However, 80% of the HBoV DNA-positive patients were coinfected with common human respiratory viruses (8, 10, 17). Thus, whatever role HBoV plays in lower respiratory tract infection remains unclear.
both the BEV-infected insect cell lysates and purified VLPs, three capsid proteins were observed, not two as predicted from the HBoV genomic sequence. Rabbit antiserum produced in response to immunizations with purified VLP developed high-titer immunoglobulins (Igs) specific for HBoV. Thus, we developed an ELISA and tested 404 serum samples from adults. The data obtained from these sera produced a broad range of titers, suggesting that the prevalence of prior exposure to HBoV in the United States adult population is 59 to 67%.

MATERIALS AND METHODS

Bocavirus sequence and modification. The open reading frame of the bocavirus capsid proteins (or virus proteins), encoded by the gene, is based on the previously published HBoV-st2 sequence (GenBank accession number DQ000496) (2). By introducing several sequence modifications into the capsid protein genes, a bicistronic mRNA was utilized to produce VP1 and VP2 in the baculovirus system (Fig. 1). A single open reading frame encodes both the large and small capsid proteins, with the larger capsid protein (VP1) and internal translational initiation producing the smaller, major capsid protein. To prevent translation initiation at a noninitiating AUG triplet, the three out-of-frame ATG triplets in the VP1 unique sequence were altered without changing the amino acid. Translation initiation at a noninitiating AUG, three out-of-frame ATG triplets in the VP1 unique sequence were altered without changing the amino acid. Optimization of the HBoV genome for production of virus proteins in a baculovirus system. The VP1 unique region and a VP1 gene, is based on the HBoV genomic sequence. Rabbit antiserum produced in response to immunizations with purified VLP developed high-titer immunoglobulins (Igs) specific for HBoV. Thus, we developed an ELISA and tested 404 serum samples from adults. The data obtained from these sera produced a broad range of titers, suggesting that the prevalence of prior exposure to HBoV in the United States adult population is 59 to 67%.

VLP production and purification. The capsid or virus proteins of HBoV were produced by infecting 100 ml of SF9 cells (2 × 10^6 cells/ml) with clonally isolated baculovirus at a multiplicity of infection of 3 PFU/cell. At 72 h postinfection, cells and supernatant were separated by centrifugation (900 × g × 15 min). The VLP in the supernatant was recovered by precipitation in 2.5% (final concentration) polyethylene glycol (PEG) (5 ml of 50% PEG 8000 [Sigma-Aldrich, St. Louis, MO] added to 95 ml of supernatant) and incubated for 3 h at 4°C with gentle agitation. Cell pellets were resuspended in 7 ml of phosphate-buffered saline with 2 mM MgCl₂ and disrupted using a 7-ml Dounce homogenizer. The cell lysates were sedimented by centrifugation at 900 × g for 15 min and resuspended by precipitation in 2.5% PEG as described above. The PEG-precipitated material was recovered by centrifugation (45 min at 2,600 × g), and the pellets were resuspended in 11 ml of CsCl solution (refractive index [RI] = 1.372 or ρ = 1.41 g/cm³). The CsCl solutions were centrifuged to equilibrium (72 h at 222,000 × g) in a swinging-bucket rotor (SW41 rotor; Beckman Coulter, Inc., Fullerton, CA). Each centrifuge tube was fractionated dropwise via bottom puncture using a 28-gauge butterfly needle set, 0.5 ml fractions were collected, and the RI of each fraction was measured with a digital refractometer (AR 200; Leica Microsystems, Inc., Buffalo, NY). Typical parovirus empty-particle densities ranged from 1.30 to 1.32 g/cm³, corresponding to an RI of 1.362 to 1.364 (21). Size exclusion column chromatography (Sephadex 200, 10/300; GE Healthcare BioScience Division, Piscataway, NJ) provided the final purification step. The homogeneity of the HBoV VLP was assessed by silver-staining sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Larger amounts of VLP were produced using 5-liter bioreactors (Wave mixer; GE Healthcare BioScience Division, Piscataway, NJ). The purification protocol described above was modified for scale but otherwise remained the same. The theoretical, 280-nm molar extinction coefficient (117,855 M⁻¹ cm⁻¹) was determined using ProtParam software (18) and used to calculate the protein concentration by UV absorption.

Antibody production and purification. Rabbit anti-bocavirus VLP immune serum was produced using a standard 70-day prime-boost regimen. In brief, 200 µg of purified HBoV VLP was administered intramuscularly to the rabbit followed by three intramuscular boosts at 21, 35, and 49 days. Initially, a preimmunization sample was obtained, and subsequently, serum samples were collected at 44, 59, and 63 days postimmunization. The mean specific antibody concentration was estimated to be 0.15 to 0.5 ng/ml.

