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Human Bocaviruses Are Not Significantly Associated with Gastroenteritis: Results of Retesting Archive DNA from a Case Control Study in the UK

Sameena Nawaz, David J. Allen, Farah Aladin, Christopher Gallimore, Miren Iturriza-Gómarad

Introduction

In 2005 a novel parvovirus was discovered in respiratory secretions of young children and was termed Human Bocavirus (HBoV-1) [1]. Other important members of the parvoviridae family include B19 which causes fihth disease and human parvovirus 4 (Parv 4) which has not yet been associated with a disease [2]. Parvoviruses in animals are generally associated with systemic disease but also with respiratory and enteric symptoms [3,4]. Since the discovery of HBoV-1 three other HBoV genotypes have been described, HBoV-2, HBoV-3 and HBoV-4. The association between HBoV-1 and respiratory disease has previously been well established [5,6,7,8,9,10,11,12,13,14,15,16,17,18,19]. Although all HBoVs have also been detected in stool samples with prevalences ranging from <1% to 20%, only HBoV-2 has been reported to be associated with symptoms of gastroenteritis [20]. Nevertheless, the role of HBoV2 as an aetiological agent of gastroenteritis has not been clearly confirmed, furthermore, to date, no clear association between the presence of HBoV-3 and HBoV-4 and disease has been established [21,22].

Recent seroepidemiological studies indicate that exposure to HBoVs occurs early in life and 90% of the population are seropositive by the age of 5, although differences were reported in the seroprevalence of type-specific antibodies to the different HBoVs, which suggested that HBoV-1 infections are more prevalent [23].

The Infectious Intestinal Disease Study (IID Study) was a large case control study of gastroenteritis carried out in the UK between 1993–1996 [24] with the aim to determine the burden and aetiology of sporadic cases IID in the UK population. Initially, the use of classical microbiology diagnostic methods and electron microscopy (for virus detection) failed to detect a potential aetiological agent or toxin in 49% of the cases [25]. Retesting of the archived samples from this study using molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common molecular methods for the detection of enteric viruses, bacteria and protozoa revealed viruses to be the most common
Materials and Methods

Samples
A total of 4,380 archived DNA from the IID study [26,27] were tested for the presence of HBoV DNA. This archive comprised DNA extracted from stool samples from 2,256 cases and 2,124 controls.

Pan- HBoV Detection Assay
The qPCR assay targeted the NS1 gene (Ratcliff et al., unpublished method, personal communication) and was performed using an ABI Taqman7500. Oligonucleotide primer and probe sequences and positions are described in table 1.

The reaction consisted of 0.1 M DDT (Invitrogen), 1X Platinum Quantitative PCR Supermix-UDG (Invitrogen), Pan-HBoV-F and Pan-HBoV-R primers each at a concentration of 100 μm, Pan-HBoV-NS1 probe at 10 μm concentration, ROX 25 μm (Invitrogen) 2.5 μl of template DNA and RNase free water to a final reaction volume of 25 μl. The amplification consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 60°C for 45 sec.

HBoV1, 2 and 3 Genotyping qPCR Assays
HBoV-1, 2 and 3-specific primer pair and probes were designed in house through alignment of sequence data available in GenBank. The reaction conditions are as follows; 1X Platinum Quantitative PCR Supermix-UDG (Invitrogen), HBoV-NS1-1F, HBoV-1R, HBoV2-R and HBoV3R primers each at a concentration of 20 μm, the HBoV1,2 and 3 probe at 10 μm concentration, ROX 25 μm (Invitrogen), 2.5 μl of template and RNase free water was added to a final reaction volume of 25 μl. The amplification conditions for the typing assay are the same as those described in the HBoV NS1 detection assay above.

Plasmid Controls
Plasmids containing a 1773 bp and a 1737 bp region of the NS1 encoding gene of HBoV-1 and HBoV-3 respectively were used for assay optimisation and as controls. Control material was kindly provided by R. Ratcliff, Adelaide, Australia.

The controls were also used in order to generate a standard curve for use with the pan-HBoV assay in order to allow for normalisation of the data generated including the comparison of relative sensitivities of the different assays and for quantitation of DNA present in each of the positive samples. The standard curve was generated using the plasmid containing a genome segment of the HBoV-1 and consisted of a series of 10 fold dilution containing from 300,000 copies/μl down to 3 copies/μl. Inter- and intra-assay reproducibility was analysed by performing replicate testing of the standards in a single run (X11) and repeated runs (X2), and the standard curve was also included in each assay run for quality control and normalisation of results.

