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ELISAs USING HUMAN BOCAVIRUS VP2 VIRUS-LIKE PARTICLES FOR DETECTION OF ANTIBODIES AGAINST HBoV

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Abstract

Human bocavirus (HBoV) has been identified worldwide in children with lower respiratory tract infections with an incidence of approximately 2% −11%. The role of HBoV in pathogenesis, however, is largely unknown, and little is known about the epidemiology of the virus. To study the seroepidemiology of HBoV infection, the capsid protein was expressed in insect cells. Expression of the putative major capsid protein VP2 in insect cells led to the formation of virus-like particles exhibiting the typical icosahedral appearance of parvoviruses with a diameter of approximately 20 nm. The expressed particles were used to establish an ELISA method, and serum samples from groups of children of various ages in China were tested for IgG antibodies against HBoV. HBoV antibodies were detected in as high as 36% of healthy children under 9 years. Of children hospitalized with lower respiratory tract infections, 31% were seropositive, and all age groups of these children showed a significantly higher level of HBoV IgG antibody than their healthy counterparts. When divided into age cohorts, results showed that more than 48% of healthy children had seroconverted by age of 4. Thus, HBoV appears to be a common infection in children. The potential pathogenesis of this virus, especially its role in lower respiratory tract infections in children warrants further investigation.

Keywords

Human Bocavirus (HBoV); Parvovirus; Virus-Like Particles (VLPs); Antibody

1. Introduction

Human bocavirus (HBoV) is a member of the genus *Bocavirus* of the subfamily *Parvovirinae* (Cotmore et al., 2006). The *Bocaviruses* so far identified include bovine parvovirus type 1 (BPV) (Chen et al., 1986), canine minute virus (CnMV) (Schwartz et al., 2002) and the recently identified human bocavirus (HBoV) (Allander et al., 2005). BPV causes diarrhea and mild respiratory symptoms in calves inoculated intranasally (Via et al., 2006).

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CnMV is associated with fetal infections leading to reproductive failure and neonatal respiratory disease (Parrish, 2006). HBoV was first cloned from pooled human respiratory tract samples collected in Sweden and was classified provisionally into the genus *Bocavirus* based on sequence comparisons (Allander et al., 2005). However, similar to the bovine bocavirus BPV, HBoV was recently reported to be associated with acute gastroenteritis (Lau et al., 2007;Lee et al., 2007;Mackay, 2007).

The HBoV genome has been detected in respiratory tract infections. The incidence of HBoV has been reported to be between 1.5% to 11.3% based on tests of respiratory samples from individuals with acute respiratory illness (Allander et al., 2005;Arnold et al., 2006;Bastien et al., 2006;Choi et al., 2006;Foulongne et al., 2006;Lin et al., 2007;Ma et al., 2006;Qu et al., 2007;Sloots et al., 2006;Weissbrich et al., 2006). HBoV appears to be associated with lower respiratory tract infections, and in many cases, co-infection with other respiratory viruses (Allander et al., 2007;Fry et al., 2007). The frequency of detection suggests that HBoV is less common than respiratory syncytial virus and probably also rhinoviruses in infants with respiratory illnesses. However, the virus is approximately as common as influenza viruses, human metapneumovirus, parainfluenza virus 3, and adenoviruses and is probably more common than coronaviruses and the other parainfluenza viruses (McIntosh K, 2006).

The exact role of HBoV in pathogenesis of lower respiratory tract infections is unknown and requires further study. At this time, neither virus isolation nor infectious clone has been reported. Serological study of HBoV infection in Japan has shown an overall seroprevalence rate of 71.1% against the VP1 protein of HBoV using an immunofluorescence assay in a population aged from 0 months to 41 years (Endo et al., 2007). Currently, detection of human bocavirus in children with lower respiratory tract infections relies on DNA amplification by PCR, however, PCR assays do not reflect the course of HBoV infection. Therefore, a method to detect HBoV infection in a large scale is required to diagnose and characterize the role of HBoV in human disease. Purified HBoV proteins are needed to develop an accurate and efficient enzyme-linked immunosorbent assay (ELISA) to help characterize potential HBoV etiology of lower respiratory tract infections.

A 5,299 segment of the HBoV genome has been sequenced (Allander et al., 2005). This segment lacks both the left and right-hand palindromic hairpin termini, and therefore, the plasmid containing this sequence of HBoV is not infectious. The genomic organization of HBoV closely resembles that of BPV (Allander et al., 2005;Qiu et al., 2007). There are two major ORFs encoding a nonstructural protein (NS1) and at least the two capsid proteins VP1 and VP2, respectively. The recombinant VP1 protein was expressed in insect cells, and used successfully in detection of specific antibodies against HBoV infection by an immunofluorescence assay (Endo et al., 2007).

In this study, the major capsid gene VP2 of HBoV was expressed in insect cells. Expression of VP2 in insect cells led to the formation of virus-like particles (VLPs) which have the typical icosahedral appearance of parvoviruses with a diameter of approximately 20 nm. Purified VLPs were used to establish an ELISA for detection of IgG antibodies against HBoV in human sera from groups of children of various ages in China. These results show that HBoV infection appears to be common in children.

