

## Development of a Real-Time PCR Assay for Detecting and Quantifying Human Bocavirus 2<sup>▽</sup>

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**Human bocavirus 2 (HBoV2) is a parvovirus that has been recently identified in stool samples from children. Any association between the virus and clinical disease is unclear. A rapid, reliable diagnostic method is necessary to address this issue. In this study, we developed a sensitive and specific HBoV2 quantitative real-time PCR assay that targets the HBoV2 *NP-1* gene, based on the TaqMan method. The assay could reproducibly detect 10 copies of a recombinant DNA plasmid containing a partial region of the HBoV2 genome, with a dynamic range of 8 log units ( $10^1$  to  $10^8$  copies). A clinical evaluation detected HBoV2 in 85 (24.6%) of 345 children with gastroenteritis, with viral loads ranging from  $1.67 \times 10^2$  to  $4.27 \times 10^9$  copies per ml of stool specimen.**

The family *Parvoviridae* consists of the subfamilies *Parvovirinae* and *Densovirinae*. The subfamily *Parvovirinae* is classified into five genera: *Parvovirus*, *Erythrovirus*, *Dependovirus*, *Amdovirus*, and *Bocavirus*. Parvovirus B19, in the genus *Erythrovirus*, is the only parvovirus known to cause human disease (11). Human bocavirus (HBoV), in the genus *Bocavirus*, was discovered recently and is detected frequently in nasopharyngeal aspirate, fecal, urine, and blood samples from children with respiratory or gastrointestinal tract infections (1, 2, 15, 18, 20). Serology studies demonstrated that most children and elderly people have been exposed to HBoV (4, 9, 12, 14). Although current data suggest that HBoV is the second parvovirus pathogenic in humans, it is very difficult to prove this without animal and cell models of HBoV.

In 2009, Kapoor et al. discovered human bocavirus 2 (HBoV2) in stool specimens from Pakistani children with acute flaccid paralysis (13). Subsequently, Arthur et al. reported that HBoV2 was the third most-prevalent virus in children with acute gastroenteritis (3). They found an association between HBoV2 infection and gastroenteritis using multivariate conditional logistic regression analysis. However, the exact clinical significance and epidemiological characteristics of HBoV2 remain to be determined, as discussed here. While it is frequently detected in fecal samples, HBoV2 is rarely detected in respiratory samples (7, 10).

To date, HBoV2 has been detected only by using nested PCR assays, which are laborious and prone to false-positive results. A sensitive and specific real-time PCR assay is necessary to address the epidemiology and disease association of HBoV2. In the present study, we developed a real-time PCR assay targeting the HBoV2 *NP-1* gene and used it to detect HBoV2 in stool specimens from acute gastroenteritis patients in Taiyuan, China.

### MATERIALS AND METHODS

**Clinical specimens.** Stool specimens were collected from 345 children with acute gastroenteritis who were hospitalized in Taiyuan Children Hospital, Taiyuan, Shanxi, China, between November 2007 and October 2008. Informed consent was obtained from the parents of all of the children who provided specimens. The study protocol was approved by the hospital ethics committee. The stool specimens were transported to the China Center for Disease Control (CDC) on dry ice and stored at  $-20^\circ\text{C}$  until further analysis. Viral DNA was extracted into 50  $\mu\text{l}$  of elution buffer manually from 140  $\mu\text{l}$  of a 10% fecal suspension in phosphate-buffered saline using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

**Primers and probe.** A conserved region of the HBoV2 *NP-1* gene distinct from that of HBoV was identified from alignments of nucleotide sequences available from GenBank (for HBoV, these were DQ000495, EF203921, EF203922, EF450721, EF450723, EF450727, and NC\_007455; for HBoV2 these were FJ170279, FJ170278, FJ170280, GU138990, GU138991, and GU138992) using CLUSTAL W (6). Primer and probe sets were designed for the conserved region using the Primer Express software (version 2.0.0; Applied Biosystems, Foster City, CA). The best primer and probe set selected by the software that showed no major nonspecific homologies in a BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was synthesized by Takara Biotechnology (Dalian). The TaqMan probe was labeled at the 5' end with the reporter molecule 6-carboxyfluorescein and at the 3' end with eclipse dark quencher (Table 1).