Purification of HBoV-specific antibody from human serum and rabbit serum was performed with either an affinity column prepared by covalently attaching HBoV-VLP to a 1-mI HiTrap N-hydroxysuccinimide-activated HP Sepharose column (GE Healthcare Biosciences) or using a 1-mI protein G-Sepharose column (GE Healthcare Biosciences) as indicated.

PAGE and Western blotting. Polyacrylamide gel electrophoresis (PAGE) was used for determining the apparent molecular mass and estimating VLP homogeneity. Ten microliters of loading buffer and 4 µl of reducing agent (NuPage system; Invitrogen Corp.) were added to 26 µl of sample and heated at 70°C for 10 min prior to electrophoresis. Samples were applied (10 µl per lane) to a precast 4 to 20% polyacrylamide gel and electrophoretically fractionated in a morpholineethanesulfonic acid-SDS buffer system (NuPage; Invitrogen) at 150 V (constant voltage) for 60 min. Protein bands were visualized with either Coomassie brilliant blue (SimpleBlue SafeStain; Invitrogen) or silver staining (SilverQuest; Invitrogen) according to the recommendations of the manufacturer. For Western blot analysis, proteins were electroblotted from the gel onto a nitrocellulose membrane according to the manufacturer's protocols (iBlot system; Invitrogen).
Electron microscopy. The Electron Microscopy Core Facility of the National Heart, Lung, and Blood Institute of the National Institutes of Health (NIH) (Mathew P. Daniels, Director) supported the ultrastructure analysis of the HBoV VLP specimens using a transmission electron microscope (JEM1200EX; Jeol, Ltd., Tokyo, Japan) equipped with an AMT XR-60 digital camera (Advanced Microscopy Techniques, Danvers, MA). The VLP samples were diluted, and a small aliquot, e.g., 10 to 20 μl, was pipetted onto parafilm film. An aliquot of each sample (5 μl) was placed onto a carbon-coated 200-mesh copper grid for 1 min and then washed with 10 drops of distilled water. Staining was achieved by adding 5 drops of 2% (wt/vol) uranyl acetate. Excess staining solution was immediately wicked away with blotting paper, and the grids were then air dried. The grids were examined with a transmission electron microscope, and micrographs of randomly selected fields were taken at various magnifications.

VLP-based ELISA. The optimum conditions for ELISA, including coating concentration of the VLP protein, serum dilution, conjugate dilution, incubation times, temperature, and blocking reagent, were determined in preliminary checkerboard titration experiments. To optimize ELISA, bocavirus-VLP proteins were diluted to 2 mg/ml in 0.2 M transmission electron microscope (JEM1200EX; Jeol, Ltd., Tokyo, Japan) equipped with an AMT XR-60 digital camera (Advanced Microscopy Techniques, Danvers, MA). The VLP samples were diluted, and a small aliquot, e.g., 10 to 20 μl, was pipetted onto parafilm film. An aliquot of each sample (5 μl) was placed onto a carbon-coated 200-mesh copper grid for 1 min and then washed with 10 drops of distilled water. Staining was achieved by adding 5 drops of 2% (wt/vol) uranyl acetate. Excess staining solution was immediately wicked away with blotting paper, and the grids were then air dried. The grids were examined with a transmission electron microscope, and micrographs of randomly selected fields were taken at various magnifications.

Statistical testing. Chi-squared or Fisher’s exact test was performed to determine differences between females and males and as a function of age of the donors (1, 15).

**RESULTS**

**HBoV VLP purification.** Modifying the capsid protein sequence (Fig. 1) allowed us to produce HBoV capsid proteins using a single BEV in SF9 cells. In BEV-infected SF9 cells, the capsid proteins assemble into particles that are physically separable from other cellular components. Using CsCl-isopycnic gradients, an obvious opalescent band appeared approximately midway in the gradient at a density of 1.33 g/cm3 (Fig. 2a). Assuming that the VLP consists of approximately 60 capsid proteins, the molecular mass is therefore approximately 4 MDa, making the VLP much larger than other soluble cellular components, allowing separation by size exclusion chromatography. The VLP fraction elutes from a Sephadex 200 sizing column in the void volume well separated from the smaller cell and baculovirus proteins and CsCl salt, as indicated by the UV (VLP) and conductivity (salt) traces (Fig. 2b). The protein composition of the column fractions was assessed by SDS-PAGE and silver staining (Fig. 2c). Three distinct bands appeared in the VLP fractions, with masses of 72 kDa, 68 kDa, and 62 kDa, corresponding to VP1, VP2, and VP3, respectively.
the first residue of VP2 is the alanine corresponding to VP1 residue 92.

