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Detection of Human Bocavirus in Respiratory, Fecal, and Blood Samples by Real-Time PCR

Sarah J. Tozer,[1,2] Stephen B. Lambert,[1,2] David M. Whiley,[1,2] Seweryn Bialasiewicz,[1,2] Michael J. Lyon,[3] Michael D. Nissen,[1,2,4,5] and Theo P. Sloots[1,2,4,5]*

[1]*Queensland Pediatric Infectious Diseases Laboratory, Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital and Health Service District, Herston Road, Herston, Queensland, Australia*
[2]*Clinical Medical Virology Centre, University of Queensland, Queensland, Australia*
[3]*Public Health Virology Laboratory, Queensland Health Forensic and Scientific Services, Queensland, Australia*
[4]*Department of Paediatrics and Child Health, University of Queensland, Queensland, Australia*
[5]*Microbiology Division, Queensland Health Pathology Service Central, Brisbane, Queensland, Australia*

Human bocavirus (HBoV) has been detected worldwide in respiratory samples. Two real-time PCR assays, targeting the non-structural protein (NP-1) and viral protein (VP-1) genes, were designed and validated to detect HBoV in patients with respiratory disease, gastroenteritis, or systemic illness. Sensitivity of the NP-1 and VP-1 assays were equal to the conventional PCR assay previously described by Allander et al. [2005: Proc Natl Acad Sci USA 102: 12891–12896] being 100%, and giving specificity of 94% and 93%, respectively. There was no cross-reaction identified with unrelated respiratory agents, or to human DNA. The limits of detection were 10 copies of genomic DNA equivalents per reaction for both assays. The assays were used to screen three different sample populations, combined nose, and throat swabs (n = 96) from children with acute respiratory disease, fecal samples (n = 375) from adults, and children with gastroenteritis and whole blood (n = 229) collected from 31 immunocompromised children taken over an 18-month period. In total 17 (18%) respiratory samples and 18 (4.8%) fecal samples were identified as having HBoV present. Of the pediatric whole blood specimens investigated, HBoV was detected in six (2.6%) samples from four patients. In summary, two real-time PCR assays targeting different genes were designed and validated for use as screening methods for the detection of HBoV. HBoV was found in three different specimen types: parent-collected combined nose–throat swabs, fecal samples collected from symptomatic individuals and whole blood from immunocompromised children. ***J. Med. Virol. 81:488–493, 2009.***

KEY WORDS: virus; real-time PCR; blood; fecal; respiratory illness; gastroenteritis

INTRODUCTION

In 2005, a previously undetected parvovirus, human bocavirus (HBoV), was identified in respiratory samples obtained from infants in Stockholm, Sweden, using a random amplification and cloning technique [Allander et al., 2005]. Since then, HBoV has been detected in more than 17 countries in pediatric respiratory tract samples [Arden et al., 2006; Arnold et al., 2006; Bastien et al., 2006; Choi et al., 2006; Kesebir et al., 2006; Ma et al., 2006; Manning et al., 2006; Sloots et al., 2006; Smuts and Hardie, 2006; Allander et al., 2007; Fry et al., 2007; Naghipour et al., 2007; Qu et al., 2007]. These data demonstrate prevalence rates ranging from 1.5% to 19% in patients with acute respiratory illness (ARI).

HBoV is classified in the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Bocavirus* based on

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*Correspondence to: Theo P. Sloots, Associate Professor, Queensland Pediatric Infectious Diseases Laboratory, Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital & Health Service District, Herston Road, Herston, Queensland 4029, Australia.
E-mail: t.sloots@uq.edu.au

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sequence homology and is closely related to bovine parvovirus 1 and canine minute virus. Both these viruses are associated with gastroenteritis in puppies and calves [Carmichael et al., 1980; Durham et al., 1985]. HBoV has been identified in fecal samples from children with acute gastroenteritis (AGE) in Italy [Maggi et al., 2007] and Hong Kong [Lau et al., 2007]. Further, findings of HBoV in serum from patients with acute wheezing have suggested an association with systemic infection [Allander et al., 2007].

To date most studies investigating HBoV have been prevalence studies without addressing disease association. Many studies are reporting HBoV coupled to large incidences of co-infection in both respiratory and fecal samples, increasing the complexity of disease association. Not being able to apply Koch's postulate due to the inability to culture this virus at this stage, or to be able to apply it to an animal model [Fredericks and Relman, 1996] is hampering efforts to investigate the role this virus plays in disease. Improved diagnostic assays are required to document the extent of HBoV's presence in clinical specimens and its potential for causing disease.

