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Short communication

# Identification of human bocaviruses in the cerebrospinal fluid of children hospitalized with encephalitis in China

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ABSTRACT

*Background:* Encephalitis is a major cause of death and disability in adults and children; different members in the family *Parvoviridae* are known to be associated with encephalitis to some extent.
*Objectives:* To determine the presence of human bocaviruses (HBoVs) and corresponding HBoV-specific immunoglobulins (Igs) in cerebrospinal fluid from children with suspected viral encephalitis.
*Study design:* Employing real-time PCR and nested touchdown PCR, 67 cerebrospinal fluid (CSF) samples from children with suspected viral encephalitis were screened for HBoV and routine encephalitis-causing viruses. Using ELISA, Western blot and IFA, HBoV-specific Ig were determined in the samples.
*Results:* Nine samples (134%) were HBoV1 DNA-positive, while one sample (15%) was HBoV2 DNA-positive. HBoV-specific IgM and IgG antibodies were detected in the CSF samples from three children; two samples were HBoV1 DNA-positive and one sample was negative. One death was recorded; CSF from this child was the only HBoV-IgM-positive CSF samples among the 67 CSF tested.
*Conclusion:* We screened CSF samples obtained from children with encephalitis for the presence of HBoV1 and HBoV2 and specific IgM- or IgG-responses. Detection of viral DNA and/or immunological response to HBoV1/HBoV2 highlights the significance of these viruses as causes of encephalitis in children. Further studies are needed to examine the role of HBoV infection in children encephalitis.


## 1. Background

Encephalitis is a globally important cause of death and disability in human. It may be caused by myriad infectious agents, including rabies, herpes simplex, poliovirus, toxoplasmosis, and malaria.[1,2] Despite the use of various diagnostic methods, the cause of an estimated 70–85% of encephalitis cases remain unknown,[3] indicating the absence of a known agent, lack of assay sensitivity, or the presence of a novel agent not considered in conventional assays.

Recent studies have shown family *Parvoviridae* members, parvovirus B19, human parvovirus 4, and human bocavirus, are to some extent associated with human encephalitis.[4–6]

## 2. Objectives

This study examined the presence of HBoVs and corresponding specific IgM and IgG-antibody responses in CSFs from children with suspected viral encephalitis to estimate the relationship between HBoV presence and encephalitis.

## 3. Study design

### 3.1. Samples

Sixty-seven CSF samples were collected from hospitalized children aged less than 12 years with suspected viral encephalitis in Hunan Province People's Hospital during June 2010 and October 2011. Children presenting with a headache, fever, lethargy, convulsions, or unconsciousness, with or without signs of meningeal irritation,[5] and CSF samples negative for bacterial and fungi culture were considered suspected viral encephalitis cases. Cranial computed tomography and magnetic resonance imaging were used to exclude intracranial vascular and space occupying lesions. One CSF sample was obtained from each patient and all samples were collected within 48 h of hospitalization. The male-to-female ratio was

*Abbreviations:* HBoV, human bocavirus; CSF, cerebrospinal fluid; Ig, immunoglobulin; ELISA, enzyme-linked immunosorbent assay; IFA, indirect immunofluorescence assay; FITC, fluorescein isothiocyanate.

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1.47:1; mean age was 52.4 months (range 4–132 months). Informed consent was obtained from the parents of each patient. The study protocol was approved by the hospital ethics committee.

### 3.2. Detection of viruses

All specimens were screened for 13 encephalitis-causing viruses: enterovirus (EV), adenovirus (AdV), Epstein–Barr virus (EBV), mumps virus (MuV), cytomegalovirus (CMV), human herpes virus 6 (HH6), measles virus (MeV), human parechovirus (HPeV), varicella zoster virus (VZV), Japanese encephalitis virus (JEV), human parvovirus 4 (PARV4), herpes simplex virus 1 and 2 (HSV1, HSV2). Primers used are listed in Supplementary Table 1. The presence of HBoV1–4 was first assessed using real-time PCR targeting of the partial 5′ untranslated region to NS1[7] and confirmed by nested touchdown PCR targeting the VP1 region of HBoV1–4.[8] Products from the touchdown PCR were sequenced. Strict laboratory procedures were performed to prevent contamination and negative controls were included in every run.

### 3.3. Detection of HBoV-specific antibodies

The VP2 genes of HBoV1 (GenBank NC_007455) and HBoV2 (GenBank NC_012042) were expressed using a baculovirus expression vector system (Life Technologies, USA), and VP2 virus-like particles (VLP) were purified from infected sf9 cells. An enzyme-linked immunosorbent assay (ELISA) was established based on the VLP to detect HBoV-specific IgM and IgG antibodies in the CSF samples.[9] Furthermore, a Western blot using VLPs as antigen was used to determine the threshold OD value for ELISA-positive CSF samples.

### 3.4. Indirect immunofluorescence assay (IFA)

CSF from the IgM- and IgG-positive samples was applied to a plate coated with sf9 cells expressing HBoV1–2 VLPs, covered with FITC-labeled, goat antihuman IgG conjugate in PBS-BSA. Specific fluorescence was examined with an ultraviolet epifluorescence microscope (BM-21AY, Shanghai).

