MAJOR ARTICLE

Human Bocavirus in Children Hospitalized for Acute Gastroenteritis: A Case-Control Study

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Background. Human bocavirus (HBoV) was recently discovered in children with respiratory tract disease and gastroenteritis. The causative role of HBoV in human gastroenteritis remains uncertain, and, to our knowledge, no previous case-control study has studied the relationship between HBoV and gastroenteritis.

Methods. We conducted a case-control study that examined stool samples from 397 children with diarrhea and from 115 asymptomatic control subjects. HBoV was detected using polymerase chain reaction. Real-time polymerase chain reaction was used to quantify the HBoV loads in case and control groups. Common enteric viruses were examined using enzyme-linked immunosorbent assays, polymerase chain reaction, and reverse-transcription polymerase chain reaction.

Results. At least 1 viral agent was discovered in 60.2% of cases. HBoV was detected in 14 samples, and 9 were coinfected with either rotavirus (7 of 14 samples) or human calicivirus (2 of 14). Many (8 [57.1%] of 14) of the HBoV infections occurred during September–December 2006. Most (12 [85.7%]) of the HBoV-infected children were 7–18 months of age. The percentage of children with HBoV infection did not differ significantly between case patients and control subjects (3.5% vs. 3.5%), and the statistical analysis did not support a correlation between HBoV infection and more-severe clinical symptoms. The viral load differences between the 2 groups were not statistically significant ($P = .09$, by log-normal Student's t test). In addition, the *VP1/VP2* partial gene of HBoV from case patients and control subjects showed minimal sequence variation.

Conclusions. A single genetic lineage of HBoV was revealed in persons in China. Despite its high prevalence in stool samples, our study does not support a causative role of HBoV in gastroenteritis.

Gastroenteritis is a major cause of childhood morbidity and mortality worldwide. Between 1.5 and 2 million infants and young children die of gastroenteritis-related diseases or complications annually [1]. Pathogenic enteric bacteria are important etiologic agents of this disease, but with the improvement of molecular detection methods, a large proportion of cases of gastroenteritis is found to be associated with viral etiological agents [2, 3], such as rotaviruses, human calicivirus (HuCV), adenoviruses, and astroviruses [4]. However, the etiologic agents are still not identified in some patients with gastroenteritis, despite improvements in diagnostic technology.

In 2005, Allander et al. [5] reported the detection of a new human parvovirus in children with acute respiratory tract infections. This virus belongs to the genus *Bocavirus* in the subfamily *parvovirinae* of the family *parvoviridae* and is most closely related to bovine parvovirus and minute virus of canines. Therefore, it was named "human bocavirus" (HBoV). Subsequently, HBoV has been detected frequently in children with respiratory tract infections and asthma exacerbation worldwide [6–11]. Several studies confirmed that HBoV infection is indeed associated with respiratory tract symptoms [12–15]. Recently, HBoV has also been implicated in diarrhea, and its detection rates in children with gastroenteritis have a range of 0.8%–9.1%. Therefore, this newly identified virus has been suggested to be an enteric pathogen, as well as being a respiratory

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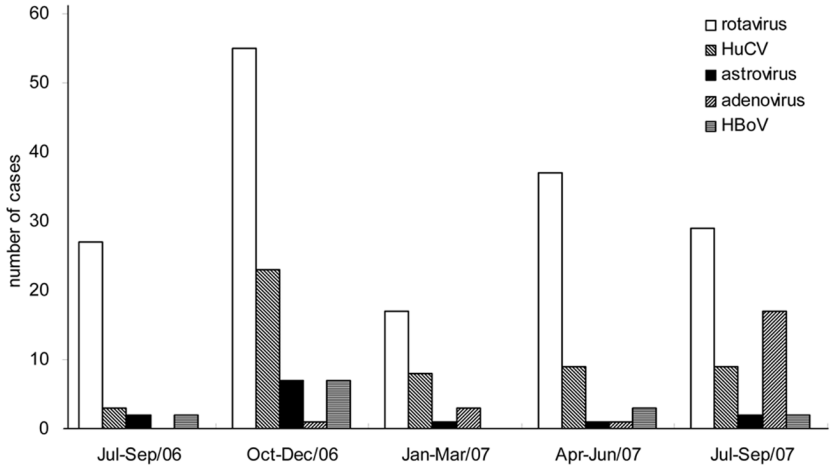

