Detection and quantification of human bocavirus in river water

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Human bocavirus (HBoV) was recently discovered in children with respiratory-tract infection and has been detected frequently in faecal specimens from children with gastroenteritis. The present study addresses for the first time, to our knowledge, the prevalence of HBoV in river water. By using a newly developed real-time PCR targeting a conserved region of the NP1 gene of HBoV, virus levels in water samples were determined. Moreover, partial sequence analysis of the NP1 gene of HBoV and comparative phylogenetic analysis were performed. HBoV was detected in 40.8 % of collected water samples. The virus level ranged between $3 \times 10^1$ and $2 \times 10^3$ genome equivalents l$^{-1}$. Therefore, the present study suggests that river water could play a role in the spread of HBoV. However, further work should be done to determine the actual risk of infection via surface water.

Virus contamination of water resources is a public-health concern. Water may play a meaningful role in the transmission of many enteric viruses that are transmitted via the faecal–oral route. Viruses enter water resources through direct or indirect discharge of treated and non-treated wastewater.

Human bocavirus (HBoV) was first described in 2005 in respiratory-tract samples (Allander et al., 2005). HBoV is a member of the family Parvoviridae (subfamily Parvovirinae, genus Bocavirus). The virus is most closely related to minute virus of canines (MVC) and bovine parvovirus (BPV), animal viruses associated with respiratory symptoms and enteritis of young animals (Allander et al., 2005; Durham et al., 1985). HBoV infections have been detected frequently in young children and may also contribute to severe respiratory infections in adults (Chow et al., 2008; Kupfer et al., 2006). The virus has also been detected in faecal and urine samples (Campe et al., 2008; Lee et al., 2008; Pozo et al., 2007; Tozer et al., 2008; Vicente et al., 2007).

Detection of HBoV in faeces of children with gastroenteritis and no signs of respiratory infection raised the possibility of enteric replication and spread via faeces (Vicente et al., 2007). Although its role in gastroenteritis patients is under discussion (Campe et al., 2008; Ziegler et al., 2008), excretion in faeces/urine and/or presence in water used for drinking or recreation purposes increases the potential risk of infection through water, as has been suggested for other enteric viruses (Hamza et al., 2009; Kukkula et al., 1997; Lee & Kim, 2002; Lodder & de Roda Husman, 2005; Pina et al., 1998). Therefore, the present study aimed to determine the prevalence of HBoV in river water, which could be a vehicle for virus transmission. To our knowledge, this is the first study that addresses the prevalence of HBoV DNA in environmental water.

To address this issue, 120 water samples (10 l each) were collected over a 1 year period (January–December 2008) from the Ruhr and Rhine rivers, Germany. Water samples were concentrated by using the previously modified virus adsorption–elution method, which has been evaluated for the recovery of a large panel of enteric viruses (Hamza et al., 2009). Briefly, a type HA negatively charged membrane filter (Millipore) with a 0.45 μm pore size and a 142 mm diameter was used for virus adsorption. For recovery of the adsorbed viruses, a non-organic elution buffer [0.05 M KH$_2$PO$_4$, 1.0 M NaCl, 0.1 % (v/v) Triton X-100, pH 9.2] was used, followed by a PEG-6000 precipitation step.

Viral nucleic acids were extracted by using a QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer’s instructions.

To obtain a DNA standard for HBoV quantitative (q)PCR, a 354 bp fragment of the HBoV genome was amplified from an HBoV-positive sewage sample by using the primers 188F (5′-GACCTCCTGAATCTATTAC-3′) and 542R (5′-CTCTGTGTGACTGAATACAG-3′),
described previously (Allander et al., 2005). The PCR fragment was sequenced and cloned into the pSC-Amp/kan vector by using a StrataClone PCR cloning kit according to the manufacturer’s instructions (Stratagene). The concentration of purified plasmid DNA was determined by using the Quant-iT dsDNA HS assay (Invitrogen) and fluorescence was measured by using a Qubit fluorometer (Invitrogen).

HBoV DNA in the concentrated water samples was quantified by using a newly designed real-time PCR using the following primers and Taqman probe: HBoV-NP1-F2421 (5’-TGGCAGACAACTCATCACAG-3’), HBoV-NP1-R2544 (5’-TCTTCCAAGCAGTGCAAGAC-3’) and HBoV_Probe-2494 [5’-(6FAM)ATCATCAGGACACCC-AATCAGCCfAC(BHQ1)-3’]. The position of each nucleotide is relative to the sequence of the NP1 gene of HBoV Stockholm strain ST1 (GenBank accession no. DQ000495).