## 4. Results

In total, ten CSF samples were positive for HBoV-1 ($n = 9$; 99–100% nucleotide match with GenBank JN632519.1) or -2 ($n = 1$; 100% nucleotide match with GenBank GU301644.1) DNA by real-time and nested-touchdown PCR; identification of the specificity of the PCR was confirmed by DNA sequence analysis. The mean age of the HBoV-DNA positive children was 68.3 months (range 11–120 months), and the sex ratio was 1:1. Nine CSF samples were HBoV1-DNA positive, while only one CSF sample was HBoV2-DNA positive. Three of the HBoV1 DNA-positive samples were detected with other encephalitis-causing viruses; two with EV and one with CMV. The HBoV2 DNA-positive CSF was also positive for AdV and CMV. The viral loads in the nine HBoV1-DNA positive CSFs ranged from $1.14 \times 10^4$ to $2.34 \times 10^5$ copies/mL by real-time PCR; CSF positive for HBoV2-DNA had $5.51 \times 10^4$ copies/mL (Table 1).

For ELISA, the optimized dilution of CSFs was 1:10. ELISA absorbance values (OD450) for IgM and IgG ranged from 0.061 to 1.516 and 0.094 to 0.642, respectively. CSFs with different absorbance values were selected for further identification of negative-CSF samples by Western blot. The cut-off value was calculated as the mean of the OD values for the negative samples plus three standard deviations, the values for HBoV1/2 IgM and IgG were 0.274/0.268 and 0.253/0.261, respectively.

Due to limited sample volumes, among the ten HBoV-DNA-positive samples, ELISA could only be performed on five samples.

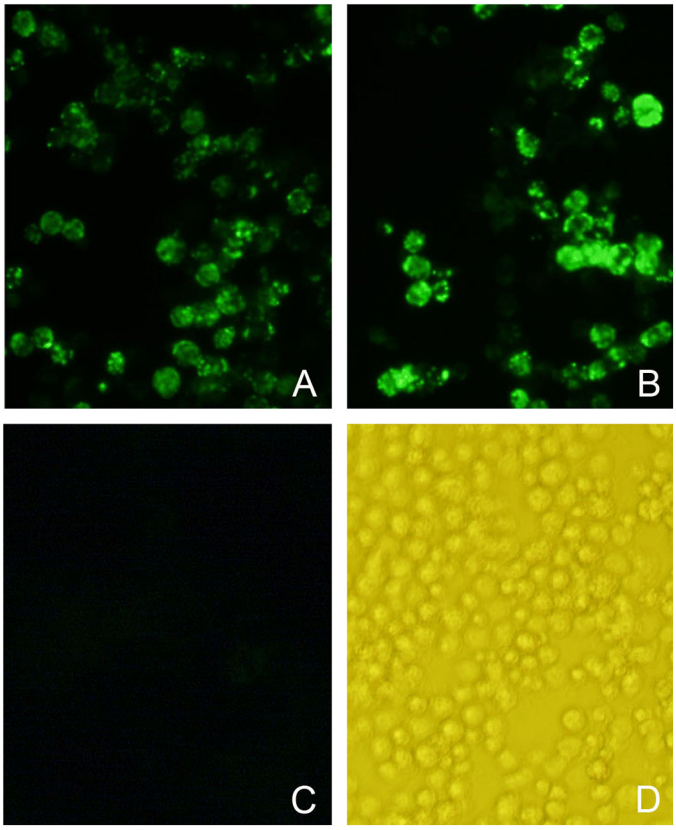


**Fig. 1.** Images of HBoV-specific IgG and IgM and normal sf9 cells. (A) IgG in the immufluorescence microscopy, (B) IgM in the immufluorescence microscopy, (C) normal sf9 cell in the immufluorescence microscopy, and (D) sf9 cell in the light microscopy.

Two samples had an HBoV-1-specific IgG response (OD = 0.642 and 0.425, respectively), while no sample had a specific IgM response. After examining the 57 HBoV DNA-negative samples, one sample, patient ID 16, was IgM-positive for both HBoV1 and HBoV2 IgM (OD = 1.516 and 1.461, respectively); none of the HBoV-DNA-negative samples had a HBoV-specific IgG response. IFA was used to confirm the specificity of IgM and IgG (Fig. 1). None of the HBoV IgM- or IgG-positive samples were PCR-positive for any of the 13 additional encephalitis-causing viruses.

The profiles of the patients with available laboratory investigations, treatments and outcomes are shown in Table 1.

## 5. Discussion

Four species of HBoV are known, HBoV1–4, of which HBoV1 is prevalent in respiratory and enteric infections; HBoV2 is associated with gastrointestinal disease, while the pathogenesis of HBoV3–4 are unknown.[10] Recently, Mitui's study suggested HBoV is a cause of encephalitis,[5] the detection of HBoV1–2 and their specific IgM or IgG in CSFs of children with encephalitis in this study supports Mitui's hypothesis.

The prevalence of HBoV in this study was higher than previously reported (14.9% and 5.8%, respectively),[5] which may suggest regional differences. Common encephalitis-associated viruses were co-detected in HBoV-DNA-positive CSFs; co-detection of HBoV with other viruses is also common in gastrointestinal (e.g., rotavirus and norovirus)[11] and respiratory (e.g., respiratory syncytial virus and AdV)[12] tract sites. Co-infected children had similar clinical symptoms compared to children in which only HBoV-DNA was detected, which suggest co-infection does not have a synergistic