To date, the methods of detecting HBoV have been conventional PCR [Allander et al., 2005; Arden et al., 2006; Bastien et al., 2006; Kesebir et al., 2006; Kupfer et al., 2006; Manning et al., 2006; Sloots et al., 2006] and real-time PCR [Choi et al., 2006; Manning et al., 2006; Smuts and Hardie, 2006; Allander et al., 2007; Esposito et al., 2007; Qu et al., 2007] due to the limited success of serological and viral culture techniques. Real-time PCR offers greater sensitivity, increased specificity with the addition of oligoprobes, and the added benefit of a closed detection system, reducing the likelihood of false positive results due to contamination with amplicon [Chieochansin et al., 2008]. Real-time platforms also offer rapid turnaround times with the ability to test for a number of gene targets with multiplexing. With PCR leading the way for the detection of viral agents [Lipson, 2002], we have developed and validated two real-time assays targeting the non-structural protein (NP-1) gene and the viral protein (VP-1) gene for the detection of HBoV in three different sample types, combined nose–throat swabs (NTS), fecal samples, and whole blood.

MATERIALS AND METHODS

Samples

Three separate banks of specimens were used in this investigation. The first set consisted of 96 community acquired NTS specimens collected from children less than 5 years of age by their parents or carers in Melbourne, Australia, between January 2003 and January 2004 with acute respiratory symptoms. The samples had previously been screened for a range of respiratory viruses [Lambert et al., 2007]. The specimens were extracted using the Corbett X-tractor Gene (Corbett Research, Australia) and tested using the HBoV PCR assays.

The second specimen bank comprised of 375 fecal samples from two different patient groups. Set A, 136 samples, were from children with AGE symptoms under the age of six presenting to the Royal Children's Hospital Brisbane between May and September 2002. These samples were extracted using the Roche MagNA Pure LC Instrument (Roche Diagnostics, Australia) and screened for other viral agents including adenovirus, astrovirus, norovirus, rotavirus, WU polyomavirus, KI polyomavirus, JC, and BK polyomavirus using PCR following standard conditions and published methods [Gouvea et al., 1990; Allard et al., 1992; Noel et al., 1995; Hirsch et al., 2001; Kageyama et al., 2003; Pal et al., 2006; Bialasiewicz et al., 2007]. Set B were 239 fecal samples collected by Queensland Public Health Units between June and October 2006 from patients with AGE linked to undiagnosed institutional outbreaks. These patients were children, the elderly, or staff from day-care centers, nursing homes, hostels, or hospitals throughout Queensland. The mean age was 40.3 years with the median age being 39.9 years and the age range was 1 month to 97.6 years. These samples were extracted using the Qiagen DNA Stool kit (Qiagen, Clifton Hill, Australia) and were screened using PCR for norovirus, WU polyomavirus, KI polyomavirus, JC, and BK polyomavirus using previously published methods [Hirsch et al., 2001; Kageyama et al., 2003; Pal et al., 2006; Bialasiewicz et al., 2007].

The final bank was 229 whole blood samples collected from 31 immunocompromised children with acute leukemia or post-stem cell transplant recipients. These were specimens collected at the Royal Children's Hospital Brisbane, taken over an 18-month period beginning in July 2004. Samples were collected on consecutive weeks over an average of 9 weeks, with the longest period for consecutive sampling from one patient being 24 weeks. These children did not present with respiratory or gastrointestinal symptoms at the time of blood collection. Nucleic acids were extracted from whole blood using the Roche High Pure Viral Nucleic Acid kit (Roche Diagnostics, Castle Hill, Australia).

Real-Time PCR Assay Design

The primers and Taqman probes for HBoV real-time PCR were designed using Bioedit 7.0.5.3 [Hall, 1999] and Primer Express 2.0 software (Applied Biosystems Pty. Ltd., Scoresby, Australia). Briefly, Bioedit software was used to screen full length and partial genome sequences obtained from Genbank in order to identify highly conserved regions. Genbank accession numbers used were: AB248271-2, AB257721-2, AM689298, DQ000496, DQ677523, DQ457414, DQ494200-4, DQ471802-11, DQ499604, DQ267761-75, DQ513330-1, DQ344465-83, DQ340570, and DQ296618-35. Primer Express was then used to investigate the conserved regions and identify primer and probe targets for each gene target selected. Two assays were designed one targeting the Non-structural Protein 1 (NP-1) region and the second targeting the VP-1 (Table I).