Untypable Strains
A subset of 17 samples positive in the Pan-HBoV assay but which failed to amplify in the type-specific assays were confirmed using an alternative method published elsewhere [28,29], and 6 were further confirmed though direct sequencing of the amplicons obtained after purification either from solution or agarose gels using Agencourt AMPure (Beckman Coulter, USA) and GeneClean Spin kit (QBiogene), respectively, following manufactures protocols.

Statistical Analysis
The chi-squared test was used in order to evaluate the significance of differences observed between groups. For comparison of median values (analysis of CT values) the Mann Witney U-test was used. Prevalence Odds Ratio (POR = Pcases/(1-Pcases)/Pcontrols/(1-Pcontrols)) was calculated in the total cohorts and by age group.

Table 1. HBoV-specific oligonucleotide primers and probes (all located at the NS gene).

| Primers | Sequence (5’-3’) | Nt positions | reference |
|---------|------------------|--------------|-----------|
| Pan qpcr primers | | | |
| Pan-HBoV-F | ATA AAG TTC CAA ACT CAT TTC CTC TTG | 1994–2020 | Ratcliff et al* |
| Pan-HBoV-R | AGT GCA GWA TCC GTT TTC GTG | 2079-2059 | Ratcliff et al* |
| Pan HBoV1-F | TCT CC GGC GAG TGA ACA TC | 201–219 | This study |
| Type-specific qpcr primers (anti-sense) | | | |
| HBoV1-R | CAT CCG GAT GAG GAG CGC | 424-407 | This study |
| HBoV2-R | CTT CAG GAT GTG GTG CGC | 427-410 | This study |
| HBoV3-R | CAT CCG GAT GAG GA CAC | 405-392 | This study |
| Generic PCR primers for sequencing | | | |
| HBoV01.2F | TAT GGC CAA GGC AAT CGT CCA AG | 2091 | [29] |
| HBoV02.2R | GCC GCG TGA ACA TGA AAG ACA GA | 1791 | [29] |
| Probes | | | |
| pan HBoV-NS1 | 6FAM-CCT TTG TCC TAC WCA TTC-MGBBNFQ | 2025–2042 | Ratcliff et al* |
| HBoV-1 | 6Fam- TAT CAT AGA TTG TCC AGT TGC AGC-MGBBNFQ | 393-372 | This study |
| HBoV-2 | 6Vic- TT GGA TCA TGA GAC GTT CAG TCC C-MGBBNFQ | 399-376 | This study |
| HBoV-3 | 6Ned- CTG CAT GTC GAG TTG CTC GGT A-MGBBNFQ | 377-351 | This study |

*Unpublished method, personal communication. doi:10.1371/journal.pone.0041346.t001
Results

Prevalence of Infection with HBoVs

A total of 7.4% of the samples tested were positive for HBoV. No statistically significant differences were seen in the prevalence of HBoV between cases and asymptomatic controls, POR = 0.79 (Table 2). Peak HBoV infection was observed in children under the age of 5, both in cases and controls, with significantly higher HBoV incidence in children between 1 and 4 in asymptomatic controls than in the cases of gastroenteritis (POR = 0.6; p < 0.02). The number of HBoV positives in older age groups was too small for meaningful statistical analysis.

Viral Load

The average CT values were 34.5 and 34.8, and the median CT values were 36.3 and 37.4 in cases and controls respectively (see distribution in Figure 1). The majority of HBoV-positives in both cases and controls had copy numbers ranging between 30 and 299 copies/reaction (or between $4.5 \times 10^3$ and $4.5 \times 10^4$ copies/ml of feces). The distribution of HBoV viral loads between cases and controls was comparable and the median CT values between cases and controls were not significantly different (U-test; z = 0.458139, p > 0.05).