**VLP physical characterization.** Electron microscopy performed on purified samples confirmed the formation of VLPs (Fig. 3a). Consistent with the *Parvoviridae*, the coexpressed VP polypeptides assembled into uniformly shaped particles with apparent icosahedral symmetry. According to the electron microscopy analysis, the estimated particle diameter is between 18 and 25 nm. Dynamic light-scattering (DLS) analysis provides a means for measuring nanoparticle sizes in solution and characterizing the state of the VLP in solution (model number 802; Viscotek, Houston, TX). The DLS data indicate that the solution contains a range of particles that average 27.7 nm in diameter. Although the peak is not perfectly symmetrical, the steep leading edge suggests that monodisperse particles are a prominent component in the solution. The more gradual trailing edge suggests that particle dimerization and possibly higher-order interactions exist in solution, at least under these conditions (Fig. 3b).

**Polyclonal antibody cross-reactivity.** Rabbit immune serum was tested for cross-reactivity to AAV, an unrelated parvovirus. Using single-lane PAGE, AAV or HBoV capsid proteins were transferred onto a membrane and incubated with serially diluted rabbit immune sera in a multilane blotting apparatus. The HBoV and AAV antisera were tested for cross-reactivity to either AAV (Fig. 4a) or HBoV (Fig. 4b) capsid antigens using serum dilutions of 1/1,000 to 1/250,000. The Western blots indicate that the antisera did not react with the heterologous virus capsid antigens. A sequence alignment of HBoV capsid protein and AAV capsid proteins indicates that there are three regions of similarity greater than 66% consisting of 49, 33, and 11 amino acids. The largest region of similarity is located in a unique region of VP1 that overlaps with (or contains) the phospholipase A2 motif (49 to 53 residues). The phospholipase A2 motif is required for infectivity by facilitating endosomal escape (19) and is highly conserved among the *Parvoviridae* (53). Structural analysis predicts that these residues are located internally (25) and are unlikely to interact with circulating neutralizing Igs. The two other regions are located in VP3, 91 and 471 residues after the VP3 starting codon.

**VLP-based ELISA optimization.** To determine the optimal concentration of antigen for the ELISA, serially diluted HBoV-VLP (0.1 to 15 µg/ml) was used to coat the plate. The optimum concentration, defined as the antigen concentration producing the maximum signal in the colorimetric assay using the least amount of antigen, was 2 µg/ml (Fig. 5a). Conditions for detecting anti-HBoV antibodies in human serum samples were established using rabbit immune serum as a surrogate. Microtiter plates were coated with HBoV VLP (2 µg/ml), and using a twofold dilution series of rabbit immune serum (1:100 to 1:12,800), a single concentration of rabbit serum was added to each row of wells. Each dilution of the secondary antibody goat anti-rabbit IgG-HRP (1:5,000, 1:15,000, or 1:30,000) was then added to a column of wells, incubated for either 5 or 15 min, and processed as described in Materials and Methods. The best response over the greatest range of immune serum dilutions (Fig. 5b) without saturation of the color and in a reasonable incubation time was obtained using a serum dilution of 1:12,800 and a secondary antibody dilution of 1:5,000.

![FIG. 3. Physical characterization of VLPs.](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5654630/)
produced using a 1:30,000 dilution of secondary antibody with a 15-min incubation time.

**VLP-based ELISA of human samples.** Serum samples from 404 adults were tested for the presence of HBoV-Ig using an ELISA method. The response ranges were uniformly distributed between the highest serum response and the lowest serum response and are presented graphically in Fig. 6. Every clinical sample value fell between values for the rabbit preimmune serum (negative control) and the rabbit immune serum (positive control). From these results, we categorized the higher values obtained with human sera as positive samples and, conversely, the lower values obtained with human sera as negative samples. The test produced typical sigmoid ELISA curves with an end point (optical density value below 0.3) at a 1:1600 dilution for negative sera and an end point at 1:25,600 for positive sera. With these parameters and under these conditions, in a cohort of 404 adults, we found that 63% were serologically positive for HBoV. In Table 1, we categorized the response results by age and sex. The prevalence tended to increase with age and was slightly higher in women. Age groups of men between 18 and 65 years of age had 59% to 65% seropositivity, whereas women between 18 and 65 years of age had 57 to 73% seropositivity.