**Plasmid standard construction.** A 587-bp DNA subgenomic fragment of HBoV2 was amplified from a positive stool specimen using primers bracketing the real-time PCR target region in the *NP-1* gene (forward 5'-ATGAGCTCCG AATCTATG-3' and reverse 5'-CCTCTGATTCTGTGAAG-3'). The product was cloned into plasmid vector pGEM-T Easy vector (Promega, Madison, WI) and sequenced for verification (GenBank accession no. GU138990). The pGEM-T NP-1 HBoV2 plasmid was purified by using a QIAprep miniprep kit (Qiagen) and quantified by using an Eppendorf 6131 nuclear detector (Eppendorf). To generate standard curves for quantitative determinations and to assess the amplification efficiency, replicate serial 10-fold dilutions of the pGEM-T NP-1 HBoV2 plasmid were prepared in 10 mM Tris-EDTA buffer containing 100  $\mu\text{g}$  of herring sperm DNA (Promega, Madison, WI)/ml and stored at  $-20^\circ\text{C}$  until use. To minimize the potential for contamination, the HBoV2 plasmid DNA was prepared in a separate laboratory.

**HBoV2 real-time PCR assay.** The real-time PCR assays were performed using TaqMan Gene Expression Master Mix (Applied Biosystems); each 25- $\mu\text{l}$  reaction mixture contained 0.5  $\mu\text{M}$  concentrations of forward and reverse primers, a 0.1  $\mu\text{M}$  concentration of probe, and 2.5  $\mu\text{l}$  of nucleic acid extract. The Rotor-Gene 3000 real-time thermal cycling system (Corbett Research, Mortlake, New South Wales, Australia) was used for DNA amplification. The thermocycling conditions consisted of 10 min at  $95^\circ\text{C}$  for activating the AmpliTaq Gold, UP enzyme, and 45 cycles of 15 s at  $95^\circ\text{C}$  and 1 min at  $60^\circ\text{C}$ . Each run included serial 10-fold dilutions of the pGEM-T NP-1 HBoV2 plasmid as a positive control and

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TABLE 1. HBoV2 primers and probe used in the real-time PCR assay for gene target *NP-1*

Primer or probe <sup>a</sup>	Sequence (5'–3')	Amplicon length (bp)	Position <sup>b</sup>
Primer, forward	TCAGACCAAGCGACGAAGAC	145	2531–2550
Primer, reverse	CTCTAGCAAGYCTAGTAGAATGCC	145	2675–2652
Probe	AACCCACACCATCCAGGAGCATCTG	145	2646–2622

<sup>a</sup> The probe was 5' end labeled with 6-carboxyfluorescein and 3' end labeled with eclipse dark quencher.

<sup>b</sup> Nucleotide numbering is based on HBoV2 isolate strain PK-5510 (accession no. NC\_012042).

for the standard curve. A duplicate no-template control was extracted and amplified for every run.

**Conventional HBoV2 PCR.** The real-time HBoV2 PCR assay was compared to a nested-primer PCR using conditions described by Kapoor et al. (13). The first-round PCR primers were HBoV2-sf1 (AACAGATGGGCAAGCAGAAC) and HBoV2-sr1 (AGGACAAAGGTCTCCAAGAGG); the second-round PCR primers were HBoV2-sf2 (TGCTTCAACAGGCAAAACAA) and HBoV2-sr2 (TCCAAGAGGAAATGAGTTTGG), which amplified a 455-nucleotide (nt) region within NS1. The PCR products were analyzed by agarose gel electrophoresis, and the positive products were sequenced directly.