HBoV in the Presence of Other Enteric Pathogens

HBoV DNA was found in 149 (46%) samples in the absence of other co-pathogens (Table 3). No statistically significant differences were observed in the proportion of cases or controls in which HBoV was found as a single organism or in the presence of one or more pathogens in the cohort as a whole, however in the 1–4 years of age group, HBoV in the absence of any other enteric pathogens

| Table 2. Age distribution of HBoV positive samples in cases and controls of IID. |
|-----------------|-----------|----------|-----------|----------|-----------|----------|-----------------|
| Age Group       | CASES     |          | CONTROLS  |          | ALLTOTAL  |          | POR             |
| (years)         | HBoV pos % | TOTAL    | HBoV pos % | TOTAL    | HBoV pos % | TOTAL    |
| <1              | 34        | 26.2     | 130       | 62       | 34.8      | 178      | 0.66514        |
| 1–4             | 58        | 12.2     | 476       | 91       | 18.0      | 506      | 0.633          |
| 5–9             | 9         | 6.9      | 131       | 8        | 6.0       | 134      | 1.16112        |
| 10–19           | 5         | 4.4      | 114       | 2        | 1.9       | 103      | 2.37635        |
| 20–29           | 7         | 2.4      | 286       | 2        | 1.1       | 177      | 2.21088        |
| 30–39           | 8         | 2.2      | 365       | 3        | 1.0       | 294      | 2.22699        |
| 40–49           | 11        | 4.4      | 249       | 2        | 0.8       | 240      | 5.70711        |
| 50–59           | 8         | 4.0      | 199       | 1        | 0.5       | 192      | 8.29167        |
| 60–69           | 5         | 2.9      | 175       | 3        | 1.7       | 177      | 1.72696        |
| >70             | 4         | 3.1      | 131       | 1        | 0.8       | 123      | 3.96698        |
| TOTAL           | 149       | 6.6      | 2256      | 175      | 8.2       | 2124     | 0.79109        |

POR = prevalence odds ratio.
doi:10.1371/journal.pone.0041346.t002

Figure 1. HBoV detection assays, CT distribution in cases and controls.
doi:10.1371/journal.pone.0041346.g001
was found in 29% of the cases, but in 56% of the controls ($p<0.05$).

**Temporal Distribution of HBoV Infections**

HBoV infections were detected year round although a peak was observed in the spring/early summer months, between April and June 1994 (Figure 2).

**Distribution of HBoV by Gender**

HBoV DNA was found in 48.8% and 45.7% of female cases and controls, respectively. The distribution of HBoV among females and males was not significantly different from the distribution of females and males in the entire cohort which was 53% and 47%, respectively.

**Distribution of HBoV Genotypes**

A total of 106 (32.7%) HBoV positives were genotyped, whilst 218 (67.3%) remained untyped after testing in the HBoV types 1, 2 or 3 specific assays (Table 4). HBoV-1 detection was found predominantly in controls, ($p<0.001$) and HBoV-2 was predominantly associated with cases ($p<0.01$). The prevalence of HBoV-3 was not significantly different between cases and controls.

![Figure 2. Temporal distribution of HBoV infections.](doi:10.1371/journal.pone.0041346.g002)
HBoV-1 and -3 were predominantly found in children (Table 4). HBoV-2 in the absence of any other pathogen was detected in 17 (81.3%) of the cases, compared to 9 (47.4%) of the controls. In cases, HBoV-2 was found across the age groups, although more frequently in children <5, whereas in controls they were found predominantly in children <5 with only 1 example in an adult (Table 4). The prevalence of HBoV-2 in children <5 years old was however not significantly different between cases and controls, 2.8% and 2.6%, respectively.

A subset of HBoV that were negative in the type 1,2 or 3-specific assays were confirmed in an alternative pan-HBoV PCR, and sequencing of a small number confirmed them as types 1, 2 or 3. The majority of the untyped samples (70%) had a CT value of >37 in the screening pan-HBoV PCR, indicative of low viral loads being present in the samples.

