RESEARCH NOTE

Detection of human bocavirus in children with Kawasaki disease

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ABSTRACT

Human bocavirus (HboV) is an emerging virus that has been implicated as a cause of acute upper and lower respiratory tract infection in children. As no serological assay is available, PCR was used to screen nasopharyngeal, serum or stool samples from 16 patients with Kawasaki disease for HBoV nucleic acid. HBoV was identified by PCR in five (31.2%) patients, suggesting that this emerging virus may also play a pathogenic role in some cases of Kawasaki disease.

Keywords Childhood infections, diagnosis, human bocavirus, Kawasaki disease, PCR

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Human bocavirus (HBoV) is a new member of the Parvoviridae family that has recently been described by Allander *et al*. [1]. Initial studies revealed that this virus is a common cause of acute upper and lower respiratory tract infection in children, with a prevalence of 1.5–8.2% [2–7]. An initial study using nasopharyngeal aspirates (NPAs) from children hospitalised with fever also revealed HBoV nucleic acid in two patients hospitalised with Kawasaki disease (KD). Therefore, the present study retrospectively screened NPA, serum or stool samples from patients with KD for HBoV.

All NPA, serum and stool specimens were collected in the 48-h period following admission. NPAs had been tested previously for common viral respiratory pathogens by direct immunofluorescence assays using monoclonal antibodies for respiratory syncytial virus, influenza A and B viruses, parainfluenza virus types 1, 2, and 3, and human adenovirus. NPAs were also inoculated on HUH7 and A549 cell monolayers for virus isolation. The specimens were then stored frozen at either −80°C (for NPAs) or −30°C (serum and stools) before being tested for the presence of HBoV DNA.

DNA was extracted from 200 μL of each sample using a QIAamp DNA Blood Mini Kit (Qiagen, Courtabeuf, France) according to the manufacturer's instructions, with an elution volume of 110 μL. DNA extracts of NPA were tested for HBoV with a conventional in-house PCR assay. Extracted DNA was stored at −80°C between performing the conventional PCR and a subsequent real-time PCR assay.

The conventional in-house PCR targeted the VP2 gene using forward primer 5′-CAGTGGTACCAGACACCAGAAG and reverse primer 5′-GCCAGTTCTTTGTTGCGTATCT. The corresponding sequences are found in all the bocavirus sequences deposited in GenBank. PCRs comprised 0.5 μL of 10 mM dNTP mix, 1.5 U of Ampli*Taq* Gold DNA polymerase, 10 pmol of each primer, 2 μL of 10× PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, gelatin 0.01% w/v), 1 μL of glycerol and 2 μL of nucleic acid extract in a final volume of 20 μL. Amplification comprised 94°C for 5 min, followed by 40 cycles of 94°C for 15 s, 60°C for 15 s and 72°C for 30 s, and was performed using a GeneAmp PCR System 9600 (Perkin Elmer, Warrington, UK). The resulting 403-bp amplicon was detected using conventional agarose gel electrophoresis. Amplicons (403 bp) from each positive specimen were sequenced in both directions to confirm the sequence specificity.

The real-time PCR assay targeted the HBoV NS1 gene. Specific primers and probes were based on the reported sequences of the NS1 gene at positions 953–1029 of HBoV strain st1 (GeneBank accession no. DQ000495), and are available commercially as part of the Bocavirus r-gene kit (Argene, Varilhes, France). The real-time PCR

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Table 1. Clinical and biological data for patients with Kawasaki disease from whom human bocavirus DNA was detected

Gender, age, month of hospitalisation	Clinical presentation	Coronary aneurysms	WBC/mm^3	CRP (mg/L)	PCT (ng/L)	Detection of HBoV DNA by PCR	IFN-α (IU/mL)	Co-infection
M, 8 years, February	Fever, cervical adenopathy, pharyngitis, conjunctivitis,	No	7900	3.3	0.2	Serum +	75 at day 6	Influenza B in NPA at day 6
M, 12 months, December	Fever, cervical adenopathy, pharyngitis, exanthema	Aneurysm of the left coronary artery	9000	327	20.1	NPA + Serum –	<2	
M, 21 months, January	Fever, cervical adenopathy, pharyngitis, conjunctivitis, exanthema, oedema and desquamation of the extermities	No	5800	273	16.3	NPA + Serum + Stools +	2 at day 6	Rotavirus in stools at day 6
M, 19 months, February	Fever, cervical adenopathy, pharyngitis, exanthema, oedema of the extremities	No	24 000	279	5.4	NPA + Serum – Stools –	<2	
M, 21 months, December	Fever, cervical adenopathy, cheleitis, conjunctivitis, exanthema	No	15 900	122	3	NPA + Serum + Stools – CSF +	Serum <3 CSF <2	

