Seroepidemiology of Human Bocaviruses 1–4

Kalle Kantola,1 Lea Hedman,1,2 Jane Arthur,6 Abdiwahab Alibeto,1 Eric Delwart,5,6 Tuomas Jartti,3 Olli Ruuskanen,3 Klaus Hedman,1,2 and Maria Söderlund-Venermo1

1Department of Virology, University of Helsinki, 2Helsinki University Central Hospital Laboratory Division, and 3Department of Pediatrics, Turku University, Finland; 4Microbiology and Infectious Diseases Laboratories, SA Pathology, Adelaide, Australia; 5Blood Systems Research Institute, and 6Department of Laboratory Medicine, University of California, San Francisco

Background. Recently, 3 new members of the genus Bocavirus, human bocavirus 2 (HBoV2), human bocavirus 3 (HBoV3), and human bocavirus 4 (HBoV4), were discovered. HBoV2–4 occur mainly in the gastrointestinal tract but rarely in the respiratory tract, contrary to human bocavirus 1 (HBoV1).

Methods. To investigate HBoV1–4 seroepidemiology among 195 adults and 252 wheezing children, we conducted immunoglobulin G (IgG) and immunoglobulin M (IgM) enzyme immunoassays with recombinant viruslike particles (VLPs). The children’s sera were also tested for HBoV1–4 DNA by quantitative polymerase chain reaction (qPCR).

Results. Both rabbit and human antibodies to HBoV1–4 VP2 VLPs were found to be cross-reactive. After depletion of HBoV1-reactive antibodies, the HBoV2–4 approximate seroprevalences in adults were 34%, 15%, and 2% and in children aged 1–2 years 25%, 10%, and 5%, respectively. After depletion of HBoV2–4-reactive antibodies, the HBoV1 seroprevalence among adults decreased from 96% to 59%. No cross-reactivity of human anti-HBoV IgG was observed with bovine parvovirus1, parvovirus B19 or PARV4. No child was HBoV2–4 viremic.

Conclusions. HBoV2–4 infect humans less commonly and elicit weaker B-cell responses than HBoV1. In our study HBoV2–4 did not seem to have a major etiological role in wheezing. Cross-reactivity with HBoV2–4 IgG partially accounts for the high HBoV1 seroprevalences previously reported. Correction for cross-reactivity is a prerequisite for VLP-based HBoV seroepidemiology.

Human bocavirus 1 (HBoV1) was discovered in 2005 [1] and has since been shown to circulate globally [2–5] and to be associated with acute respiratory disease in young children (reviewed in [6]). HBoV1 DNA is commonly found in feces [7, 8] and serum [2, 9], in addition to respiratory secretions.

In 2009–2010, 3 more human bocaviruses, HBoV2–4, were identified in feces [8, 10, 11]. In the HBoV1–4 strains used here, the amino acid sequences of the major structural virus protein 2 (VP2) of HBoV2–4 are 22%–23% divergent relative to HBoV1 and ~11% divergent relative to each other. HBoV2 and HBoV4 diverge from each other the most the most (11.3%) and HBoV2 and HBoV3 diverge from each other the least (10.6%). HBoV2 has been encountered more widely than HBoV3–4 and is likely to circulate globally [10–17]. Contrary to that of HBoV1, the tropism of HBoV2–4 seems to favor the intestines over the respiratory tract [8, 12, 13], although HBoV2 DNA has been detected also in the latter [14]. A case control study indicated a correlation of HBoV2 DNA detection in stool and acute gastroenteritis [10].

The persistent or intermittent presence of HBoV1 in the nasopharynx emphasizes the possibility that the detection of HBoV2–4 DNA in stool may not always be a sign of acute infection but may be due to prolonged shedding [18–20]. The relatively frequent detection of HBoV2 in the stool of asymptomatic participants [8, 10] also supports persistent shedding or reinfections. This would render qualitative polymerase chain reaction (PCR)–based diagnostics problematic, impede the assessment of the clinical role of these viruses, and underline the need for antibody assays for the dating of acute HBoV2–4
infections. On the other hand, the cocirculation of HBoV2–4 may influence serological tests for HBoV1.