2. Materials and Methods

2.1 Expression of capsid protein VP2 in Sf9 cells

The HBoV VP2 gene (nts 3443–5068) of the Stockholm 1 isolate (ST1) (Allander et al., 2005) was synthesized using an overlapping PCR strategy based on the published sequence (GenBank accession no.: NC_007455). This synthetic VP2 gene was confirmed by sequencing,

and was inserted into the baculovirus expression transfer vector (pFastBac1) (Invitrogen) with or without an HA tag. Generation of recombinant baculoviruses (BacHBoVCap and BacHBoVCapHA) was followed according to a commercially available kit (Bac-to-Bac, Invitrogen).

2.2 SDS-PAGE and Immunoblot

SDS-PAGE and Immunoblot analysis were essentially done as previously described (Qiu et al., 2006).

2.3 Purification of VLPs

Sf9 cells were infected with BacHBoVCap1 at a multiplicity of infection (m.o.i) of 10 and harvested 2 days later by low speed centrifugation. After removing the supernatant, the cells were resuspended in 25mM NaHCO3 solution at 2×10^7 cells/ml and left on ice for 20 min. The resulting cells were lysed, and the VLPs were recovered from the suspension. Debris was removed by centrifugation. To the supernatant was added saturated $(NH_4)2SO_4$ to a final concentration of 20%, and the pellet was collected by low-speed centrifugation. This VLP-containing solution was adjusted to a density of 1.40 g/ml by adding solid CsCl, which spun at 36, 000 rpm (Sorvall TH641) for at least 36 hrs at 20ºC. Fractions (~0.5 ml) were taken from the bottom, and their refractive index was measured using an ABBE refractometer. Fractions contained an approximate density of ~1.32 g/ml, as expected for empty parvovirus capsids, and were dialyzed against PBS.

2.4 Observation of VLPs by electron microscope

Negative staining was performed using a modification of the standard two-step drop method (i.e., adsorption followed by negative staining). The final VLPs preparation was stained with 1% aqueous uranyl acetate, pH 4.5 (Hayat, 1986), and images were resolved under the JEOL 1200EX Transmission Electron Microscope at the Electron Microscope Core Facility, University of Missouri-Columbia.

2.5 Establishment of ELISA for detection of HBoV IgG

The VLPs formed by HBoV VP2 capsid proteins were employed to establish the ELISA to detect IgG antibodies that react to HBoV. Briefly, purified VLPs were coated onto 96 well plates in coating buffer (100mM NaHCO3, pH9.6 buffer or neutral PBS, pH7.2 buffer). The coating concentrations of VLPs were determined at various concentrations (0.5 μg /ml, 1 μg / ml, 2 μg /ml, 2.5 μg /ml, 5 μg /ml and 10 μg /ml). After coating, plates were washed three times with PBS-T (0.05% Tween 20 in PBS) and blocked by adding 200 μl of 5% non-fat milk in PBS-T at 37 °C for 60 min. Serum samples were added in duplicate at a 1:200 dilution or serial dilution in PBS-T for 60 min of reaction at 37 °C. Following three washes with PBS-T, a goat anti-human IgG HRP conjugate (Sigma), 1:10,000 diluted in PBS-T, was added for 60 min at 37 °C, followed by adding a substrate of O-phenylendiamine dihydrochloride (Sigma, St. Louis, MO). The absorbance of each serum at OD 450nm was read and the mean value of the duplicates was calculated.

2.6 Antibody detection of other agents that can cause acute respiratory infection

Total antibody detection of influenza virus A and B, respiratory syncytial virus, parainfluenza virus and adenovirus was performed using the complement-fixation test on the automated Seramat system with the manufacturer's diagnosis kit (Diesse, Monteriggioni, Italy). Titers of 1:8 or greater as detected by the Seramat system were considered positive.

2.7 Quantitative PCR detection of HBoV genome in nasopharyngeal aspirate samples

nasopharyngeal aspirate samples for HBoV genome detection were collected simultaneously, and real-time quantitative PCR was performed as previously described (Lin et al., 2007). 200 μl of nasopharyngeal aspirate samples were used for extraction of DNA by the Blood DNeasy kit (Qiagen, Valencia, CA).

2.8 Hemagglutination assay

The hemagglutination assay was performed in 96-well U-bottom plates (Becton Dickinson, Franklin Lakes, NJ). Briefly, human "O" type red blood cells were centrifuged at 2,500 rpm for 10 min. Red blood cells were then washed twice and resuspended in Alsever's buffer (20mM sodium citrate, 72 mM NaCl, 100mM sucrose, pH 6.5) at a final concentration of 0.5%. Serial twofold dilutions of BPV (a gift from Dr. F. Brent Johnson) (Johnson et al., 2004) or HBoV VLPs were prepared in Alsever's buffer in a volume of 50 μl. Then, 50 μl of red blood cells were added into each well and mixed. Plates were incubated at 4°C overnight. The hemagglutination titers were expressed at the minimum virus concentration required to hemagglutinate the red blood cells suspension completely.