Figure 1. Seasonal distribution of viral gastroenteritis in pediatric patients hospitalized in the First Hospital of Lanzhou University (Lanzhou, China). HBoV, human bocavirus; HuCV, human calicivirus.

pathogen [16–19]. However, because most studies assessed the prevalence of HBoV in children with gastroenteritis in the absence of defined control children without enteric disease, the clinical spectrum of HBoV and the role that it plays in gastroenteritis remain to be clarified. The present case-control study investigated the prevalence of HBoV and other major viral etiologic agents in children hospitalized for gastroenteritis from July 2006 through September 2007 in Lanzhou, China, and the relationship between HBoV and gastroenteritis.

MATERIALS, PATIENTS, AND METHODS

Study participants and sample collection and processing. From July 2006 through September 2007, we conducted a case-control study to explore gastroenteritis-associated viral agents in hospitalized children with diarrhea and in healthy children in Lanzhou, China. Stools from 397 hospitalized children with diarrhea and from 115 asymptomatic control subjects were collected. All of the children were <5 years of age, and their parents were interviewed to determine the symptoms. Consecutive cases were collected from among children who were hospitalized for gastroenteritis in the Department of Pediatrics, The First Hospital of Lanzhou University (Lanzhou, China). Diarrhea was defined as ⩾3 loose stools in the previous 24–72 h [20]. Patients were excluded from the study if insufficient stool samples were available for a complete evaluation of viral agents, their stools had clear blood streaks, or they had another diagnosed illness, such as pneumonia. Control subjects were asymptomatic children who visited the First Hospital of Lan-

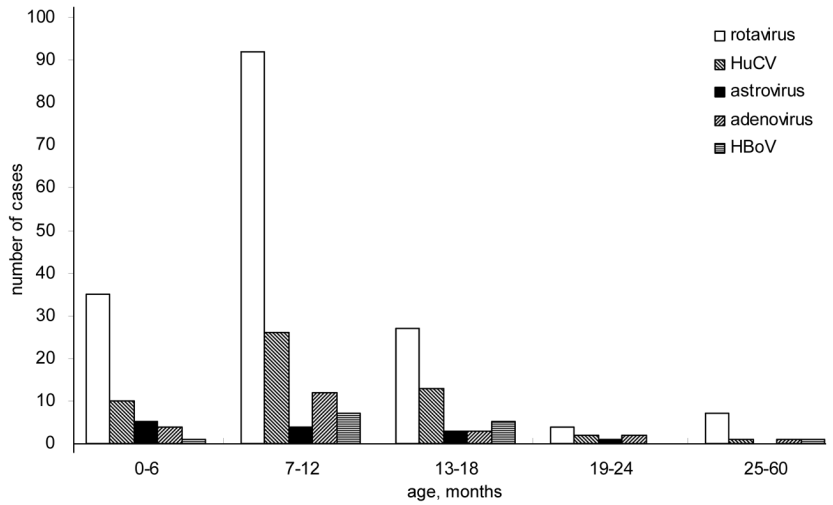

Figure 2. Age distribution of pediatric patients with viral gastroenteritis who were hospitalized in the First Hospital of Lanzhou University (Lanzhou, China). HBoV, human bocavirus; HuCV, human calicivirus.

Table 1. Clinical characteristics and viral loads of the 18 case and control children who were positive for human bocavirus.