To address these issues, we expressed recombinant VP2s of all 4 currently known human bocaviruses for immunoenzymatic examination of adult and child sera for immunoglobulin G (IgG) antibodies. The pediatric sera were also tested for HBoV1–4 DNA by quantitative PCR (qPCR), and some also for immunoglobulin M (IgM). Polyclonal rabbit antisera generated toward HBoV1 and HBoV2 viruslike particles (VLPs) and competition assays for human sera were used to assess the antigenic relatedness and to compensate for serological cross-reactivities.

METHODS

Clinical Specimens
We obtained serum samples from 115 healthy Finnish medical students (median age, 23 years; range, 21–32 years) [21, 22] and 80 Pakistani blood donors (median age, 20 years; range, 18–20 years) with informed consent. In addition, acute-phase (at the time of admission) and convalescent-phase (2 week later) serum samples were available from 252 (median age, 1.6 years; range, 20 years) with informed consent. In addition, acute-phase (at the time of admission) and convalescent-phase (2 week later) serum samples were available from 252 (median age, 1.6 years; range, 0.2–15 years) of 259 children with acute respiratory wheezing [2]. Paired serum samples from 4 and 1 serum sample from each of 3 children were not available for enzyme immunoassay (EIA) experiments because of depletion of sera. All 252 pediatric convalescent-phase sera initially underwent HBoV1–4 IgG EIA, and when results were positive, both paired samples were reexamined in parallel for IgG antibodies. Furthermore, 30 paired serum samples from the same 252 children were examined by HBoV2–4 IgM EIA, and 248 paired samples were examined by HBoV2–4 multiplex real-time qPCR [21, 23]. From 14 children, 17 sera (acute- or convalescent-phase or both) were unavailable for qPCR analysis. The selection criteria for HBoV2–4 IgM testing were a previously determined acute HBoV1 infection [21] or IgG seroconversion or IgG increase for HBoV2–4 observed in this study. All children had previously been tested for HBoV1 DNA by qPCR as well as for HBoV1 IgG and IgM by EIA without antigen competition [21, 23]. Real-time qPCR testing for HBoV2–4 was done as described [16], except that primers targeting HBoV1 were excluded from the assay. The study was reviewed and approved by the Ethics Committees of Turku and Helsinki University Hospitals.

Recombinant Protein Expression and Polyclonal Antisera
The bovine parvovirus1 (BPV1) VP2 gene (GenBank ABC69731) was PCR-amplified from a bovine serum sample and cloned in the baculovirus expression vector pAcUW51 (Becton Dickinson Biosciences). The HBoV1 [21], B19 [24], Merkel cell polyomavirus (MCPyV) [25], and PARV4 [26] constructs have been described previously. Protein purification methods, generation of polyclonal sera, and the creation of recombinant baculoviruses encoding for HBoV2, HBoV3, and HBoV4 VP2 are described in the online supplementary material.

Serologic Testing of Human Sera
The HBoV IgG and IgM EIAs were conducted as described for HBoV1 [21] and PARV4 [26]. We performed immunoblotting as described [23]. We initially examined all sera, either in this study or previously [21], by EIA for HBoV1 IgG without VLP competition. Samples without HBoV1 IgG were not retested for the virus with competition assays.

To calculate cutoff values for the EIAs, we ranked optical densities (ODs) from the HBoV2 IgG assay and determined the inflection point (Figure 1) as described [27]. For comparison, we did the same with the HBoV1 IgG EIA. For HBoV1, the inflection points were 0.08 with and 0.06 without competing (preincubating) the sera with HBoV2–4 VLPs to account for antibody cross-reactivity. For HBoV2, the inflection points were 0.07 with and 0.06 without competition with HBoV1 VLPs. Cutoff was determined as the inflection point plus 4 standard deviations (SDs) of ODs below the inflection point. The cutoffs for EIA without competition were 0.15 for HBoV1 and 0.11 for HBoV2. With competition the corresponding cutoffs were 0.13 and 0.12. Because of antibody cross-reactivity, the HBoV2-based cutoff was used also for the HBoV3 and HBoV4 VP2 assays.