2.9 Clinical samples

From January to December 2006, 161 serum and nasopharyngeal aspirate samples were collected from children with a clinical diagnosis of lower respiratory tract infections on the day they were hospitalized in the Wenling Hospital of Wenzhou Medical College, Zhejiang Province, China. The standard clinical diagnosis of lower respiratory tract infections was made by x-ray chest examination and by the presence of typical acute respiratory tract infection symptoms, including cough, dyspnea, wheeze, and hypoxia. A total of 233 control serum samples were collected from healthy children, as determined by standard physical examination in theWenling Hospital of Wenzhou Medical College, Zhejiang Province, China. All the nasopharyngeal aspirate samples and serum samples were taken after informed consent was obtained from parents or other legal guardians.

20 serum samples from healthy US blood donors aged 18–65 were purchased from, and were certified as negative for HBV, HCV, HIV and HTLV-I and II by, Innovative Research, Inc. (Southfield, MI).

3. Results

3.1 Expression of HBoV VP2 ORF in Sf9 cells and assembly of VLPs

The putative ORF of the major capsid protein VP2 of HBoV (nts 3443–5071) of the Stockholm 1 isolate (ST1) (Allander et al., 2005) was synthesized using an overlapping PCR strategy and inserted into the baculovirus expression transfer vector, pFastBac1 (Invitrogen), with or without an HA tag (YPYDVPDYA). Generation of recombinant baculoviruses was performed according to the protocol of the Bac-to-Bac kit (Invitrogen). Finally, 4 recombinant baculoviruses (BacHBoVCap1&2 and BacHBoVCapHA1&2) were generated. Sf9 cells were infected with the third-passage stock of these viruses at an m.o.i.=1, and two days later, cell lysates were prepared and subjected to SDS-PAGE and immunoblot analysis. Results showed that expression of VP2 reached approximately 60% of total cellular proteins (Fig. 1A, lanes 1–4). The identity of the VP2 band, which has a molecular weight of approximately 60 KDa, was confirmed by immunoblot probed with an anti-HA antibody (Fig. 1B., lanes 1 and 2).

BacHBoVCap1, which had no HA tag, was used as a seed for follow-up preparation of VLPs. The final VLPs preps were analyzed by standard negative staining under an electron microscope (Foulongne et al., 2006). A typical icosahedral appearance of parvoviruses with a diameter of approximately 20 nm was uniformly seen (Fig. 2), indicating that the HBoV VP2

protein can form a parvovirus-like particle. Isolation of the naturally circulating human *Bocavirus* has not been successful as yet.

The human B19 parvovirus and the bovine bocavirus BPV, or VLPs formed by expressed capsid protein of these viruses, have the capability to hemagglutinate human red blood cells (Brown et al., 1992;Brown et al., 1994; Johnson et al., 1973;Johnson et al., 2004). Surprisingly, as shown in Fig. 3, the VLPs of HBoV did not hemagglutinate human red blood cells even at a high concentration (0.5 mg/ml), whereas purified BPV (0.5 mg/ ml) had an hemagglutination titer as high as of 1:81920. The result from the hemagglutination assay suggested that HBoV may be more like the human adeno-associated virus type 2 (AAV2) (Walters et al., 2001) in that they do not hemagglutinate human red blood cells (Walters et al., 2001). That HBoV cannot be detected by hemagglutination assays is representative of the difficulties in detecting or isolating this virus from clinical specimens.

3.2 Establishment of an ELISA for detection of HBoV IgG antibody

Because adults have often been exposed to many respiratory viruses, as well as the human parvoviruses B19 (Anderson et al., 1986) and AAVs (Chirmule et al., 1999;Peden et al., 2004), 20 serum samples from blood donors from the United States (Innovative Res. Inc., Southfield, MI) were used to test reactivity to the HBoV VLPs. First, we optimized the concentration of the coated VLPs for our assay. A concentration of 2.5 μg/ml in 100 mM NaHCO3, pH 9.6, was determined to develop an OD value of approximately 1.0 at 450 nm with two strong reactive serum (Serum #2 and #17) at a dilution of 1:100 in PBS (Fig. 4A). Serum #2 and #17 were also confirmed to be strongly reactive with the VP2 protein by immunoblot (Fig 4B, lanes 1 and 2). Next, ELISAs were performed on serially diluted sera from selected healthy blood donors. The test produced typical ELISA curves with an end point (with an OD value below 0.2 at 1:400 dilution for negative sera and 1:6400 for positive sera) (Fig. 4A). The presumed negative serum #13 and #14 were also confirmed to be so by immunoblot (Fig. 4B, lanes 3 and 4). Since all strongly reactive and presumed negative sera gave discriminatory results at 1:200 dilutions, this dilution was chosen to analyze all subsequent serum samples.