Patient	Sex	Date of hospitalization	Age, months	Coinfection	Fever	Duration of diarrhea, days	Frequency of diarrhea, stools per day	Vomiting	Viral load, no. of copies
49316	M	July 2006	12	Rotavirus	+	3	8	+	9.26×10^5
50383	M	September 2006	16	Rotavirus	+	1	8	+	2.57×10^4
50410	F	October 2006	17	–	+	1	15	+	1.53×10^6
50429	F	October 2006	15	Human calicivirus	+	1	5	+	8.54×10^5
52756	M	November 2006	40	–	–	2	6	+	1.19×10^7
52762	M	November 2006	10	Rotavirus	–	2	10	+	8.70×10^6
52772	M	December 2006	12	Rotavirus	–	4	6	–	7.88×10^6
52773	M	December 2006	8	–	–	7	5	–	3.46×10^6
52780	F	December 2006	18	–	–	3	4	+	7.57×10^6
52886	F	May 2007	9	Rotavirus	–	3	5	+	6.09×10^6
52914	F	June 2007	7	Rotavirus	+	3	4	+	3.63×10^6
52924	M	June 2007	16	Rotavirus	+	3	6	+	1.25×10^6
53784	M	July 2007	8	Human calicivirus	+	16	7	+	1.05×10^6
53819	M	August 2007	6	–	–	6	10	–	7.33×10^5
53040	M	March 2007	6	–	–	–	–	–	1.87×10^6
53047	F	May 2007	4	–	–	–	–	–	1.03×10^7
53055	F	June 2007	6	–	–	–	–	–	1.75×10^6
53889	M	August 2007	3	–	–	–	–	–	1.32×10^9

NOTE. Duration of diarrhea indicates the time from the first day with diarrhea to the day of hospitalization. +, Present; –, absent.

zhou University Pediatric Primary Care Center for a routine examination and did not have fever, diarrhea, vomiting, or a respiratory illness in the previous 3-week period [21]. All of the control subjects in this study were selected by the frequency-matching method. Control subjects received follow-up by telephone, and the children who had had the above-mentioned clinical symptoms during the week after the initial examination were excluded.

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. All specimens were stored at −70°C until further analysis. Viral RNA and DNA were extracted from 140 μL of 10% fecal suspension in phosphate-buffered saline with use of the QIAamp Viral RNA Mini Kit (Qiagen), which is supposed to extract viral RNA and DNA simultaneously, according to the manufacturer's instructions.

Rotavirus detection. Rotavirus antigen was detected using a commercial ELISA kit (IDEIA Rotavirus; Dako), according to the manufacturer's instructions. Positive specimens were then G and P genotyped with use of nested PCR with type-specific primers, as described elsewhere [22, 23].

Detection of common enteric viruses. Six enteric viruses were identified using multiplex RT-PCR and PCR with 3 sets of primers [24], as follows: set A, for detecting noroviruses GI and GII, sapoviruses, and astroviruses; set B, for all adenoviruses; and set C, for group B and C rotaviruses. The multiplex RT-PCR and PCR were performed in accordance with a protocol described elsewhere [24].

HBoV detection. HBoV was detected in the extracted DNA by PCR amplification of a 291–base pair fragment of the *NS1* gene with forward primer HBOV-1 (5′-TATGGCCAAGGCAATCGTCCAAG-3′) and reverse primer HBOV-2 (5′-GCCGCGTGAACATGAGAAACAGA-3′), as described elsewhere [11]. To acquire the partial *VP1/VP2* gene, we used forward primer VP1/2-1 (5′-GGACCACAGTCATCAGAC-3′) and reverse primer VP1/2-2 (5′-CCACTACCATCGGGCTG-3′), targeting an 820–base pair fragment [13]. The reaction mix contained 20 pmol of each primer and 2.5 units of ExTaq DNA polymerase (Takara Bio). After 5 min at 94°C, 35 cycles of amplification (94°C for 1 min, 54°C for 1 min, and 72°C for 2 min) were performed, followed by a 10-min extension at 72°C. Positive PCR products were purified using a QIAquick PCR purification kit (Qiagen) and were sequenced by Invitrogen.

Sequence analysis and accession numbers. The nucleotide and deduced amino acid sequences of the *NS1* gene and the *VP1/VP2* partial gene were compared with those of HBoV strains available at the GenBank site. Phylogenetic analyses were conducted with MEGA, version 3.1 [25]. The 12 partial sequences of the *VP1/VP2* gene were submitted toGenBank (accession numbers EU400116–EU400128).