VLP-Based Competition Assays
To study IgG cross-reactivity between 2 or more VLPs or to measure antibodies specific to an individual antigen (HBoV1) or group of antigens (HBoV2–4), we competed serum samples with ≥1 VLPs. We used varying concentrations of homologous VLPs to determine the concentration (30 μg/mL) of VLPs required for exhaustive antibody competition of all 4 human bocaviruses (Figure 2). For the detection of HBoV1-specific antibodies, we diluted the sera in phosphate-buffered saline (PBS) containing soluble unbiotinylated HBoV2–4 VLPs at 30 μg/mL each, or vice versa, and preincubated them for 1.5 hours at +4°C before transferring them to wells containing immobilized heterologous HBoV VLPs.

We assessed efficient competition of human sera by testing the samples in parallel with competing VP2 that was homologous to the immobilized antigen. These residual ODs were typically very low (median 0.05; 90th percentile, 0.11). The net ODs were calculated by subtracting the residual OD from the raw OD read at 492 nm. We designated the competed samples IgG positive or negative based on the net OD.

RESULTS

Purification of HBoV1–4 VLPs
CsCl-gradient purification of VP2s from insect cell lysates yielded highly pure recombinant protein preparations with an apparent molecular weight of ∼62 kDa. Analysis of the purified proteins by electron microscopy (EM) showed the presence of VP2 VLPs with an approximate diameter of 21–24 nm in all 4 protein stocks (data not shown).
Specificity of IgG Assays With Rabbit Antisera

Polyclonal rabbit sera produced against HBoV1 or HBoV2 VP2 VLPs were tested for HBoV1–4 VP2 reactivity in EIA by limiting dilution analyses (Figure 3). As expected, both HBoV1 and HBoV2 antisera showed the highest levels of reactivity with the homologous antigens. However, both antisera also showed reactivity, albeit $40$-fold less, with the heterologous HBoV antigens. No significant reactivity was observed with the phylogenetically unrelated MCPyV VLPs [25], used here as a measure of unspecific antibody binding.

The HBoV1 rabbit antiserum showed no reactivity with BPV1 VLPs and a modest reactivity with both PARV4 and B19 VLPs (Figure 3). The corresponding rabbit preimmune serum was unreactive with all of these antigens. Likewise, the HBoV2 antiserum showed only nonsignificant reactivities with the parvovirus relatives (Figure 3). Interestingly, despite the notably closer phylogeny of HBoV2–4 relative to HBoV1, the HBoV2 rabbit antiserum was more reactive with HBoV1 VLPs than with HBoV4 VLPs. Already the preimmunization serum sample of the HBoV2 rabbit showed a significant level of reactivity for
Figure 2. Immunoglobulin G (IgG) seroreactivity (mean ± range) with human bocavirus (HBoV) virus protein 2 (VP2) viruslike particles (VLPs) after competition with various concentrations (x axes) of soluble heterologous HBoV VLPs indicated in the captions. Sera from 3 children and 4 adults positive.
HBoV2 VLPs, which increased the HBoV2 reactivity approximately 100-fold.

Strikingly, the HBoV2 rabbit antiserum was very poorly reactive in immunoblotting with all tested (denatured) human parvovirus antigens, including the homologous HBoV2 VP2, despite the same gamma globulin concentration as that of the HBoV1 antiserum and very high EIA reactivity with non-denatured HBoV2 VLPs (data not shown). In immunoblotting, the 2 rabbit antisera at a 1:250 dilution did not show any observable reactivity with B19, PARV4, or BPV VLPs.