Next, 8 of the 20 serum samples from blood donors were tested under two different coating conditions: 100mM NaHCO3, pH 9.6 *vs.* PBS, pH7.2. No significant difference between these conditions was detected for any of the 20 samples tested (Table 1). The higher pH buffer of 100mM NaHCO3, pH 9.6 may have been able to disassemble VLPs (Wang et al., 2005), and thus more antigenic epitopes may have been exposed. For this reason, in order to be as inclusive as possible, we chose the high pH buffer (100mM NaHCO3, pH 9.6) to use for our large-scale analysis. To determine the specificity of the assay, the purified bovine bocavirus BPV (a gift from Dr. F. Brent Johnson) (Johnson et al., 2004) was coated at a concentration of 2.5 μg/ml. All these tested sera showed very low OD values (under 0.14), indicating that IgG antibodies in these positive sera (#2, 10, 16 and 17) were specific for the HBoV VP2 antigen (Table 1).

To define a cut-off value, the positive to negative (P/N) ratio was calculated, where P=OD reading with the HBoV-VLP antigen, and N=OD reading with the control BPV antigen. The mean (N) of these 20 sera from healthy blood donors was 0.116. Therefore, a cut-off value of 0.3, which is equal to a P/N value of 2.5, was used in our data analysis. Thus, all the serum samples with an OD of 0.30 were putatively considered IgG positive for HBoV in this study. When this assay was applied to the remaining adult serum samples it was determined that 50% (10/20) of the sera from these healthy adult US blood donors aged 18–65 years were positive for HBoV IgG (cut-off=0.3), some with a high titer [e.g., serum #2 with a titer of 1:1600 (Fig. 4A)].

3.3 Detection of HBoV IgG in serum samples by the HBoV VLPs-ELISA

All the serum samples used for detection of HBoV IgG antibody were taken from children hospitalized with lower respiratory tract infections, and which had been previously screened, using the automated complement-fixation test, to be antibody negative (<1:8) for influenza virus A and B, respiratory syncytial virus, parainfluenza virus and adenovirus.

HBoV IgG antibodies in all the sera were detected by ELISA using VLPs coated with buffer containing 100mM NaHCO3. The OD values of all the sera were categorized in groups based on age: <6 months, 7–12 months, 13–24 months, 25–36 months, and >3 years (but no older than 9 years) for sera from children with lower respiratory tract infections (Fig. 5A); and 0–12 months, 13–24 months, 25–36 months, 4–9 years and >9 years for sera from healthy children (Fig. 5B). In both groups, the levels of HBoV IgG antibody, as detected by reactivity to the VLPs and expressed as an OD value at 450nm, increased as age increased. This is similar to the seroprevalence of human B19, the first human parvovirus identified to cause disease (Anderson et al., 1986;Brown et al., 1994). In the group of healthy children over 9 years of age, nearly half (48%) had apparently been previously infected with this virus, as the level of IgG antibody was high in this group (Fig. 5B). Therefore, HBoV infection in children clearly occurs frequently as the virus specific antibodies last longer than the viruses in patient after an acute viral infection.

A significant difference in the OD values was observed when the sera from healthy children were compared to sera from children with lower respiratory tract infections in similar age groups ($P<0.05$) (Fig. 5, compare the symbols above the cut-off line in A with those in B). This suggested that HBoV infection had occurred more frequently in children with lower respiratory tract infections. In children with lower respiratory tract infection younger than 3 years of age, 11/134 samples were found to be strongly positive (OD>0.8), while no serum sample (out of 66 tested) from healthy children of this age group had an OD value over 0.8. Only five serum samples from healthy children of any age had an OD over 0.8, and these samples were from children older than 3 years of age.

Interestingly, of the nasopharyngeal aspirate samples collected from 161 children with lower respiratory tract infections, only 5 were found to be HBoV genome positive by quantitative PCR with a range of genomic copies from 6.6×10^3– 2.6×10^6/ml. Of these 5 samples, 3 that had genomic copies over 1×10^6/ml were strongly serum IgG positive, with an OD value of 1.5, 1.2 and 0.7, respectively; 2 were mildly positive with an OD of 0.5 and 0.4 (Table 2). These results suggest an acute or recent infection of HBoV in these patients.

4. Discussion

In this study, HBoV VLPs, purified from Sf9 cells that expressed the putative HBoV VP2 capsid protein, were employed successfully in an ELISA to detect IgG antibodies in human serum samples. This ELISA showed specificity for the HBoV VP2 protein; no cross-reactivity was observed with the closely-related bovine *Bocavirus* BPV capsid. This is the first report to demonstrate that the HBoV VP2 protein is capable of forming VLPs, and the second to show the presence of HBoV specific IgG antibody in human sera. Importantly, a significantly higher level of HBoV IgG antibody in relative age groups of children with acute respiratory infection was found compared with that of children without acute respiratory infection, suggesting that HBoV may be an agent causing or playing a role in acute respiratory infection in children. Nevertheless, characterization of the presence of IgM antibody or IgG in double serum samples is required in subsequent studies for precisely evaluating the status of infection of HBoV in acute respiratory infection. In addition, a high positive rate of HBoV IgG antibody in sera from healthy U.S. blood donors (aged 18–65 years) was observed in this study, indicating HBoV infection is widespread in the U.S.. Together, these results also indicate that HBoV is likely

first encountered in childhood, which is consistent with previous suggestions (Allander et al., 2005;Arnold et al., 2006;Bastien et al., 2006;Choi et al., 2006;Foulongne et al., 2006;Lin et al., 2007;Ma et al., 2006;Qu et al., 2007;Sloots et al., 2006;Weissbrich et al., 2006).