Real-time PCR for HBoV. To quantify the HBoV loads, real-time PCR assays were performed. Each 25-μL reaction mixture consisted of 0.5 μL of forward primer 5′-TGC AGA CAA CGC YTA GTT GTT T-3′ and reverse primer 5′-CTG TCC CGC CCA AGA TAC A-3′ for the 88–base pair *NS1* target, 0.125 μL of probe 5′-CCA GGA TTG GGT GGA ACC TGC AAA-3′ [26],

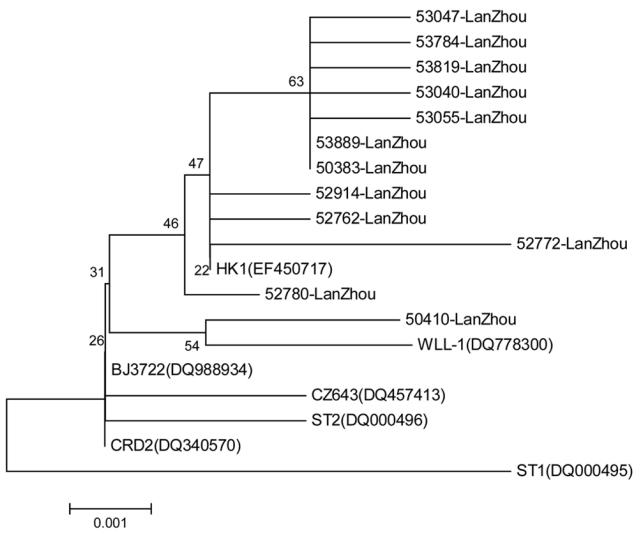

Figure 3. Phylogenetic tree of the *VP1/VP2* gene sequences of 12 human bocavirus strains from fecal samples. Strains 53040-LanZhou, 53047-LanZhou, 53055-LanZhou, and 53889-LanZhou were from asymptomatic children; the other strains were from children with gastroenteritis. Human bocavirus strains ST1 and ST2 from Sweden are the 2 prototype strains. Strains HK1, BJ3722, WLL-1, and CZ643 are from China, and strain CRD2 is from the United States.

and 2.5 μL of sample DNA. PCR was conducted at 50°C for 2 min and at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and at 60°C for 1 min. Serial dilutions of the pGEM-T Easy vector (Promega) containing the HBoV *NS1* gene were used as a quantification standard. Positive and negative controls were included. The Rotor-Gene 3000 real-time thermal cycling system (Corbett Research) was used, and the data were analyzed using Rotor-Gene software (Corbett Research), with the help of a standard curve. The minimum viral load that would allow reproducible quantification was 10 copies per reaction.

Statistical analysis. The statistical significance of rates between various groups was tested using the χ^2 test and Fisher's exact test. The statistical significance of means between various groups was tested using the Student's *t* test and the log-normal Student's *t* test. Analyses were performed using SPSS, version 11.5 (SPSS).

RESULTS

Patient characteristics. The ages of children with diarrhea ranged from 15 days to 60 months (mean age ± SD, 11.18 ± 9.3 months). The majority of patients (74.8%) were 0–12 months of age. The ratio of boys to girls was 1.8:1.

Virological findings in children with diarrhea. Of the 397 specimens, 239 (60.2%) contained at least 1 viral agent. Rotaviruses were identified in 165 (41.6%) of the 397 samples: G1 was the most common G serotype (91 of 165; 55.2%), and P was the most common P genotype (112 of 165; 67.9%) [8]. We detected HuCV in 52 (13.1%) of 397 samples, astroviruses in 13 samples (3.3%), adenoviruses in 22 samples (5.5%), and HBoV in 14 samples (3.5%). Group B and C rotaviruses were not detected in this study. Detection of rotaviruses, HuCV, astroviruses, and HBoV peaked during the winter. HBoV was not detected during spring 2007. Of the adenovirus-positive samples, 77.3% (17 of 22 samples) were collected during July–September 2007, indicating a possible outbreak of adenovirus infection during that season (figure 1). The majority of patients who were positive for rotaviruses, astroviruses, and adenoviruses were 0–12 months of age, whereas HuCV and HBoV were found mostly in children aged 7–18 months (figure 2). Of the 14 HBoV-positive samples, 64.3% (9) were coinfected with either rotavirus (7) or HuCV (2). Of the patients with HBoV-positive samples, 9 were boys and 5 were girls. Of these 14 patients, 7 had fever and 11 were vomiting, and the mean duration and frequency of diarrhea were 4.6 days and 6.5 times per day, respectively (table 1).