## RESULTS

**Real-time PCR amplification efficiency and limit of detection.** An optimized PCR assay targeting a conserved region of the *NP-1* gene was evaluated for amplification efficiency and the limit of target detection. PCR amplification of serial 10-fold dilutions of the pGEM-T NP-1 HBoV2 plasmid achieved >95% efficiency, with linear amplification over an 8-log dynamic range, from 10<sup>1</sup> to 10<sup>8</sup> copies per reaction (Fig. 1 and Table 2). To assess the limit of detection of the assay, 1, 5, and 10 copies of the pGEM-T NP-1 HBoV2 plasmid per reaction were tested in 12 replicates. At 10 copies 100% of the replicates were positive, at 5 copies eight (67%) replicates were positive, and at 1 copy three (25%) replicates were positive.

**Real-time PCR reproducibility.** To assess the intra- and interassay reproducibility, 10-fold serial dilutions of the pGEM-T *NP-1* HBoV2 plasmid from 10<sup>1</sup> to 10<sup>8</sup> copies per reaction were tested in triplicate on three different days. Over

TABLE 2. Efficiency of the HBoV2 real-time PCR assay

Estimated pGEM-T <i>NP-1</i> HBoV2 plasmid DNA copy no.	Mean <i>C<sub>T</sub></i> ± SD <sup>a</sup>
10 <sup>1</sup>	41.32 ± 0.80
10 <sup>2</sup>	37.62 ± 0.37
10 <sup>3</sup>	35.06 ± 0.07
10 <sup>4</sup>	32.47 ± 0.07
10 <sup>5</sup>	30.13 ± 0.11
10 <sup>6</sup>	26.03 ± 0.10
10 <sup>7</sup>	21.69 ± 0.06
10 <sup>8</sup>	17.18 ± 0.04

<sup>a</sup> The values shown are the means of triplicate samples. The slope, −3.31, was determined from the formula  $y = y \text{ intercept} - \text{slope} \log_{10}$ . The slope was calculated from 10<sup>1</sup> to 10<sup>8</sup> copies per reaction. The percent efficiency was 100.5. Efficiency =  $10^{(-1/\text{slope})} - 1$ .

the linear range of the assays, the coefficient of variation of the mean cycle threshold (*C<sub>T</sub>*) values within runs ranged from 0.21 to 1.92% and from run to run ranged from 0.36 to 1.72% (Table 3).

**Real-time PCR specificity.** The specificity of the real-time PCR assay was assessed by testing nucleic acid extracts of stool specimens containing human and microbiological flora DNA, as well as some enteric viruses, including rotavirus, norovirus, sapovirus, astrovirus, enteric adenovirus, and HBoV. We analyzed three or four positive samples for every enteric virus listed above. No specific amplification was detected for any of these samples.

**Real-time and conventional nested PCR results from patients with gastroenteritis.** As determined by real-time PCR, 85 (24.6%) of the 345 stool specimens were positive. The median viral load of the 85 positive samples was  $1.02 \times 10^4$  copies per ml of starting stool material (range,  $1.67 \times 10^2$  to  $4.27 \times 10^9$  copies/ml, Fig. 2). With conventional nested PCR, 57 (16.5% [57/345]) were determined to be positive by gel electrophoresis (with sequencing, 52 were confirmed as HBoV2, 4 were confirmed as HBoV, and 1 was confirmed as HBoV3; GenBank accession no. HM853485 to HM853512, HM853514, HM853515, HM853517 to HM853525, HQ012667 to HQ012677, and HQ141389 to HQ141395). The prevalence with the real-time PCR assay was higher than with the nested PCR assay (24.6% versus 16.5%,  $\chi^2 = 6.952$ ;  $P = 0.008$ ). The