Empty VP2 VLPs of B19 have been used effectively as antigen in ELISAs for evaluating B19 antibodies (Soderlund et al., 1995). The capsid formed by HBoV VP2 protein, which appears to be a characteristic icosahedral parvoviral particle, was used as antigen for detecting IgG to HBoV in this study. Different coating buffers were compared in initial optimization of the ELISA. Because no significant difference in the reactivity to serum samples was observed, a high pH (pH9.6) was used as a standard coating buffer. While HBoV VLPs might be denatured in this high pH buffer system (Wang et al., 2005), for human B19 IgG antibody studies, results from conformational antigen (capsid) or denatured VP1 and VP2 proteins did not show a significant difference (94.6% vs. 89.5%) (Manaresi et al., 2004).

Human metapneumovirus (hMPV), another newly identified respiratory virus, has been detected in patients with either upper or lower respiratory tract disease, or both (van den Hoogen et al., 2001). Seroepidemiologic studies of hMPV revealed that nearly 25% of children between 6 and 12 months of age had antibodies to hMPV, and virtually all of the children had become seropositive by the age of 5 (Ebihara et al., 2005;van den Hoogen et al., 2001). This is similar to the single study so far reported relating to the seroprevalence of HBoV infection done in the Japanese population, using a immunofluorescence assay. The positive rate was lowest in the age group of 6 to 8 months and gradually increased with age: 42.3% in the 1 year group, 83.3% in the 2–3 years group, 89.5% in the 4–5 years group and 100% in 6–19 years group (Endo et al., 2007).

In this study, a relatively lower HBoV IgG-positive rate of approximately 15% was observed in healthy children younger than 24 months, and this rate increased to approximately 42% for healthy children older than 3 years of age. The relatively lower positive rate in our study, compared to that of the Japanese group could be explained if the immuofluorescence assay used by the Japanese group also detected HBoV specific IgM in the tested sera (Endo et al., 2007). For children clinically diagnosed with lower respiratory tract infections in the study presented here, HBoV IgG seroprevalence rates were as follows: 22% in children younger than 12 months of age, 31% in children 1–2 years of age, and 60% in children 3 years of age. However, a high level of IgG was observed in positive sera from the children with lower respiratory tract infections [11% (18/161) had an OD value of higher than 0.8]. Either re-infection or acute infection of HBoV may have occurred, and HBoV may have been the causative agent for lower respiratory tract infections in these children. Only 5 of 161 nasopharyngeal aspirate samples (3.1%) from these children showed a significant level of HBoV genome (6.6×10^3 -2.6×10^6 gc/ml). Interestingly, all of these 5 children were hospitalized with lower respiratory tract infections and had a significant IgG antibody titer (OD>0.4); 3 of them had a high IgG antibody titer (OD>0.7), which suggested acute infection of HBoV. HBoV co-infection with other respiratory viruses (Allander et al., 2007;Fry et al., 2007) has been found in many cases, therefore, the role of HBoV in lower respiratory tract infections is unclear. This study was designed initially to probe the independent pathogenesis of HBoV in lower respiratory tract infections. A high level of IgG (11% had an OD value of higher than 0.8) in positive sera from children with lower respiratory tract infections suggests, but certainly does not prove, that HBoV may cause infection independently.

Although IgG antibody present in a single serum sample does not reflect an acute infection, a high rate of IgG positive sera from healthy children does suggest that HBoV infection is more common in children than genomic detection has shown previously (Allander et al., 2005;Arnold et al., 2006;Bastien et al., 2006;Choi et al., 2006;Foulongne et al., 2006;Lin et al., 2007;Ma et al., 2006;Qu et al., 2007;Sloots et al., 2006;Weissbrich et al., 2006). Infection

with B19 occurs in children in a high incidence asymptomatically. Specific IgG antibodies appear several days after IgM and persist for years. Seropositivity to B19 (IgG) has been shown to reach 80% in healthy European blood donors (Gallinella et al., 2003). Thus, infection with HBoV might also occur widely. A high seroprevalence in healthy children and adults suggests that most HBoV infections do not require medical attention in children.

In conclusion, this study shows for the first time that expression of the HBoV VP2 protein in insect cells forms parvoviral like particles, which reassemble into icosahedral structures of ~20nm. This parvoviral like particle did not hemagglutinate human "O" type red blood cells unlike the bovine *bocavirus* BPV. An ELISA based on these VLPs was established for detection of IgG antibody against HBoV in sera. A significant difference in serum reactivity to HBoV VLPs was observed; a high percentage with higher OD values were present in sera from children hospitalized with lower respiratory tract infections compared with sera from healthy children. Therefore, this VLPs-based ELISA was appropriate for early diagnosis of HBoV infection and for further study of the virus and its infection in children with lower respiratory tract infections. In addition, HBoV VLPs have the potential to be used in the future for vaccine development.