TABLE I. Primer and Probe Sequences Used in Real-Time PCR for the Detection of HBoV

Oligonucleotide	Sequence (5′–3′)	Target gene
STBoVP-1f	GGCAGAATTCAGCCATACTCAAA	VP-1
STBoVP-1r	TCTGGGTTAGTGCAAACCATGA	VP-1
STBoVP-1pr	JOE-AGAGTAGGACCACAGTCATCAGACACTGCTCC-bhq1	VP-1
STBoNP-1f	AGCATCGCTCCTACAAAAGAAAAG	NP-1
STBoNP-1r	TCTTCATCACTTGGTCTGAGGTCT	NP-1
STBoNP-1pr	FAM-AGGCTCGGGCTCATATCATCAGGAACA-bhq1	NP-1

VP, viral protein; NP, non-structural protein.

Real-Time PCR

Both assays used the same PCR mix and cycling conditions. The primers targeting the VP-1 region were BoVP-1f and BoVP-1r and for NP-1 were BoNP-1f and BoNP-1r (Table I). The respective probes were BoVP-1pr and BoNP-1pr (Table I). Briefly 12.5 μL of Qiagen Quantitect Probe Master Mix (Qiagen), 10 pmol of each primer, 4 pmol of the corresponding probe, and 2 μL of sample nucleic acid extract in a final reaction volume of 25 μL. The following cycling conditions were used: initial incubation of 15 min at 95°C, followed by 50 cycles of 95°C for 15 sec, and 60°C for 1 min, with fluorescence acquired at the end of each 60°C step.

Conventional PCR

The real-time assays were compared to previously published conventional PCR [Allander et al., 2005] using the same primer sets 188f GAGCTCTGTAAGACTATTAC and 542r CTCTGTGTTGACTGAATACAG. Briefly, each reaction mix contained 0.4 μM of each primer, 0.5 μl of 10 mM dNTPs, 2.5 μl of 10× Qiagen PCR buffer (Qiagen), 1.0 U of Qiagen HotStart Taq (Qiagen), and 1 μl of nucleic acid extract in a final volume of 25 μl. PCR cycling was performed on an DNA Engine thermal cycler (Bio-Rad, Gladesville, Australia) with the following parameters; a 15 min incubation at 95°C, followed by 50 cycles of 94°C for 20 sec, 55°C for 20 sec, and 72°C for 30 sec, followed by a final extension of 72°C for 7 min. PCR products were visualized by electrophoresis on 1.5% agarose gel with ethidium bromide staining.

Clinical Sensitivity and Specificity

Clinical sensitivity and specificity was determined using the bank of 96 parent collected, combined NTS specimens [Lambert et al., 2007] and both assays were compared to results obtained from a previously published conventional PCR method [Allander et al., 2005]. Samples that were positive by conventional PCR were deemed true positives, while all other specimens were considered true-negative specimens for real-time assay sensitivity and specificity calculations.

Nested PCR

A nested PCR targeting the bocavirus NP-1 gene was performed to further investigate samples that were positive in the real-time PCR assays but negative in the conventional method. Briefly, primers nestF 1 (AAGTACTATTACTTTCTTTAACACTTGGCA) and nestR 1 (CCCACACCACCCTGGAGC) were used in a first round of PCR amplification, following which 2 μl of first round PCR mix was added to a second round of PCR amplification using primers nestF 2 (GCACAGCCACGTGACGAAG) and nestR 2 (TTTTCCCCGATGTACTCTCCC). For both nested PCR reactions, the Quantitect Probe Master Mix (Qiagen) was used as the basis for the reaction mix and 45 cycles of thermocycling were performed on a DNA Engine thermal cycler (Bio-Rad) as described above. PCR products were visualized by electrophoresis on 1.5% agarose gel with ethidium bromide staining and then purified and DNA sequenced using the ABI PRISM™ BigDye sequencing kit (Applied Biosystems Pty. Ltd.).

Analytical Specificity

Analytical specificity was investigated by testing a number of organisms found as commensals or pathogens within the human respiratory tract (Table II). Human genomic DNA was also tested.