Specificity of Human Bocavirus VLP-Based EIAs With Human Sera
To assess the specificity of HBoV1–4 IgG and IgM EIAs, we used paired sera from 21 children with a HBoV1 IgG seroconversion and no detectable HBoV2–4 IgG in the acute-phase serum (Figure 4). These children have been thoroughly characterized as having acute HBoV1 infections [2, 21]; all tested positive for HBoV1 DNA and IgM in the acute- or convalescent-phase sera, and 17 (81%) also had HBoV1 DNA in the nasopharyngeal aspirate. IgG seroconversions for both HBoV2 and HBoV3 were observed in 5 of the paired samples (24%), whereas only marginal or no IgG increases were observed in the remaining 16 pairs (76%, Figure 4A). Of the 5 seroconversions, only 1 showed a relatively high IgG OD, whereas the remaining 4 showed low ODs in the convalescent samples. Of the 5 children with HBoV2/3 IgG seroconversions, 3 also seroconverted for HBoV4 IgG. After competition with soluble HBoV1 VLPs, none of the paired samples showed IgG seroconversions for HBoV2, HBoV3, or HBoV4. In contrast, all 21 serum pairs still showed HBoV1 IgG seroconversions after competition with soluble HBoV2–4 VLPs. The sera were without observable IgM reactivity with HBoV2–4 VLPs; IgM absorbances remained below the HBoV1 IgM-EIA cutoff (Figure 4B).

To further study the immunological relations of these viruses, we monocompeted serum samples from 3 children and 4 adults with various concentrations (0–32 μg/mL) of HBoV1, HBoV2, HBoV3, or HBoV4 VLPs (Figure 2). The children’s sera were selected on the basis of specific seroconversion (HBoV1) or IgG increases (HBoV2 or HBoV3), whereas the adults’ sera were selected on the basis of the presence of IgG for only 1 HBoV species (HBoV1–3). Such HBoV4-specific sera were not available; instead we used a serum sample from an adult that was IgG positive for both HBoV1 and HBoV4. Relatively little or no reduction in absorbances was observed in heterologous competition with the children’s sera, whereas a relatively high reduction of IgG reactivity was observed with the adults’ sera. This difference was most prominent with the HBoV3-IgG-positive sera.

No reduction of HBoV1–4 IgG reactivity was observed in heterologous competition of the adult or child sera with human parvovirus B19 VLPs, as illustrated in Figure 2. Competitions with PARV4 or BPV1 VLPs yielded results similar to those of B19 (results are not shown).

Seroprevalence Among Adults
Serum samples from 115 Finnish and 80 Pakistani adults aged 18–32 years were tested for HBoV1 VP2-reactive antibodies after competition with HBoV2–4 VLPs. HBoV1-specific IgG seroprevalences within these 2 groups were 64% and 53%, respectively. The combined HBoV2–4 IgG seroprevalences for these 2 groups after HBoV1-VLP competition were 30% and 45%, respectively.

For comparison, we also tested samples from the Finnish adults for IgG with all 4 antigens without competition. In these assays, the frequencies of HBoV1–4 IgG detection were 95%, 96%, 87%, and 78%, respectively. These significant differences in IgG detection rates between uncompetited and competed sera are demonstrated in Figure 5.

Of note, 16 of the 115 Finnish adults (14%) were found to be IgG negative for all 4 human bocaviruses on the basis of the competition assays. In contrast, without competition, all 16 of these participants were (mostly low-level) IgG positive for HBoV1 and HBoV2, whereas 12 (75%) of them showed IgG for HBoV3 and 9 (64%) for HBoV4.

Of the 20 Pakistani serum samples (25%) with concurrent reactivity for HBoV2 and HBoV3 (after HBoV1 competition), 15 (75%) lost their reactivity to both antigens after heterologous competition with HBoV2 or HBoV3 VLPs. We obtained similar results with sera showing coincident reactivity for HBoV4 and HBoV2 or HBoV3. On the basis of these results, we deemed the determination of species-specific HBoV2–4 antibodies by these assays unfeasible, and we discontinued it. The seroprevalence figures related to HBoV2–4 should therefore be considered only as quasi-specific estimates.