**Discussion**

This represents the largest study to date investigating the role and distribution of HBoVs infections in community acquired sporadic gastroenteritis and in asymptomatic controls. Although the presence of enteric pathogens, eg norovirus or rotavirus, in asymptomatic individuals is well documented, a significantly higher prevalence of the pathogen is seen in cases than in the controls [26]. Therefore, our data suggests that HBoV are not causally associated with gastrointestinal disease in the UK population as a whole, nor in children. The prevalence of detection of HBoV in stool samples in previous studies varies widely (see summary in Table 5),

Of the 324 HBoV positive samples, 106 (32.7%) were genotyped in the type-specific assays. HBoV-1 was found predominantly in controls (p<0.001) and the prevalence of HBoV-3 was similar in cases and controls. Both HBoV-1 and -3 were predominantly found in children. HBoV-2 was predominantly associated with gastroenteritis cases (p<0.01). The overall prevalence in cases was 1.4% and 0.8% in controls, however, in children <5 year of age, the prevalence in cases and controls was similar, 2.8% and 2.6%, respectively. The prevalence of HBoV-2 in children in the UK was significantly lower than that reported in gastroenteritis and in age-matched asymptomatic controls.

| Table 4. Distribution of HBoV genotypes in cases and controls. |
|-------------|-------|-------|-------|-------|-------|-------|
| COHORT | Age Group (years) | HBoV-1 | HBoV-1+HBoV-2 | HBoV-2 | HBoV-3 | Untyped | TOTAL |
| CASES | <1 | 1 | 1 | 9 | 2 | 21 | 34 |
| | 1–4 | 3 | 1 | 8 | 6 | 40 | 58 |
| | 5–9 | 1 | 1 | 7 | 9 |
| | 10–19 | 2 | 1 | 2 | 5 |
| | 20–29 | 2 | 5 |
| | 30–39 | 3 | 5 | 8 |
| | 40–49 | 2 | 9 | 11 |
| | 50–59 | 3 | 5 | 8 |
| | >70 | 2 | 2 | 4 |
| | 60–69 | 5 | 5 |
| CASES Total | 4 | 2 | 32 | 10 | 101 | 149 |
| Percent | 2.7 | 1.3 | 21.5 | 6.7 | 67.8 | 100.0 |
| CONTROLS | <1 | 10 | 1 | 10 | 4 | 37 | 62 |
| | 1–4 | 14 | 8 | 6 | 63 | 91 |
| | 5–9 | 1 | 7 | 8 |
| | 10–19 | 2 | 2 |
| | 20–29 | 2 | 2 |
| | 30–39 | 2 | 1 | 3 |
| | 40–49 | 2 |
| | 50–59 | 1 |
| | 60–69 | 1 | 2 | 3 |
| | >70 | 1 | 1 |
| CONTROLS Total | 24 | 1 | 19 | 14 | 117 | 175 |
| Percent | 13.7 | 0.6 | 10.9 | 8.0 | 66.9 | 100.0 |
| GRAND TOTAL | 28 | 3 | 51 | 24 | 218 | 324 |
| Percent | 8.6 | 0.9 | 15.7 | 7.4 | 67.3 | 100.0 |
Table 5. Summary of published studies on the prevalence of HBoV in stool samples.

| Country     | Sampling date          | Cases No | Controls No | Population studied | % Cases HBoV-pos | % Controls HBoV-pos | Main Conclusion                                                                 | Ref |
|-------------|------------------------|----------|-------------|--------------------|-----------------|---------------------|----------------------------------------------------------------------------------|-----|
| Australia   | Jan-Dec 2001.          | 186      | 186         | Paediatric         | 17.20%          | 8.10%               | HBoV-2 associated with gastroenteritis (only if cases with a concomitant bacterial infection included). [22] |
| Brazil      | Jan 2003–Dec 2005.     | 705      | ND          | Paediatric         | 2.0% (HBoV)     | ND                  | No obvious temporal clustering of the HBoV-positive patients. [39]               |
| China       | July 2006–Sept 2007.   | 397      | 115         | Paediatric         | 3.50% (HBoV)    | 3.50% (HBoV)       | HBoV not associated with gastroenteritis. Temporal patterns observed : winter months. [38] |
| China       | July 2006–June 2008.   | 632      | 162         | Paediatric         | 20.40% (HBoV-2) | 12.30% (HBoV-2)    | No statistically significant association between HBoV detection and gastroenteritis. [32] |
| Germany     | Jan-Feb and Sept–Dec 2007. | 307      | ND          | Paediatric (daycare outbreaks) | 4.60% (HBoV)   |                     | HBoV was not associated with outbreaks of gastroenteritis in children in day care. [40] |
| Hong Kong   | Nov 2004–Oct 2005.     | 1,435    | ND          | ND                 | 2.10% (HBoV)    |                     | Same virus found in respiratory and stool samples. [13]                        |
| Korea       | Jan 2005–Dec 2006.     | 962      | ND          | Paediatric         | 0.80% (HBoV)    |                     | Temporal patterns observed : summer months. [41]                               |
| South Korea | May 2008–April 2009.   | 358      | ND          | Paediatric         | 0.5% (HBoV-1)   | 3.6% (HBoV-2)       | Temporal pattern observed : winter months. [31]                               |
| Spain       | Dec 2005–Mar 2006.     | 527      | ND          | Paediatric         | 9.10% (HBoV)    |                     | Prevalence of HBoVs in respiratory and stool samples was similar. [42]         |
| Thailand    | Nov 2005–Sep 2006.     | 225      | 202         | Paediatric         | 0.90% (HBoV)    | 0                   | Low prevalence. No statistically significant difference between cases and controls (p = 0.17). [43] |
| USA         | Dec 1–March 31 2008.   | 479      | ND          | Paediatric and adult | 1.30% (HBoV2)  | 0.7%                 | 1.5% adults. [45]                                                             |