Detection Limit

Detection limits were determined using viral genomic DNA. Briefly, a plasmid control was constructed using the pGEM-T-Easy vector system (Promega, Madison, USA) containing a VP-1 gene fragment. Plasmid copy number was calculated based on the molecular weight and optical density. Using the Rotogene 6000 (Corbett Research, Brisbane, Australia), 10-fold dilutions of the plasmid were tested in order to create a standard curve, which was used to quantify HBoV viral genomic DNA obtained from a clinical specimen. Dilutions of this quantified HBoV viral genomic DNA were then tested in triplicate in each assay and the limit of detection of each assay was defined by the final dilution in which all three replicates were positive for HBoV.

RESULTS

Validation of Real-Time PCR Methods

Of the 96 respiratory samples tested, 11 samples provided positive results and 79 samples provided negative results in VP-1, NP-1 real-time and conventional PCR assays. A further six samples provided discordant results. These included five samples that were positive

TABLE II. List of Organisms Investigated in order to Determine Specificity of the VP-1 and NP-1 Real-Time PCR Assays

Viruses	
Adenovirus 14 ATCC VR15	hMPV wild-type
Cytomegalovirus wild-type	Parvovirus B19 Accurun 355(Boston Biomedica, Inc., West Bridgewater, MA)
Coronavirus HKU1 wild-type	Parainfluenza 1 ATCC VR 94
Coronavirus NL63 wild-type	Parainfluenza 2 ATCC VR 92
Coronavirus OC43 wild-type	Parainfluenza 3 ATCC VR 93
Coronavirus 229E wild-type	Polyomavirus JCV ATCC VR 45027
Epstein bar virus wild-type	Polyomavirus BKV ATCC VR 45024
Herpes simplex virus 1 wild-type	Polyomavirus KI wild-type
Herpes simplex virus 2	Polyomavirus WU wild-type
Influenza A ATCC VR 544	Rhinovirus wild-type
Influenza B wild-type	Respiratory syncytial virus ATCC VR 1400
Measles attenuated vaccine virus Ender's line	Rubella virus wild-type
Mumps vaccine virus Jeryl Lynn strain	
Bacteria	
Bartonella henselae wild-type	*Klebsiella pneumonia* wild-type
Bordetella bronchiocepacia wild-type	*Legionella pneumophillia* ATCC 43111
Chlamydia pneumoniae wild-type	*Streptococcus pneumonia* wild-type

in the VP-1 and NP-1 real-time PCR assays but were negative in conventional PCR assay (samples 1–5; Table III) and one sample that was positive in the VP-1 real-time PCR assay only (sample 6; Table III). Using the conventional assay as the reference standard, the sensitivity of both real-time NP-1 and VP-1 assays was 100%, with specificities of 94% and 93%, respectively.

All six samples providing discordant results provided high cycle threshold (C_t) values ranging from 34 to 38 (Table III), suggesting the viral loads in these samples were low and probably below the detection limit of the conventional assay. To further investigate these discordant results, these six samples specimens were retested using a nested PCR targeting the bocavirus NP-1 gene and two of the six samples provided positive results. DNA sequencing of the nested PCR product for both specimens provided sequences with 100% homology to bocavirus NP-1 sequences on the Genbank database (accession numbers include EU984096.1, EF450739.1, DQ988934.2).

Analytical specificity was assessed by testing the assays against 25 viruses and 6 bacteria (Table II). No cross-reactions were identified with these organisms or human genomic DNA in either real-time assay.

The reliable detection limit of the VP-1 and NP-1 assays was 10 copies per reaction. The conventional PCR assay had a reliable detection limit of 100 copies per reaction.

Detection of Bocavirus in the Various Sample Types

A summary of bocavirus detection in the three sample types is provided in Table IV. Of the 17 positive respiratory samples there were four samples that showed evidence of co-infection with another virus; one had both HBoV and human metapnuemovirus (hMPV) detected and the other three contained HBoV and WU polyomavirus.

Of 136 fecal specimens collected from children, 13 (10%) were positive for HBoV by both real-time PCR assays and 8 of these were from children 1 year of age or younger. HBoV was the only pathogen detected in 62% of the positive subjects. In three specimens (23%), HBoV was co-detected with rotavirus, and in a further specimen HBoV was co-detected with rotavirus, norovirus, and adenovirus. In one case, HBoV was co-detected with adenovirus plus astrovirus. The other 239 fecal samples collected from institutional outbreak patients were also

TABLE III. The Six Specimens Providing Discordant Results Following Testing of the 96 Respiratory Samples by Both Real-Time PCR and Conventional Reference PCR Assays

Sample no.	NP-1 RT PCR	VP-1 RT PCR	Conventional PCR	NP-1 nested PCR and sequencing
1	37.65	36.17	ND	ND
2	37.98	35.99	ND	ND
3	35.22	34.93	ND	Detected[a]
4	36.66	36.42	ND	ND
5	37.98	34.74	ND	Detected[a]
6	ND	33.88	ND	ND

A bocavirus NP-1 nested PCR and DNA sequencing was also performed for further investigation of these specimens. Cycle threshold (C_t) values are shown for real-time PCR assays.
ND, not detected.
[a]Bocavirus NP-1 confirmed by DNA sequencing of second round nested PCR product.