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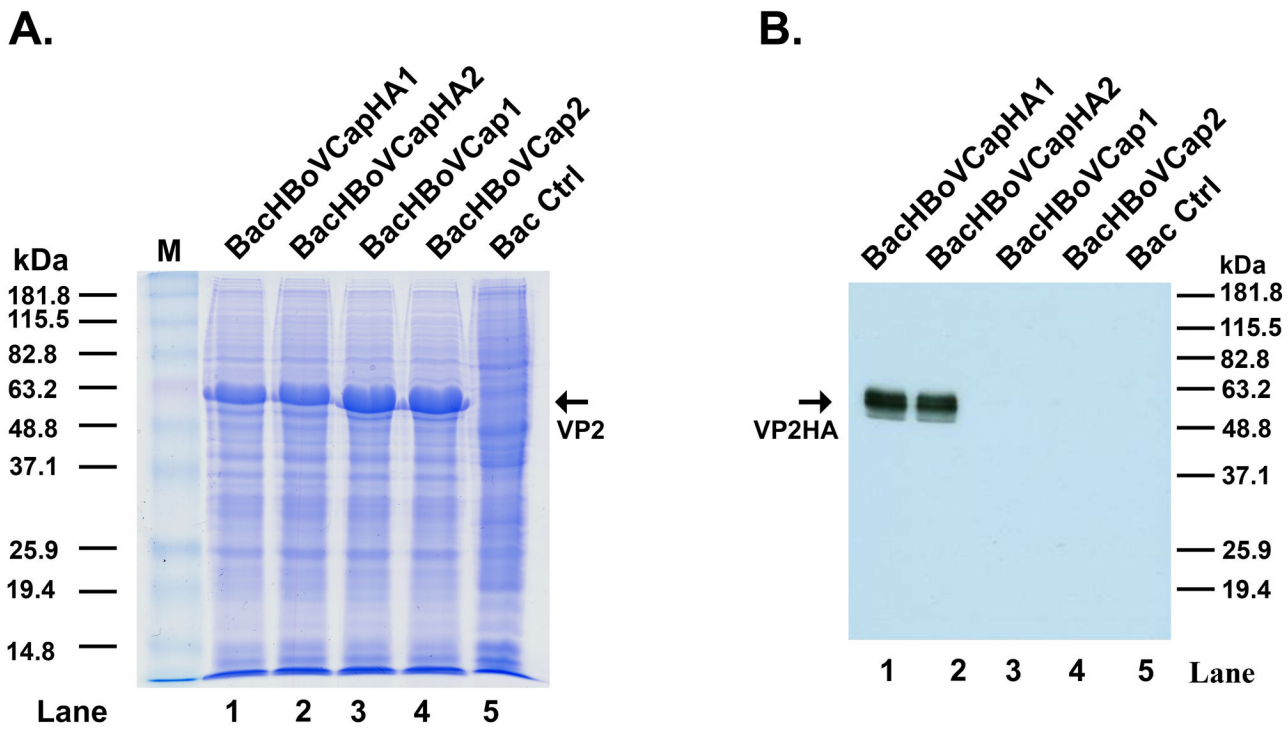

Fig. 1. Characterization of HBoV VP2 protein expression in baculovirus

Recombinant baculovirus that expressed HBoV VP2 and the control baculovirus (Bac Ctrl) that was made using the non-inserted pFastBac 1 vector were infected into Sf9 cells at 1 m.o.i., and 2 days later, equivalent numbers of cells were lysed in SDS-PAGE loading buffer. The lysates were subjected to SDS-PAGE analysis (A) or immunoblot analysis with an HA monoclonal antibody (B). Arrows show the identity of the VP2 bands (A, lanes 1–4) and the bands detected by the HA antibody (B, lane 1 and 2).

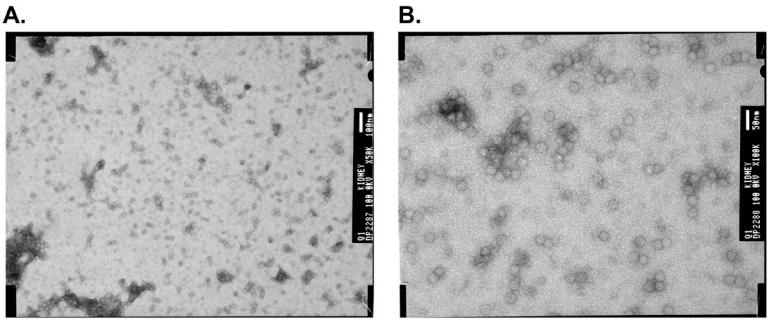

Fig. 2. HBoV VP2 assembled into virus-like particles (VLPs) of approximately 20 nm

Electron micrographs of negatively stained VLPs at 50,000 × (A) and 100,000 (B) × magnification. Scale bars are shown on the right (A=100 nm; B=50 nm).