**ELISA using purified polyclonal antibody.** To confirm that specific HBoV antibodies were present in human sera, we fractionated total Ig antibodies or specific HBoV-Ig antibodies using a protein G column or an HBoV-VLP affinity column, respectively. The eluted Ig fractions were tested for the presence of HBoV-Ig, AAV2-Ig, and AAV8-Ig using plates coated with HBoV-VLP, AAV2-VLP, or AAV8-VLP. In Fig. 7, we plotted the ELISA results for the crude human serum, the elution profiles of protein G Ig purification, and the elution profiles of the HBoV-VLP affinity column for the three different viral-protein-coated plates. Crude human serum was positive for HBoV, AAV2, and AAV8. After protein G purification, the samples remained positive for the three different
viral proteins transcriptionally or posttranscriptionally was a critically important development for producing capsids with a defined stoichiometry of structural proteins (11, 50). Therefore, the regulation of posttranscriptional protein levels was adapted for HBoV VLP production. Members of the Dependovirinae naturally regulate the stoichiometry of the capsid proteins by alternative splice acceptors and translational initiation at non-AUG codons (49). The relatively inefficient translational initiation of VP2 at a non-AUG codon caused by rRNA readthrough produces high levels of VP3 translation from the initiating AUG codon. We adapted this strategy for HBoV VLP production by substituting an ACG for the predicted VP1 AUG starting codon. Although there are no consensus Kozak elements bordering this presumptive initiation codon, it is the upstream AUG codon proximal to the Parvoviridae invariant phospholipase-like domain. Unexpectedly, we found that in addition to VP1 and the major coat protein, a third intermediate-size capsid protein was present in the VLP. Protein sequence determination of the first 8 amino acids allowed us to characterize the amino-terminal residue of this protein. This capsid protein, VP2, starts at codon 91 of VP1, which codes for alanine. The preceding codon is a GTG, which in a Kozak context has the potential to initiate protein translation as follows, where “seq” is sequence and “Prot” is protein:

\[
\text{DNA seq:} \quad \text{gcc GTG gct cct gct ctg gga aat aga gag} \\
\text{Prot seq:} \quad \text{M? A P A L G N K E}
\]

However, because no mammalian tissue culture system for HBoV propagation is available and there are no sources of wild-type HBoV, the possibility that VP2 expression results from idiosyncratic baculovirus expression remains. Thus, the strategy for regulating the expression levels of the capsid proteins remains unresolved. Alternative splicing is a common method used by cells to produce two or more proteins from the same primary transcript. However, we were not able to identify a splicing region between VP1 and VP2, nor were sequence-specific protease cleavage sites identified at the site of the new capsid protein, limiting the possibility of posttranslational modification. Typically, AAVs use a noncanonical starting codon to produce several proteins in the same reading frame. In the case of HBoV, the same strategy is possibly used to produce two or three proteins derived from a common open reading frame. A weak AUG VP1 initiation codon lacking both the upstream and downstream Kozak flanking motifs followed by the putative VP2 GUG initiation codon in a Kozak

### TABLE 1. Categorization of ELISA results by age and sex

| Age range | Men | Women |
|-----------|-----|-------|
|           | Avg age (yr) | Total no. of patients | No. of positive patients | % Positive patients | Avg age (yr) | Total no. of patients | No. of positive patients | % Positive patients |
| Total     | 31  | 246   | 147  | 59.76   | 30.79  | 158   | 106  | 67.09  |
| 18–25     | 22  | 92    | 55   | 59.78   | 22.25  | 71    | 40   | 56.34  |
| 26–35     | 30.21 | 82    | 47   | 57.32   | 29.94  | 48    | 36   | 75     |
| 36–45     | 39.26 | 55    | 34   | 61.82   | 40.09  | 27    | 22   | 81.48  |
| 46–65     | 53.91 | 17    | 11   | 64.71   | 51.75  | 11    | 8    | 72.73  |

*a Curves with an end point (optical density value below 0.3) at a dilution 1:1,600 are scored as negative sera, whereas curves with an end point at a dilution of 1:25,600 are scored as positive sera. Between 59 and 81% of ELISAs were found to be positive, increasing with age and being slightly higher in women.
context and, finally, the VP3 AUG initiation codon might be used. If there is one mature transcript derived from the capsid protein gene, this strategy may result in the production of three capsid proteins at a ratio of 1:(1 to 5):10.

The BEV constructs allowed us to produce and purify a stable HBoV VLP composed of VP1, VP2, and VP3 for use as an antigen and ELISA substrate. Using this VLP, we conducted a seroepidemiology study of a sample of 404 adults living in the United States. We observed a broad distribution of randomly distributed serology results. As observed in others studies using VP2 or VP1 HBoV ELISA (14, 23, 29), we found a high level of seropositivity: 63% of the human samples that we tested were positive for HBoV antibody, suggesting prior exposure, e.g., infection, with HBoV. The small sample size for the female cohort, ages 46 to 65 years, is not statistically representative. However, the seropositivity prevalence tended to increase with age and was statistically slightly higher in women. Because we have no information about the medical history of the serum donors, it is impossible to correlate the serology results with disease. However, the development of an ELISA using an HBoV VLP should enable a prospective study to develop correlations between HBoV infection and human disease or associations with other viral infections.

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