Prevalence of HBoV in asymptomatic children. The ages of 115 asymptomatic control children ranged from 1 to 60 months (mean age ± SD, 14.1 ± 17.2 months). The majority of these children (78.3%) were 1–18 months of age. The ratio of boys to girls was 1.2:1. The differences in mean age ($P = .08$, by Student's *t*-test) and sex ($P = .07$, by χ^2 test) between the case group and the control group were not statistically significant. HBoV was detected in 4 (3.5%) of 115 samples derived from the control subjects; 4 HBoV-positive cases were confirmed in subjects aged ⩽6 months. Samples were collected in March, May, June, and August. The ratio of boys to girls was 1:1.

Phylogenetic analyses of HBoV. All PCR products of the *NS1* gene were confirmed by sequencing. Because a previous study demonstrated that the greatest variation in the HBoV genome was in the *VP1/VP2* gene, especially at its 3′ end [13], 12 HBoV-positive specimens were randomly selected to amplify *VP1/VP2* partial gene sequences (nucleotides 4370–5189 of the HBoV genome). Phylogenetic analyses indicated that all 12 *VP1/VP2* genes in our study were in the same cluster as other strains from China (with >99% sequence homology) (figure 3).

Quantitative analysis of HBoV DNA. We used real-time

Table 2. Comparison of detection rates and human bocavirus (HBoV) loads between case group and control group.

Group	No. of HBoV-positive participants	Viral load, copies/mL
Case	14 of 397[a]	2.0×10^{6}[b]
Control	4 of 115	1.5×10^{7}

[a] $\chi^2 = 0.00$; $P = 1.00$, by χ^2 test.
[b] $t = 1.79$; $P = .09$, by log-normal Student's *t* test.

Table 3. Comparison of detection rates between children with gastroenteritis of undetectable etiology and those in whom other viruses were detected.

Etiology of gastroenteritis	Proportion with human bocavirus infection
Detectable	9/239
Undetectable	5/158

NOTE. $\chi^2 = 0.10$; $P = .75$, by χ^2test.

PCR to quantify the HBoV load. The mean HBoV load in the case group was 2.0×10^6 copies/mL of extract (range, 2.6×10^4–1.2×10^7 copies/mL), and the mean HBoV load in the control group was 1.5×10^7 copies/mL of extract (range, 1.8×10^6–1.3×10^9 copies/mL). There was no statitistically signficant difference between the mean values of these 2 groups ($P = .09$, log-normal Student's t test) (tables 1 and 2).

Association of HBoV with gastroenteritis. In our study, none of the HBoV-positive patients had respiratory symptoms. HBoV was not more prevalent among children with gastroenteritis than among asymptomatic children (3.5% vs. 3.5%) (table 2). HBoV was not more prevalent among children with gastroenteritis of undetectable etiology than among those in whom other viruses were detected (3.1% vs. 3.8%; $P = .75$, by χ^2 test) (table 3). When the HBoV-positive and HBoV-negative case groups were compared, neither the rates of fever among patients nor the rates of vomiting among patients differed statistically significantly ($P = .80$, by χ^2 test; and $P = .06$, by Fisher's exact test; respectively). In addition, the mean duration and frequency of diarrhea did not differ statistically significantly ($P = .52$ and $P = .42$, respectively, by Student's t test). Because the seasonal and age distributions of HBoV were similar to those of rotavirus and because there was often coinfection with HBoV and rotavirus, we compared the clinical symptoms between the group of children infected with rotavirus alone and the group of children coinfected with rotavirus and HBoV, and we did not find a statistically significant difference between these 2 groups (all $P > .05$, by χ^2 test and Student's t test) (table 4). These results suggest that infection with HBoV did not exacerbate the clinical symptoms of gastroenteritis.

DISCUSSION

This study examined common viral etiologic agents, to delineate the clinical role played by HBoV in children hospitalized with gastroenteritis. Our results revealed that enteric viruses play an important role in pediatric diarrhea and that the most common viral agents causing severe gastroenteritis consisted of group A rotaviruses, followed by HuCV. The prevalence of adenoviruses and astroviruses was similar to that reported elsewhere [3, 27–29]. Although some studies have detected high rates of groups B and C rotaviruses in stool samples from children with diarrhea [3, 30, 31], these pathogens were not found in samples from our participants.