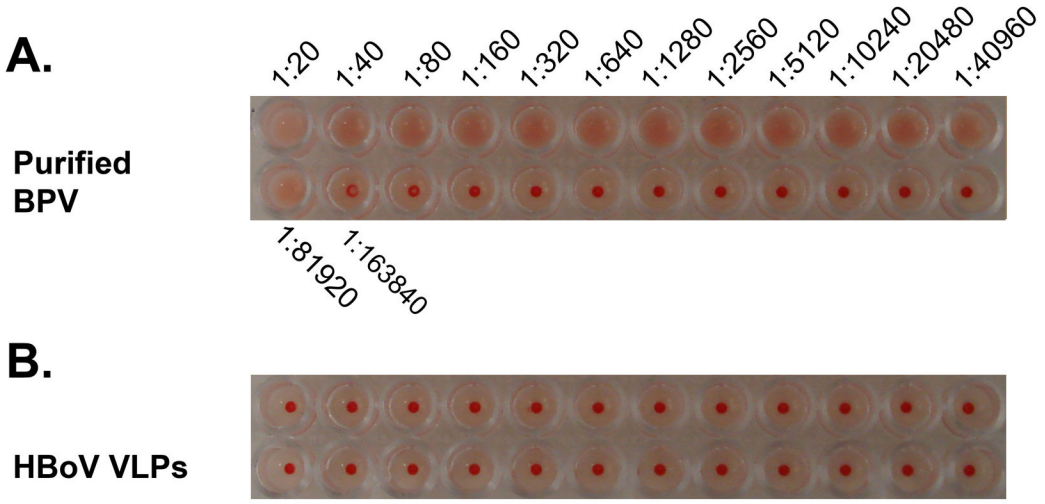

Fig. 3. Hemagglutination assay

Purified BPV (0.5mg/ml) and HBoV VLPs (0.5mg/ml) were twofold diluted in Alsever's buffer. 0.5% red cells of human "O" type were added and incubated at 4ºC overnight. Purified BPV was able to hemagglutinate human red cells at a dilution of 1:81920 (A); however, HBoV VLPs did not show any hemagglutination activity (B).

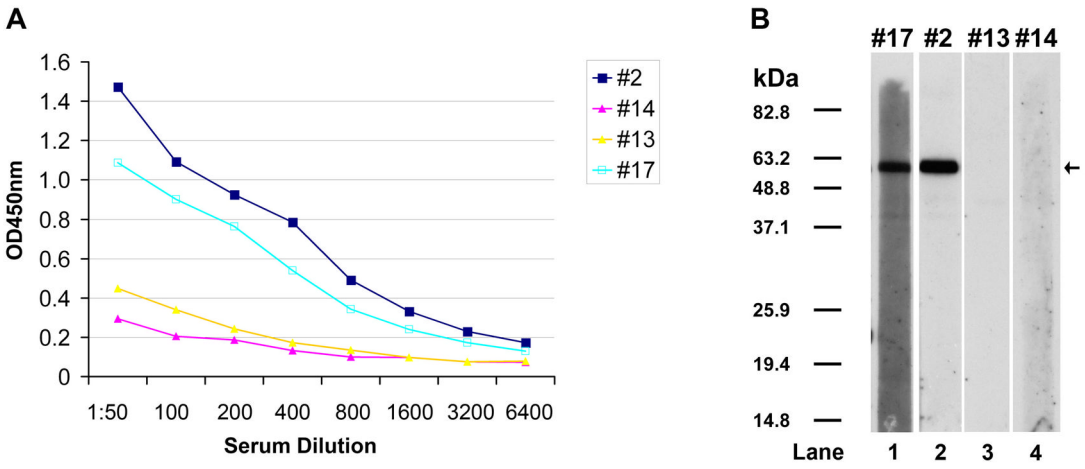

Fig. 4. Presence of HBoV-specific IgG in human sera from healthy U.S. blood donors

(A) Twofold dilution of positive sera (#2 and #17) and negative sera (#13 and #14) were tested in plates coated with purified HBoV VLPs at pH9.6. At a dilution of 1: 200, both positive and negative sera gave distinct OD values. **(B)** Positive (#2 and #17) and negative (#13 and #14) sera were further tested their reactivity to HBoV VP2 antigen by immunoblot assay. 5 μg of purified VLPs was resolved on SDS-10% PAGE and transferred onto nitrocellulose membrane. Sera diluted at 1:200 were used for blotting. Positive bands are shown as indicated by the arrow.