Detection of HBoV2–4 IgG among the exclusively male Pakistani blood donors (36 IgG+ [45%]) was significantly more frequent than among the Finnish medical students both when Finnish females were included (35 IgG+ [30%]; P = .04 by the χ² test of independence) and excluded (9 IgG+ [21%]; P = .001). Moreover, detection of HBoV2–4 IgG among adults was inversely associated with the presence of HBoV1-specific IgG (odds ratio 6.3; P < .001 by logistic regression). Only 20% of the participants with IgG specific for HBoV1 showed IgG also for HBoV2, HBoV3, or HBoV4 as opposed to 61% of participants without HBoV1-specific IgG.
Age-Specific Seroprevalence and qPCR Among Wheezing Children

Seroprevalence of HBoV1 IgG in children increased by age from 24% to 100% without assessment of cross-reactivity (Table 1). These IgG seroprevalences showed an average decrease of 12% on competition with HBoV2–4 VLPs prior to the HBoV1 assay.

The combined seroprevalence for HBoV2–4 among children aged ≤ 1 year and children aged > 2 years and ≤ 5 years increased
rapidly from 8% to 40%, respectively. As with the adults, cross-reactivity among HBoV2–4 hindered the determination of more precise species-specific seroprevalences. Among the 35 children positive for HBoV3 IgG, 30 (86%) were IgG positive also for HBoV2 after compensating for cross-reactivity with HBoV1. Detection of IgG for HBoV4 among the children was invariably associated with detection of IgG for HBoV2 or HBoV3.

Based on competition assays, 33 (13%) of the 252 children showed an IgG seroconversion for HBoV1, 5 (2%) for HBoV2, 2 (0.8%) for HBoV3, and 1 (0.4%) for HBoV4. Two pairs of sera showed concurrent seroconversions to HBoV2 and HBoV3 or HBoV4, or both. Seroconversions to HBoV3 or HBoV4 were invariably associated with a seroconversion to HBoV2. Furthermore, 3 four-fold IgG increases were noted. None of the sample pairs with seroconversions or increases were positive for HBoV2–4 IgM or DNA, and none had gastrointestinal symptoms.

None of the 487 serum samples of wheezing children showed the presence of HBoV2–4 DNA by multiplex qPCR. Of these 487 serum samples, 52 contained HBoV1 DNA [23].

**DISCUSSION**

The VLPs of all 4 human bocaviruses showed considerable cross-reactivity with HBoV1 and HBoV2 rabbit antisera. Similarly,
human sera from adults showed large reductions in HBoV1–4 ODs after competition with heterologous human bocavirus VLPs, indicating cross-reactivity. Furthermore, detection of HBoV2–4-specific IgG was shown to be inversely associated with the detection of HBoV1-specific IgG, possibly indicating some level of cross-protection or resulting from the "original antigenic sin" effect [28].

By contrast, antibody responses among children with acute primary HBoV1 infection were highly specific for HBoV1. These sera showed little or no signal reduction in heterologous competition assays and no or only a slight increase in HBoV2–4 IgG reactivity between samples with HBoV1 seroconversions. Similarly, the samples positive for HBoV1 IgM showed minimal reactivity with HBoV2–4 IgM. Furthermore, we have previously shown that diagnostic HBoV1 IgG and IgM EIA results, even without VLP competition, strongly correlate with the presence of HBoV1 DNA in serum [21, 23]. Of note, as shown here and previously [16], the qPCR is specific for HBoV1; reanalysis of the HBoV1-positive sera showed no DNA amplification with the HBV2–4 multiplex PCR.

The relatively low interspecies specificity of HBoV IgG EIAs observed here between individuals with recent versus past immunity suggests that previously reported EIA results for children with acute HBoV1 infection are likely to be accurate and predominantly HBoV1 specific, whereas reports of virtually 100% HBoV1 IgG seroprevalence rates among adults almost certainly overestimate the prevalence of HBoV1-specific antibodies. As shown here, the HBoV1 seroprevalences of 95% and 99% in Finnish and Pakistani adults measured without competition, declined to 67% and 53%, respectively, on serum preincubation with soluble HBoV2–4 VLPs. The less frequent detection of specific HBoV1 IgG among the Pakistani adults may be due to the higher prevalence of HBoV2–4, causing more cross-reactivity and, perhaps, cross-protection. Such regional variations in viral prevalences may reflect differences in environmental exposures.