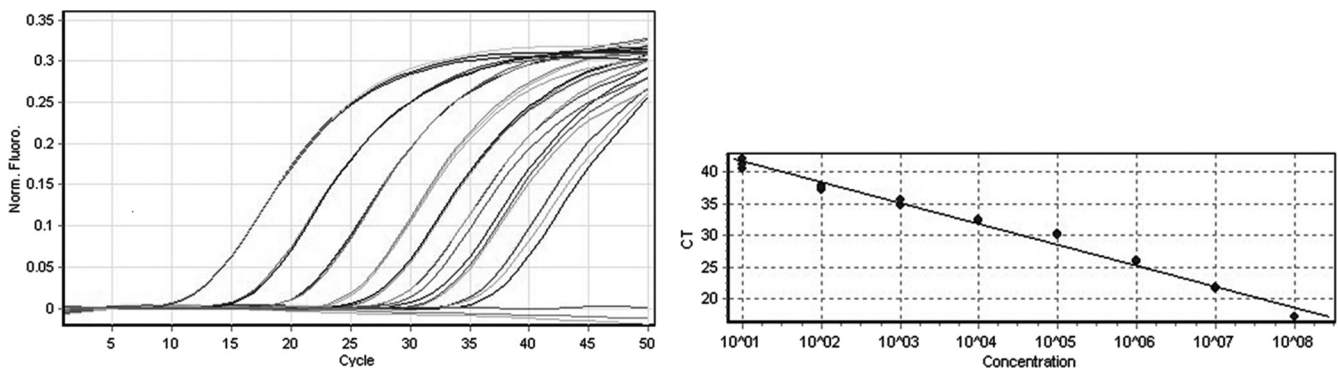


FIG. 1. *NP-1* real-time PCR assay showing representative results obtained with serial 10-fold dilutions (10<sup>1</sup> to 10<sup>8</sup> copies per reaction) of the pGEM-T NP-1 HBoV2 plasmid, run in triplicate. The left panel shows baseline subtractive curve fit views of the data with relative fluorescence units (RFU) plotted against cycle number. The right panel shows a standard curve analysis of the DNA amplification plots with cycle threshold (*C<sub>T</sub>*) values plotted against the logarithm of the input copy number. The dynamic range of the NP-1 PCR assay spanned 8 log units with a slope of −3.3 and an *R*<sup>2</sup> value of 1.0.

TABLE 3. Reproducibility of the HBoV2 real-time PCR assay

Reproducibility type	CV (%) of the no. of pGEM-T <i>NP-1</i> HBoV2 plasmid DNA for the following copies/reaction: <sup>a</sup>							
	10 <sup>1</sup>	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>	10 <sup>8</sup>
Intra-assay <sup>b</sup>	1.92	0.99	1.29	0.21	0.35	0.37	0.28	0.23
Interassay <sup>c</sup>	1.72	0.77	0.37	0.83	0.36	0.37	0.66	1.37

<sup>a</sup> Tenfold serial dilutions of HBoV2 plasmid DNA are indicated. Dilution series were thawed on three different days, and assays were performed in triplicate for each dilution. CV, coefficient of variation.

<sup>b</sup> Determined from three replicates within each assay.

<sup>c</sup> Determined from three independent assays performed on different days.

sensitivity of the conventional nested PCR was measured using dilutions of an NS1 plasmid subclone. Ten, five, and one plasmid copies were detected in 12/12, 6/12, and 2/12 PCR analyses, respectively.

Comparison of the two assays showed that 38 specimens were positive with both assays (median viral load,  $3.37 \times 10^4$  copies per ml; range,  $1.93 \times 10^3$  to  $4.27 \times 10^9$  copies per ml; Fig. 2). Of the 38 specimens, 36 were HBoV2 and 2 were HBoV according to the *NS1* gene sequences. For the two HBoV specimens, we sequenced their *NP-1* partial genes by using two sets of primers (data not shown) and found that they contained both HBoV2 and HBoV *NP-1* sequences (GenBank accession no. HQ398867 to HQ398870). In addition, 47 real-time PCR positive specimens were negative for conventional nested PCR (median viral load,  $4.01 \times 10^3$  copies per ml; range,  $1.67 \times 10^2$  to  $8.76 \times 10^4$  copies per ml, Fig. 2). Nineteen conventional nested PCR-positive specimens were negative for real-time PCR. Of the 19 specimens, 16 were HBoV2, 1 was HBoV3, and 2 were HBoV according to the *NS1* gene sequences. For the 16 HBoV2-positive specimens, we sequenced successfully *NP-1* partial regions of four specimens and found that two were HBoV2, one was HBoV, and one was HBoV3 in this region (GenBank accession no. HQ398863 to HQ398866). In addition, the *NP-1* sequences of the two HBoV2 specimens had no variation at their sites corresponding to the primers and probe. We also repeated the real-time PCR assay three times for the 16 HBoV2-positive specimens and found that 8 of them turned positive once, and the viral loads of the eight specimens

were lower than 10 copies per reaction, ranging from  $2.01 \times 10^0$  to  $6.99 \times 10^0$  copies.