HBoV, human bocavirus; M, male; WBC, white blood cell; CRP, C-reactive protein; IFN, interferon; PCT, procalcitonin; NPA, nasopharyngeal aspirate; CSF, cerebrospinal fluid; IU, International Units.

mixture comprised 15 μL of PCR Master premix (Argene Bocavirus r-gene kit), 0.4 μL of *Taq* polymerase and 10 μL of nucleic acid extract, in a final volume of 25 μL. Amplification was performed in 96-well plates on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Warrington, UK). Amplification comprised 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. Each run included a positive and negative template control. To monitor extraction quality, several extracts that tested negative for HBoV were included in each extraction batch and were tested by real-time PCR with an assay for the human HAPB gene (Applied Biosystems).

A specimen was only considered positive if the PCRs were positive for both the VP2 and NS1 regions. Specimens that were positive according to real-time PCR, but negative according to conventional PCR for the VP2 gene, were re-examined using a nested PCR for a different region of the VP2 gene with two additional pairs of primers (5′-AGACAAAACGGAAGCACAGC and 5′-TCAAAGCCAGATCCAAATCC), followed by sequencing to confirm the specificity of the amplicon.

Viral loads were measured in comparison with a plasmid containing a 799-bp sub-genomic fragment of HboV. This fragment was amplified from NPA using primers bracketing the real-time PCR target regions in the NS1 gene (forward primer 5′-AAAAAACTACCACGCAACCCTAGA and reverse primer 5′-GAGAAACAGAATTGCCACCAA). The product was cloned into plasmid pGEM T easy (Promega, Charbonnieres, France). Preparations of the purified plasmid were sequenced for verification and quantified by UV spectroscopy (10^{12} copies/mL). Measurement of interferon-α in serum was performed using a biological assay as described previously [8].

Nineteen children (mean age 4.8 years) were hospitalised between January 1999 and December 2006 with typical symptoms of KD, meeting the criteria of the American Heart Association [9], but NPAs, sera and stools were available and tested for only ten, 14 and eight patients, respectively (16 patients in all). A control population of 579 patients consisted of children aged <2 years who were hospitalised for respiratory tract infections between October 2005 and May 2006. HBoV was detected in at least one sample from five (31.2%) patients with KD. Clinical and microbiological data for these five patients are summarised in Table 1. In the control population, HBoV DNA was detected in 32 (5.5%) patients. All five patients with KD and HBoV infections were hospitalised during the winter months, whereas only eight of the 11 patients with KD who were negative for HBoV were hospitalised during winter.

All viral sequences isolated from the five HBoV-positive patients showed 98% homology with the reference sequence (GenBank DQ000496). The viral load in four NPA samples from KD patients had a median value of 6×10^3 genomic copies/mL (range 10^2 to 6×10^4 copies/mL). The median value of the viral load in NPAs from 27 HBoV-positive non-KD

patients was 3×10^4 copies/mL (range 3×10 to 1×10^{10} copies/mL). In serum samples from KD patients, the median viral load was 6×10^2 copies/mL (range 10 to 4×10^4 copies/mL), compared with 3×10 to 1.2×10^5 copies/mL in HBoV-positive non-KD patients. In stools from a KD patient, the viral load was estimated at 10^4 copies/mL.

KD is an acute vasculitis affecting small to medium-size vessels, leading to coronary aneurysms in 20% of cases. The immunopathological mechanisms involved in the pathogenesis of KD are unclear. Although its aetiology remains unknown, the clinical and epidemiological features of this disease suggest that it is infectious [10]. Epstein–Barr virus, adenovirus and cytomegalovirus have all been considered as possible agents that are involved in KD [11,12]. Members of the genus Bocavirus, e.g., HboV, could also be responsible for systemic infection, although previous studies have only tested respiratory samples. The present study detected HBoV in three sera, one stool and one cerebrospinal fluid, which supports the hypothesis that this virus could cause a systemic infection. HBoV may play a role in KD, and additional studies are required to understand the physiopathology of this new virus.

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