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a study in Australia, in which HBoV-2 was detected in 17.2% and 0.1% of the cases and controls, respectively [22]. The findings of the study in Australia lead to the proposal of HBoV-2 as an important aetiological agent of infantile gastroenteritis. It is noteworthy however, that in the Australian study, the association of HBoV-2 with gastroenteritis was only significant when cases with a bacterial co-pathogen were included in the analysis. Although in the present study HBoV-2 in the absence of other enteric pathogens was found more frequently in cases than in controls, the small numbers found in such large study suggest that the role of these viruses in IID, if any, is likely to be small. A lack of correlation between HBoVs or HBoV-2 and paediatric gastroenteritis was also reported in several smaller studies published elsewhere [30,31,32].

A total of 67% of the HBoV-positive samples could not be genotyped using the genotype-specific PCR assays. The majority of these untyped samples (70%) had CT values >37. This suggests that failure to type may be associated with low viral loads and differences in the relative sensitivities of the genotyping assays compared to the detection assay. Although under experimental conditions and using plasmid controls the sensitivities of all assays were comparable, it is likely that when applied to true clinical samples the sensitivity of the type-specific assays was inferior, possibly due to as yet not identified strain variability within genotypes. Also, a HBoV type 4-specific assay was not included in this study, therefore, any possible HBoV4 infections would not have been typed. Of the panel of samples that were tested in an alternative pan-HBoV-PCR, the strains typed through sequencing were HBoV-1 (2 samples), HBoV-2 (1 sample) and HBoV-3 (3 samples). Furthermore, the distribution of untyped HBoVs was not significantly different in cases and controls.

HBoVs in the absence of other enteric pathogens were seen in 46% of the HBoV-positive samples, and more frequently in the controls, 50.3% vs 40.9% in cases. No significant difference in HBoV load was observed between cases and controls, or between the samples positive for HBoV alone or in the presence of other pathogens. Previous studies have investigated the relationships between viral load and disease severity [33,34,35,36,37]. In respiratory infections significantly higher HBoV loads were seen in samples collected from children positive for HBoV alone than in those from children with co-infections. In respiratory infections also, viral loads >10^9 were associated with disease, whereas loads <10^9 were associated with asymptomatic children [33,38]. This lead to the suggestion that higher viral loads are indicative of a causative role of HBoV in respiratory infections [33,39]. However, Brieu et al [38] found no significant correlation between viral load and clinical symptoms or disease severity.

In conclusion, the results obtained from investigating for the presence of HBoV DNA in archived DNA samples from a large and previously well described case-control study of IID suggest that HBoV, including HBoV-2, do not appear to be a significant cause of gastroenteritis in the UK population, and particularly in the paediatric population. Although HBoVs are relatively frequent across all ages, and in particular in preschool age children, they are found just as frequently among children and adults without symptoms of gastroenteritis.

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**Author Contributions**

Conceived and designed the experiments: MIG. Performed the experiments: SN DJA FA CG. Analyzed the data: SN DJA MIG. Contributed reagents/materials/analysis tools: SN DJA CG MIG. Wrote the paper: SN DJA MIG.

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