#### Epidemiology and clinical symptoms of HBoV2 infection.

The 345 children with gastroenteritis ranged in age between 1 and 50 months (median age, 8 months). The ratio of boys to girls was 1.70:1 (217/128). According to the real-time PCR assay, the 85 HBoV2-positive cases ranged between 1 and 41 months (median age, 10 months), with no significant difference across age groups. The ratio of boys to girls was 1.36:1 (49/36). The positive cases were scattered throughout the year. There was a higher positive rate (34% [25/74]) during the period from February to April (Fig. 3). For the 85 HBoV2-positive cases, common clinical symptoms, in addition to diarrhea, included vomiting (42% [36/85]), fever (41% [35/85]), and respiratory symptoms (31% [26/85]).

#### DISCUSSION

Since the first detection of HBoV2, studies of HBoV2 infection have used conventional nested PCR assays with agarose gel electrophoresis (3, 13). To improve the diagnostic methods for HBoV2 detection, we established a real-time PCR assay in the present study. The assay was sensitive, specific, and reliable for HBoV2 DNA amplification, with a reproducible detection limit of 10 target genome copies per reaction and linear amplification over a wide dynamic range, suitable for quantitative applications.

Comparing the real-time PCR assay and the nested PCR

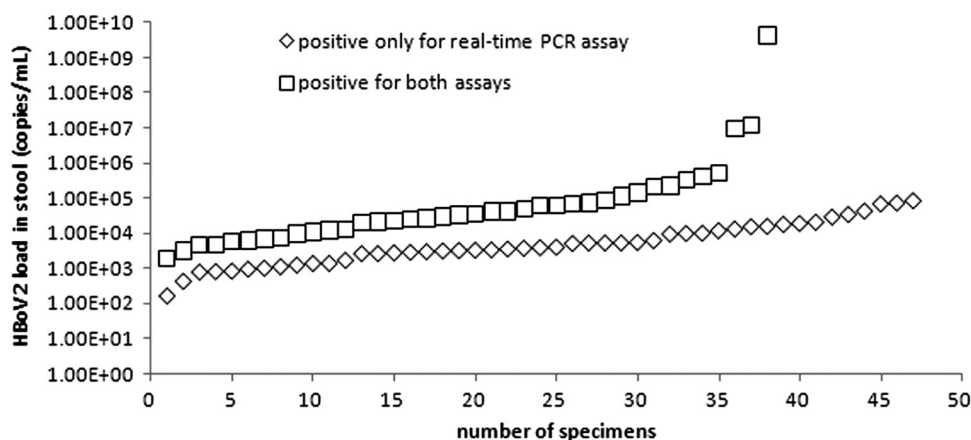


FIG. 2. Viral loads of HBoV2 in stools according to the real-time PCR assay. The median viral load of 38 positive specimens with both assays was  $3.37 \times 10^4$  copies/ml, ranging from  $1.93 \times 10^3$  to  $4.27 \times 10^9$  copies per ml. The median viral load of 47 positive specimens only by real-time PCR was  $4.01 \times 10^3$  copies/ml, ranging from  $1.67 \times 10^2$  to  $8.76 \times 10^4$  copies per ml.