Previous findings [32–34] supported the association of animal bocaviruses with both respiratory symptoms and gastroenteritis, particularly in calves and puppies. Some studies raised the question about whether HBoV is a cause of human gastroenteritis [16, 35]. HBoV is thought to be swallowed during a respiratory tract infection and subsequently excreted in the feces, without further replication in the gastrointestinal tract [35]. Recently, HBoV was found to occur frequently in stool samples from children with gastroenteritis. Vicente et al. [18] found that 48 (9.1%) of 527 stool samples from Spanish patients with gastroenteritis were positive for HBoV. Lau et al. [17] detected HBoV in 2.1% of fecal samples from children with gastroenteritis in Hong Kong, and some of these patients had respiratory tract symptoms. These studies implicated HBoV as a gastroenteritis-associated enteric virus. However, the con-

Table 4. Comparison of clinical symptoms between different groups of children with gastroenteritis.

Group	No. of children	No. of children with fever	No. of children with vomiting	Duration of diarrhea, mean days ± SD	Frequency of diarrhea, mean stools per day ± SD
Positive for HBoV	14	7[a]	11[b]	3.93 ± 3.89[c]	7.07 ± 3.00[d]
Negative for HBoV	383	178	200	4.96 ± 5.89	6.38 ± 3.11
Coinfected with rotavirus and HBoV	7	4[e]	6[f]	2.71 ± 0.95[g]	6.71 ± 2.06[h]
Infected with rotavirus alone	158	93	110	4.44 ± 4.95	7.04 ± 3.36

NOTE. HBoV, human bocavirus.
[a] $\chi^2 = 0.07$; $P = .80$, by χ^2test.
[b] $P = .06$, by Fisher's exact test.
[c] $t = 0.65$; $P = .52$, by Student's t test.
[d] $t = -0.82$; $P = .42$, by Student's t test.
[e] $\chi^2 = 0.00$; $P = 1.00$, by χ^2test.
[f] $\chi^2 = 0.24$; $P = .63$, by χ^2test.
[g] $t = -0.92$; $P = .36$, by Student's t test.
[h] $t = -0.25$; $P = .80$, by Student's t test.

current detection of HBoV and other enteric viruses, which is unusual for known pathogens, raises concern over a causative role of HBoV in human gastroenteritis. Therefore, additional evidence is required to establish a link between HBoV and gastroenteritis.

In our study, the detection rate of HBoV (3.5%) was comparable to previously reported rates [17–19]. We also found that HBoV infections occurred more frequently during the winter months (50% of cases) in children aged <2 years. Coinfection with HBoV and another virus was common (64.3% among all HBoV-positive children with gastroenteritis). The most frequent codetection of HBoV and rotavirus further supported the idea that HBoV is an "innocent bystander" in gastroenteritis. In this regard, our results were generally consistent with previous findings [11, 18, 36], and the similar rates of HBoV in our control (3.5%) and gastroenteritis (3.5%) groups also argue against a causative role of HBoV in gastroenteritis. Furthermore, the difference in the HBoV loads, as measured in our control and gastroenteritis groups, was statistically nonsignificant.

On the basis of *VP1/VP2* gene sequences, all HBoV isolates found in our study were in the same cluster as the original isolate ST2 identified by Allander et al. [5], with >99% DNA sequence homology. Moreover, the other HBoV strains identified in China—including CZ, WL, BJ, and HK—are in the same cluster. This suggests that a single genetic lineage of HBoV is circulating in humans in China and that the HBoV viruses found in both the respiratory and enteric tracts of humans in China probably belong to a single genetic lineage.

To our knowledge, this is the first case-control study to investigate the linkage between HBoV and gastroenteritis. In summary, we did not find statistically significant differences in the prevalence and HBoV loads between children with and without gastroenteritis. Concurrent detection of HBoV and other enteric pathogens, such as rotaviruses, in children with gastroenteritis was common. In addition, infection with HBoV did not significantly influence the severity of gastroenteritis. Our results do not support a causative role for HBoV in gastroenteritis, despite its frequent detection in fecal samples worldwide. To further clarify the linkage between HBoV and gastroenteritis, future priorities should include additional case-control studies that have larger cohorts of case and control samples, the establishment of serological methods to trace the viral antigens and the immune response, and the development of an animal model for HBoV infection.

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Potential conflicts of interest. All authors: no conflicts.

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