However, the apparent lack of virus-specific antibodies after VLP competition does not necessarily refute prior exposure to a virus. Competition assays by definition eliminate cross-reactive antibodies from a mixed population of specific and nonspecific antibodies. Among individuals with relatively low IgG levels, most probably due to antibody waning, it is not unlikely that this slight-to-moderate reduction in signal intensity could result in apparent IgG negativity in EIA. Indeed, 14% of the Finnish adults in this study were IgG positive for ≥ 1 HBoV without competition but became IgG negative for all 4 antigens after competition with a single heterologous antigen. In support of a frequent human exposure to HBoV1, our results show an HBoV1-specific seroprevalence of 84% among wheezing children aged 5–15 years. This apparent contradiction in

Table 1. Immunoglobulin G (IgG) Seroprevalences for the 4 Human Bocaviruses Among Adults and Wheezing Children in Convalescent-Phase Samples

<table>
<thead>
<tr>
<th></th>
<th>HBoV1,a n (%)</th>
<th>HBoV1,b n (%)</th>
<th>HBoV2–4,c n (%)</th>
<th>HBoV2,d n (%)</th>
<th>HBoV3,d n (%)</th>
<th>HBoV4,d n (%)</th>
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</thead>
<tbody>
<tr>
<td>Finnish adults (n = 115)</td>
<td>109 (95%)</td>
<td>74 (64%)</td>
<td>35 (30%)</td>
<td>33 (29%)</td>
<td>9 (8%)</td>
<td>1 (1%)</td>
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<tr>
<td>Pakistani adults (n = 80)</td>
<td>79 (99%)</td>
<td>42 (53%)</td>
<td>36 (45%)</td>
<td>33 (41%)</td>
<td>20 (25%)</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>All children (n = 252)</td>
<td>140 (58%)</td>
<td>107 (45%)</td>
<td>65 (27%)</td>
<td>59 (25%)</td>
<td>27 (11%)</td>
<td>6 (3%)</td>
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<tr>
<td>Children by age</td>
<td></td>
<td></td>
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<tr>
<td>0–6 mo (n = 25)</td>
<td>6 (24%)</td>
<td>3 (12%)</td>
<td>2 (8%)</td>
<td>2 (8%)</td>
<td>1 (4%)</td>
<td>0</td>
</tr>
<tr>
<td>6–12 mo (n = 40)</td>
<td>12 (30%)</td>
<td>9 (23%)</td>
<td>3 (8%)</td>
<td>3 (8%)</td>
<td>2 (5%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>1–2 y (n = 88)</td>
<td>51 (58%)</td>
<td>45 (61%)</td>
<td>26 (30%)</td>
<td>22 (25%)</td>
<td>9 (10%)</td>
<td>4 (5%)</td>
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<tr>
<td>2–3 y (n = 42)</td>
<td>35 (83%)</td>
<td>29 (69%)</td>
<td>16 (38%)</td>
<td>15 (36%)</td>
<td>8 (19%)</td>
<td>1 (2%)</td>
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<tr>
<td>3–4 y (n = 25)</td>
<td>20 (80%)</td>
<td>14 (56%)</td>
<td>10 (40%)</td>
<td>10 (40%)</td>
<td>7 (28%)</td>
<td>1 (4%)</td>
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<tr>
<td>4–5 y (n = 10)</td>
<td>9 (90%)</td>
<td>7 (70%)</td>
<td>4 (40%)</td>
<td>4 (40%)</td>
<td>3 (30%)</td>
<td>0</td>
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<tr>
<td>5–15 y (n = 22)</td>
<td>22 (100%)</td>
<td>17 (77%)</td>
<td>8 (36%)</td>
<td>6 (27%)</td>
<td>5 (22%)</td>
<td>1 (5%)</td>
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</table>