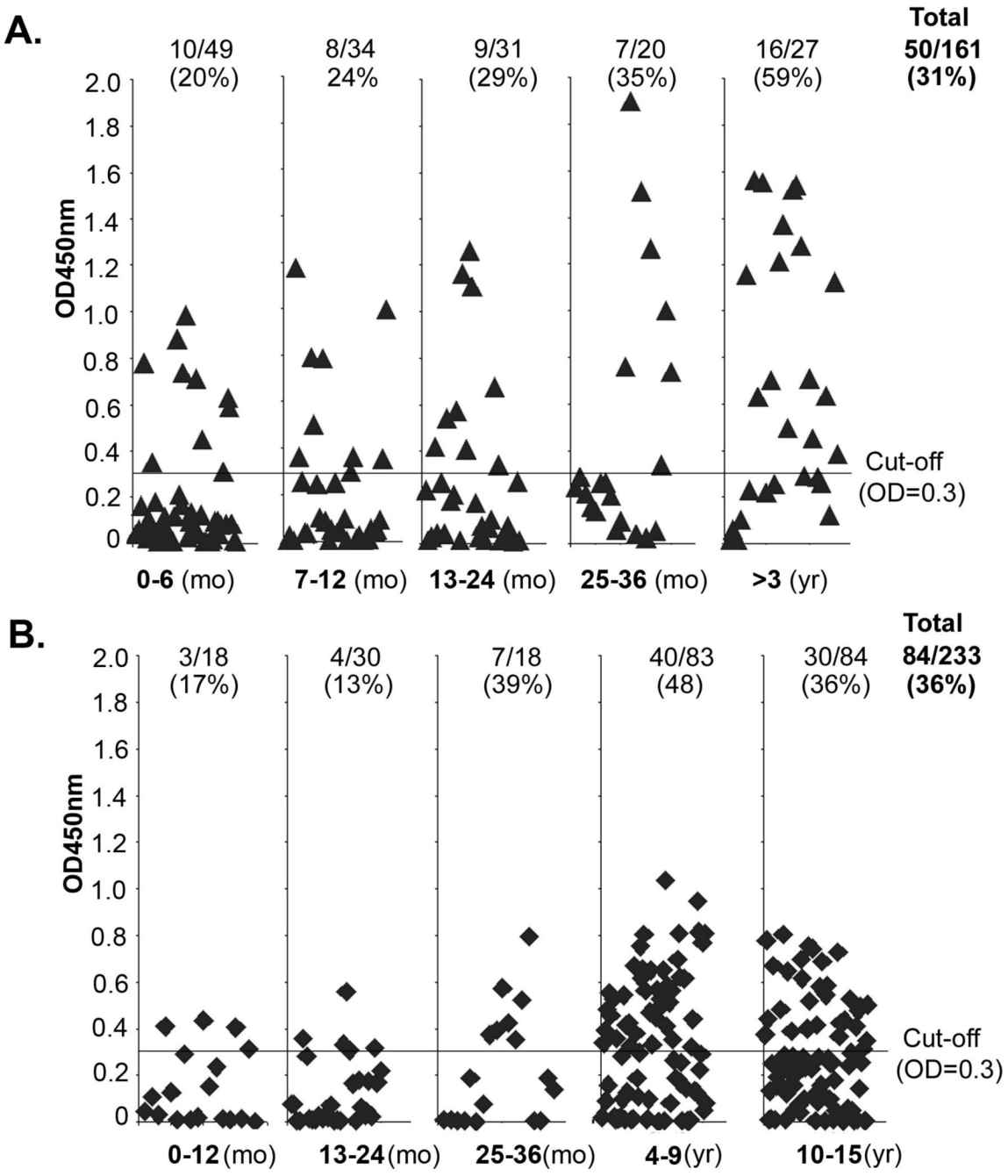

Fig. 5. Seroprevalence of IgG antibodies against HBoV

Serum samples were taken either from children with lower respiratory tract infections (A) or from healthy children (B). HBoV IgG was detected by the VLPs-ELISA method (see Materials and Methods) at a dilution of 1:200, which was expressed as an OD value at 450nm. All the serum samples were grouped according to age as indicated at the bottom of each panel. The total number, positive number and thereafter, positive rate (%) in each group are listed on top of each group panel. A cut-off line (OD=0.3) is indicated.

Table 1

Specificity of the HBoV ELISA method.

	#2	#10	#12	#13	#14	#15	#16	#17
HBoV VLPs (pH 9.6)	**1.165**	**1.031**	0.225	0.247	0.182	0.241	**0.849**	**1.012**
HBoV VLPs (pH 7.2)	**1.491**	**1.104**	0.181	0.238	0.218	0.242	**0.909**	**1.262**
BPV (pH9.6)	0.107	0.129	0.117	0.089	0.117	0.126	0.108	0.138
BSA (pH9.6)	0.080	0.079	0.087	0.077	0.076	0.077	0.083	0.077

Various antigens (HBoV VLPs, BPV and BSA as a control) were coated on a 96 well plate in a high pH buffer (pH9.6) or a neutral pH buffer (pH7.2) for HBoV VLPs only. 8 serum samples from healthy blood donors (#2, #10, #12, #14, #15, #16, #17) were incubated at a dilution of 1:200 for specific IgG detection. The OD values at 450nm and the samples are shown.

Table 2

Antibody detection in patients with high virus genomic copies in nasopharyngeal aspirate samples.

No. Specimens	Age	Genomic copies/ml	OD450nm
WL320	7 mo	6.6×10^{3}	0.503
WL399	3 ys	2.6×10^{6}	1.544
WL412	6 mo	2.0×10^{4}	0.412
WL501	3 ys	4.8×10^{6}	1.267
WL478	5 mo	1.5×10^{6}	0.736