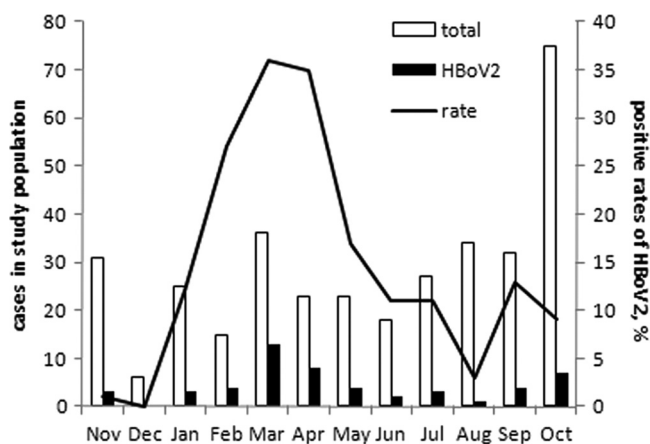


FIG. 3. Seasonal distribution of the children hospitalized with acute gastroenteritis, November 2007 to October 2008, at Taiyuan Children Hospital. The numbers of cases (bars, left y axis) and the HBoV2-positive rate (line, right y axis) varied in different months (x axis). "total" means the number of all of the cases. "HBoV2" means the number of HBoV2-positive cases.

assay of HBoV2, there was a significant difference between the detection rates (24.6% versus 16.5%,  $\chi^2 = 6.952$ ;  $P = 0.008$ ) in the clinical evaluation. However, the limits of detection of both assays determined by probit analysis were very similar. This indicates that the TaqMan assay is at least as sensitive as the nested PCR assay. Moreover, the TaqMan assay does give a quantitative measure of viral load, which could potentially correlate with clinical significance. A second advantage of the new assay is that tubes containing the amplified TaqMan product do not ever need to be physically opened in the laboratory, so that the potential for cross-contamination is theoretically much lower, making it more useful in most clinical settings.

In the specificity evaluation with positive samples, our real-time assay did not cross-amplify rotavirus, norovirus, sapovirus, astrovirus, enteric adenovirus, or HBoV. In the clinical evaluation, two HBoV-positive specimens confirmed by *NS1* sequencing were detected with the real-time assay. However, further sequence analysis showed that *NP-1* sequences of HBoV2 and HBoV coexisted in these two samples, which strengthens the specificity of the real-time assay. Using the nested PCR assay for HBoV2, an HBoV3 (*NS1* region) specimen was also detected, while it was negative using real-time PCR. This suggests that our real-time assay appeared to distinguish HBoV3 from HBoV2. However, 16 HBoV2 specimens confirmed by *NS1* sequencing after conventional nested PCR were negative for real-time PCR in our clinical evaluation. Further analysis found that this resulted from the low viral loads (lower than the detection limit) and potential recombination between *NP-1* and *NS1* in the viral genome, which had been noted in other studies (3, 13). Furthermore, as Kapoor et al. pointed out, possible divergence of the *NP-1* gene of HBoV2 may also explain this.

Note that HBoV2 had been detected using sensitive nested-PCR but not with single-round PCR in previous studies (3, 13), suggesting low viral loads in the specimens. In the present study, we found that the median viral load of the positive samples was  $1.02 \times 10^4$  copies per ml of stool

(range,  $1.67 \times 10^2$  to  $4.27 \times 10^9$  copies per ml), which was far below the viral loads in other enteric-virus-associated gastroenteritis. For example, the median viral load of norovirus was at least  $8.4 \times 10^5$  copies/g or ml of stool specimen in previous studies (5, 16). Based on the hypothesis that high viral loads are an indication of pathogenic relevance, the relatively low virus loads of HBoV2 in stool specimens do not seem to support its clinical significance. The lower viral loads even suggest that there may be a cutoff point and specimens with a viral load below this cutoff point should be classified as "negative," according to Phillips et al. (17). Further study is necessary to examine the significance of HBoV2 at low viral loads.

In our study, the HBoV2-positive rate was high from February to April. In comparison, Arthur et al. detected HBoV2 predominantly between midsummer and early spring, with no obvious peak (3). Furthermore, most HBoV2-positive patients had a fever and respiratory symptoms. Our results indicate that, like HBoV, HBoV2 is distributed worldwide (3, 8, 10, 13, 19) and may be associated with enteric or respiratory diseases. More investigations are needed to reveal the exact epidemiology of HBoV2 infection and its role in human disease.

The availability of a real-time PCR assay with the pGEM-T *NP-1* HBoV2 plasmid control developed in the present study will facilitate further studies to address these issues. In conclusion, we developed a real-time PCR assay that is more sensitive and specific than the reported conventional nested PCR. With this assay, we confirmed the high prevalence of HBoV2 with low viral loads in hospitalized children with gastroenteritis.