TABLE IV. Detections of Bocavirus in Various Sample Types

Sample type	Immune status	Age[a] mean; median	Bocavirus detections	C_t value range (mean; median)	Co-detections
NTS (n = 96)	Competent	2y3m; 2y2m	17 (18%)	14.8–36.8 (31.0; 33.9)	4 (24%)
Blood (n = 229)	Suppressed	6y8m; 5y10m	6 (2.6%)	32.8–36.9 (34.7; 34.2)	NI
Feces (n = 136)	Competent	2y11m; 1y2m	13 (10%)	18.7–40.2 (33.9; 35.2)	3 (23%)
Feces (n = 239)	Mixed	40y4m; 39y11m	5 (2%0)	25.83–39.0 (32.4; 33.4)	1 (20%)

[a]Age indicated by years (y) and months (m) C_t, cycle threshold; values for the real-time PCR assays NTS, combine nasal throat swab; NI, not investigated.

screened for HBoV, of these samples five (2.0%) had HBoV detected by both real-time PCR assays. Four of these had HBoV as the only identified pathogen, whereas one had a co-infection with rotavirus.

We tested 229 pediatric whole blood samples from immunocompromised children, with six (2.6%) positive specimens identified by both real-time PCR assays. One patient accounted for three of the positive samples (50%) identified. This patient had four consecutive blood samples taken over 4 weeks, with all but the week 3 specimen having HBoV detected.

DISCUSSION

In this study, we designed two real-time PCR assays for the detection of HBoV and applied it to three different sample populations to establish the prevalence of this virus. Two different gene targets, NP-1 and VP-1, were selected and applied in parallel to overcome potential false negative results due to sequence variants [Whiley et al., 2008]. When compared to the previously published conventional PCR method, these assays were found to have greater analytical sensitivity, with a detection limit of 10 copies per reaction for both assays. However, both real-time assays were statistically shown to have reduced clinical specificity (94% and 93%) compared to the conventional method. This was due to the increased number of detected samples, which were classified as false positive detections by the real-time assay on the basis that they were negative in the conventional assay. However, five out of the six additional positive samples were positive by both real-time PCR methods suggesting they were more likely true-positive samples. This is further supported by the accepted fact that real-time PCR generally has a superior limit of detection compared to conventional PCR methods. Analytical specificity was investigated and no cross-reactions were observed with unrelated viral or bacterial agents (Table II) or human genomic DNA.

The screening of 96 respiratory samples has produced a high rate of detection (18%) compared to other studies. This high rate may be attributed to the context of our study population (community based) or as a result of early collection of samples at the first signs and onset of respiratory illness. The presentation of HBoV in fecal samples from patients with AGE further suggests a possible link or role for this virus in gastroenteritis; however like respiratory specimens there was a high rate of co-detection.

This study detected HBoV in six blood samples from four patients, which confirms the previous findings of HBoV in an immunocompromised child [Schenk et al., 2007]. In our study, one of these immunocompromised subjects showed a prolonged viremia over a period of at least 4 weeks, which raises some important questions regarding reactivation or the pathogenic role of this virus in this patient population. It would be interesting to compare the results of serially collected blood specimens from asymptomatic children with and without an immununosuppressing illness.

In conclusion, we have developed and validated sensitive real-time PCR assays using two different gene targets for the identification of HBoV in different specimen types. By identifying HBoV in respiratory samples from the community, fecal samples from potential institutional outbreaks, and whole blood from asymptomatic immunocompromised children, we have confirmed that the virus may be widely distributed and raises further questions regarding its potential role in clinical illness. Clearly, HBoV needs to be further investigated as a possible viral causative agent associated with other clinical manifestations in addition to respiratory and gastrointestinal disease. The use of the highly sensitive and specific molecular assays described here will aid these investigations.

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