The primers and probe were designed by Primer3 software (Rozen & Skaletsky, 2000) and the size of the expected PCR product was 123 bp. The real-time PCR was conducted in a 20 μl reaction volume by using a Quantitect probe PCR kit (Qiagen), in which 5 μl template (equivalent to approx. 67 μl surface water), 5 pmol each primer and 2 pmol Taqman probe were added. DNase/RNase-free water was used as a negative control during the PCR. The temperature conditions were 95 °C for 15 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min; the amplification and data analysis were done on a Rotorgene 3000 cycler system (Corbett Research). The estimated detection limit was approximately five genome copies per reaction. The qPCR results have not been corrected for the efficiency of the concentration process.

In the present study, HBoV was detected in 40.8 % of the examined water samples. The virus level ranged between $3 \times 10^3$ and $2 \times 10^3$ genome equivalents l$^{-1}$ (Fig. 1). As concentration of virus from water can co-purify inhibitory compounds that might be present in the environmental water samples, it was important to include PCR-inhibition control. The inhibition of the PCR was determined by spiking 1000 copies of the plasmid DNA used as standard into the purified nucleic acid from river water samples. The reaction was considered to be truly negative when the cross points observed in the plasmid DNA-spiked samples were comparable to the cross points of the same DNA copies added to DNase/RNase-free water. Five samples showing inhibition of the PCR were excluded from the study.

The median level of HBoV in water samples was $1.2 \times 10^2$ genome equivalents l$^{-1}$. Thirty-eight water samples from the present study have been examined before and showed higher levels ($5.7 \times 10^2$ to $3.2 \times 10^4$ genome equivalents l$^{-1}$) of human enteric viruses (Hamza et al., 2009). One possible explanation for the low level of HBoV in surface water could be the low concentration of HBoV excreted in faeces ($7.0 \times 10^2$ to $1.75 \times 10^4$ copies ml$^{-1}$) (Campe et al., 2008). The incidence of HBoV in German children was estimated to be 10.3 %, with a higher prevalence in winter (Weissbrich et al., 2006). In river water, we detected HBoV throughout the year except in the summer months (Fig. 2). Accordingly, even though it might not cause gastroenteritis, we cannot exclude the role of contaminated water in transmission, as proposed for respiratory adenoviruses (van Heerden et al., 2005).

As this is the first report to address the prevalence of HBoV in surface water, we have no data in our hands to compare the detection rate and level of HBoV obtained in the German water with those in other aquatic environments.

The frequency of detection of HBoV in 40.8 % of the examined river water samples could be due to the extraordinary stability of parvovirus particles (Srivastava & Lund, 1980). To confirm our qPCR results and exclude the possibility of cross-reaction of the PCR with other parvoviruses, a partial NP1 fragment was amplified from representative water samples and sequenced. The sequences were compared with the sequences of reference strains of MVC, BPV, human parovovirus B19 and different strains of HBoV (ST1, WLL-1 and HK23). A phylogenetic tree was

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**Fig. 1.** Median (minimum–maximum) HBoV level for the positive river-water samples. Number of positive samples at each month can be seen in Fig. 2. In October 2008, no samples were collected.
constructed by using the neighbour-joining algorithm of MEGA4 software (Tamura et al., 2007). These analyses showed that we have different isolates (RW1–RW9 and SW) that clearly cluster with different strains of HBoV, but not with other paroviruses (Fig. 3). The nucleotide sequences showed 98–100 % identity to the reference strains of HBoV (ST1, WLL-1 and HK23) and were 99–100 % identical to each other.

To date, there is no cell-culture system available for propagation of HBoV, so it was not possible to test the infectivity of the HBoV detected in our study. Taking into account the instability of free nucleic acids and the fact that the concentration procedure is based mainly on the structure of the virus particle (Haramoto et al., 2007), the presence of infectious virus particles is suspected. Additionally, two river-water samples were treated with DNase I prior to DNA extraction and showed no reduction in the number of genome copies of HBoV in comparison to the non-treated one (data not shown). This suggests that the HBoV DNA detected is protected from DNase degradation inside intact virus particles (Klempa et al., 2009; Nuanualsuwan & Cliver, 2002).

Thus, the data obtained in our study raise a new concern about HBoV transmission. Therefore, a potential role of river water in transmission of HBoV should not be neglected. However, further work is needed to determine the actual risk of infection through contaminated surface water.

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References