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#### REFERENCES

- Allander, T., et al. 2007. Human bocavirus and acute wheezing in children. *Clin. Infect. Dis.* **44**:904-910.
- Allander, T., et al. 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc. Natl. Acad. Sci. U. S. A.* **102**:12891-12896.
- Arthur, J. L., G. D. Higgins, G. P. Davidson, R. C. Givney, and R. M. Ratcliff. 2009. A novel bocavirus associated with acute gastroenteritis in Australian children. *PLoS Pathog.* **5**:e1000391.
- Cecchini, S., et al. 2009. Evidence of prior exposure to human bocavirus: a retrospective serological study of 404 adult sera in the United States. *Clin. Vaccine Immunol.* **16**:597-604.
- Chan, M. C. W., et al. 2006. Fecal viral load and Norovirus-associated gastroenteritis. *Emerg. Infect. Dis.* **12**:1278-1280.
- Chenna, R., et al. 2003. Multiple sequence alignment with the CLUSTAL series of programs. *Nucleic Acids Res.* **31**:3497-3500.
- Chiochansin, T., A. Kapoor, E. Delwart, Y. Poovorawan, and P. Simmonds. 2009. Absence of detectable replication of human bocavirus species 2 in respiratory tract. *Emerg. Infect. Dis.* <http://www.cdc.gov/EID/content/15/9/1503.htm> doi:10.3201/eid1509.090394.
- Chow, B. D., Z. Ou, and F. P. Esper. 2009. Newly recognized bocaviruses (HBoV, HBoV2) in children and adults with gastrointestinal illness in the United States. *J. Clin. Virol.* doi:10.1016/j.jcv.2009.11.030.
- Endo, R., et al. 2007. Seroepidemiology of human bocavirus in Hokkaido prefecture, Japan. *J. Clin. Microbiol.* **45**:3218-3223.
- Han, T.-H., J.-Y. Chung, and E.-S. Hwang. Oct 2009. Human bocavirus 2 in children, South Korea. *Emerg. Infect. Dis.* <http://www.cdc.gov/EID/content/15/10/1698.htm> doi:10.3201/eid1510.090337.
- Heegaard, E. D., and K. E. Brown. 2002. Human parvovirus B19. *Clin. Microbiol. Rev.* **15**:485-505.
- Kahn, J. S., et al. 2008. Seroepidemiology of human bocavirus defined using recombinant virus-like particles. *J. Infect. Dis.* **198**:41-50.

13. **Kapoor, A., et al.** 2009. A newly identified bocavirus species in human stool. *J. Infect. Dis.* **199**:196–200.
14. **Lin, F., et al.** 2008. ELISAs using human bocavirus VP2 virus-like particles for detection of antibodies against HBoV. *J. Virol. Methods* **149**:110–117.
15. **Neske, F., et al.** 2007. Real-time PCR for diagnosis of human bocavirus infections and phylogenetic analysis. *J. Clin. Microbiol.* **45**:2116–2122.
16. **Pang, X. L., J. K. Preiksaitis, and B. Lee.** 2005. Multiplex real-time RT-PCR for the detection and quantitation of norovirus genogroups I and II in patients with acute gastroenteritis. *J. Clin. Virol.* **33**:168–171.
17. **Phillips, G., et al.** 2009. Diagnosing norovirus-associated infectious intestinal disease using viral load. *BMC Infect. Dis.* **9**:63.
18. **Pozo, F., et al.** 2007. High incidence of human bocavirus infection in children in Spain. *J. Clin. Virol.* **40**:224–228.
19. **Shan, T. L., et al.** 2009. The first detection of human bocavirus 2 infections in China. *J. Clin. Virol.* doi:10.1016/j.jcv.2009.07.012.
20. **Vicente, D., G. Cilla, M. Montes, E. G. Perez-Yarza, and E. Perez-Trallero.** 2007. Human bocavirus, a respiratory and enteric virus. *Emerg. Infect. Dis.* **13**:636–637.