Abbreviations: HBoV1, human bocavirus 1; HBoV2, human bocavirus 2; HBoV3, human bocavirus 3; HBoV4, human bocavirus 4; IgG, immunoglobulin G; VLPs, viruslike particles.

a HBoV1 seroprevalence without antigen competition.
b HBoV1 seroprevalence after competition with soluble heterologous HBoV2–4 VLPs.
c Combined HBoV2–4 seroprevalence after competition of HBoV1 IgG-positive samples with soluble HBoV1 VLPs.
d Individual HBoV2–4 seroprevalences after competition of HBoV1 IgG-positive samples with soluble heterologous HBoV1 VLPs. These values are influenced by HBoV2–4 cross-reactivity and are therefore approximations.
seroprevalences of the wheezing children and healthy adults may perhaps be explained by the positive correlation of HBoV1 infection with respiratory disease, waning IgG, or increased cross-reactivity of HBoV1 IgG among past versus acute infections.

The type of EIA assays applied in this study followed the methodology successfully used in our serodiagnosics of 3 other human parvoviruses, HBoV1 [21, 22], PARV4 [26], and B19 [24]. In future diagnostic studies it may be necessary to modify the methodology to compensate for the competition-related reduction in signal strengths. This is underlined by the inability of the current methodology to fully separate HBoV2–4 IgG detection. HBoV2–4, even though considered 3 individual species, may collectively represent a single serotype.

In the absence of negative standards to HBoV2–4, the EIA cutoffs were determined by inflection point analysis, whereby ODs are plotted against the rank of each result. Ranked random values from uninfected individuals should follow a straight line, and an exponential deviation from this line may be considered a probable sign of exposure to the immunizing agent. The inflection-point analysis for HBoV1 resulted in the same cutoff as the traditional method [20]. However, unlike in the previous study, we did not define separate cutoffs for negative and positive IgG EIA results, whereby this procedure yielded a slightly different HBoV1 seroprevalence.

In contrast to HBoV1, the lack of IgM, viremias, and the low number of diagnostic HBoV2–4 IgG among the children do not support a major etiological role of HBoV2–4 in acute expiratory wheezing, either with or without concurrent gastrointestinal symptoms. On the other hand, the low IgG ODs observed for HBoV2–4, compared with those for HBoV1, raises the possibility that antibody responses against these viruses are significantly weaker than those against systemic HBoV1 infections. This, with the lack of HBoV2–4 DNA in the 487 pediatric serum samples, may suggest local infection, such as that of the intestinal tract, rather than systemic infection. However, our children were selected based on acute expiratory wheezing and not gastroenteritis that would perhaps be more likely to be associated with acute HBoV2–4 infections.

The frequent detection of HBoV2 DNA in the feces of children as opposed to adults [8, 11, 12, 16] suggests a difference in infections. The type of EIA assays applied in this study followed the methodology successfully used in our serodiagnosics of 3 other human parvoviruses, HBoV1 [21, 22], PARV4 [26], and B19 [24]. In future diagnostic studies it may be necessary to modify the methodology to compensate for the competition-related reduction in signal strengths. This is underlined by the inability of the current methodology to fully separate HBoV2–4 IgG detection. HBoV2–4, even though considered 3 individual species, may collectively represent a single serotype.

In conclusion, we showed that the 4 known HBoVs are serologically cross-reactive. Our results support earlier PCR findings that the HBoVs most frequently infecting humans are, in descending order, HBoV1, HBoV2, HBoV3, and HBoV4. Furthermore, our results support prior data on the ubiquity of acute HBoV1 infections among children, yet indicate that previous studies may have overestimated the prevalence of HBoV1-specific antibodies, especially among adults. Our data further suggest that acute HBoV2–4 infections do not play a major role in wheezing. The data and tools in this study will help us to better evaluate previous studies on HBoV1 serology, provide building blocks for future studies, and improve our understanding of the basic epidemiology and biology of these newly discovered human viruses.

**Supplementary Data**

Supplementary data are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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