# Indictment by Association: Once Is Not Enough

**Philip E. Pellett**

Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan

Early life infections with human bocavirus 1 (HBoV-1) are common, and the virus is readily detected in the upper respiratory tract, often during acute upper respiratory tract illness. Its etiological role in respiratory tract infections has been uncertain because of its presence in asymptomatic children and frequent concurrent detection of other respiratory viruses. Longitudinal cohort studies, in which sampling is done at intervals that include periods of illness and health, have begun to bring clarity to the story. In this issue of the *Journal*, Martin et al [1] performed noninvasive weekly sampling and collected health data during the first 2 years of life and provide substantial evidence that HBoV-1 is indeed a respiratory pathogen in young children.

This story is an excellent example of the power of longitudinal cohort study designs in identifying etiologic roles for viruses that embody combinations of being highly prevalent, being able to reinfect previously exposed individuals and to persist for long periods in the infected host in the absence of symptoms, and, often, causing mild disease.

## HBoV-1

HBoV-1 was discovered in 2005 by sequencing nonhost DNA present in nasopharyngeal aspirates that were collected during respiratory tract infections [2]. HBoV-1 is a DNA virus of the family *Parvoviridae*, genus *Bocaparvovirus* (the name is derived from the hosts of the first characterized bocaviruses, bovines and canines), and species *Primate bocaparvovirus 1*, which also includes HBoV-3. The *Primate bocaparvovirus 2* species includes the somewhat more distantly related HBoV-2 and HBoV-4. There is very low sequence variability within the major strain groups. HBoV-1 VP1/2 amino acid sequences differ from their HBoV-2, HBoV-3, and HBoV-4 homologs by approximately 20%, whereas HBoV-2, -3, and -4 differ from each other by approximately 10% [3]. The 4 HBoV strain groups appear to represent distinct virus species, but this awaits formal consideration by the International Committee for Taxonomy of Viruses. HBoV have a wide geographic distribution [3]. HBoV-1 is most commonly found in respiratory tract specimens, while HBoV-2, HBoV-3, and HBoV-4 are more frequently detected in stool specimens [4].

Laboratory diagnosis of HBoV is made on the basis of type-specific quantitative polymerase chain reaction [5] or serologic analysis. The close relationship among the viruses leads to serologic cross-reactivity that complicates their seroepidemiology. Serologic methods are based on recombinant virus-like particles (VLPs) prepared for each of the 4 types of HBoV [6]. Type-specific VLPs can be used to deplete specimens of antibodies against a particular HBoV type; when this is not done, the seroprevalence of a particular HBoV type is likely to be overestimated. Combinations of immunoglobulin M (IgM), immunoglobulin G (IgG) avidity, and changes in IgG titers can be used to identify recent infections [7].

Early childhood infections with HBoV-1 are common, leading to a seroprevalence of >80% by 4 years of age [8]. The absence of HBoV-1 in umbilical cord blood DNA indicates that congenital infections are uncommon. While it is much less frequently detected in specimens from adults, HBoV-1 was present at low levels in approximately 6% of Italian blood donors [9], and its activity in adults can contribute to patterns of transmission within families [10]. Seasonality has been observed, albeit inconsistently [1, 7, 8, 11].

HBoV-1 DNA has been detected in respiratory (nasopharyngeal swab specimens), serum, and stool specimens, as well as in tonsil, adenoid, and heart tissue specimens, often in the presence of other respiratory viruses [11–14]. The virus can persist in oral secretions for months. Viral DNA was not detected in serum during serologically identified secondary

HBoV-1 events, and it appears that persistence in serum is shorter than in saliva. In paired specimens, HBoV-1 DNA was detected more frequently in saliva than in nasal swabs [15]. To better understand the significance of viral activity in various compartments, comparisons are needed of temporally matched, longitudinally collected blood, saliva, and respiratory specimens.

The spectrum of diseases associated with HBoV-1 extends beyond acute upper respiratory tract illnesses. HBoV-1 was detected in 3%–6% of acute otitis media cases (the leading cause of acute visits to pediatricians) [8, 16–19]. Serologic methods were used to identify primary or acute HBoV infections in children hospitalized with community acquired pneumonia [7]. Not just a pathogen in children, HBoV-1 genomes were present at >$10^9$ copies/mL in sputum and tracheal specimens from a 61-year-old immunocompromised man who died from a severe respiratory disease [20].

Comprehensive reviews of HBoV biology and molecular biology are available elsewhere [21–23].

## CRITERIA FOR CAUSALITY AND THE EXTRA CHALLENGES IN STUDYING PREVALENT AND PERSISTENT AGENTS

Koch's postulates and their several subsequent refinements and extensions provide rational criteria for assessing the etiologic relationship between an infectious agent and a given disease [24, 25]. An important element of etiologic proof—the criterion of temporality—is demonstration that the infection in question precedes development of the associated disease.

Much of what we know of HBoV-1 biology, such as its prevalence in various populations, comes from cross-sectional studies. Such studies involve comparison of otherwise similar populations on the basis of a variable that differs between the groups, such as the presence or absence of a particular disease. This can

answer the question of whether a particular infectious agent (or other variable, such as age) is associated with the disease of interest, but it cannot answer the critical question of temporality. In addition, such studies can be confounded if the infectious agent is common, is persistent, or can reinfect—all of which pertain to HBoV-1. The persistence and presence of HBoV-1 in asymptomatic children (cross-sectional controls) have made it very difficult to discriminate between the virus being a genuine respiratory pathogen or an apathogenic commensal in young children.

Temporality (ie, whether the infection precedes the disease) is addressed in longitudinal cohort studies, during which specimens and clinical data are collected at intervals from individuals who are followed over time. In such studies, each participant serves as his/her own control. Discriminatory power is enhanced if the time series begins before the initial exposure of interest and includes sampling during periods of health, as well as during periods of illness. If sampling begins during the acute phase of the disease being studied, critical information about temporality can be lost; fortunately, useful information can sometimes be gleaned from serologic responses that develop in the days and weeks that follow the initial event.

A key parameter in longitudinal studies is the sampling interval. Short intervals can be highly informative but can be expensive and logistically challenging. Home- and clinically-based daily sampling protocols were developed in Seattle for studies of genital herpes simplex virus 2 (HSV-2) infections [26, 27]. This work has helped to redefine our concept of HSV latency from one in which periods of virologic quiescence (organismal latency) are intermittently punctuated by relatively brief periods of overt viral activity that produce visible lesions and readily transmissible virus, to the more refined understanding that, in addition to readily visible reactivations, HSV frequently asymptomatically reactivates from neuronal latency, releasing small amounts of virus on what can be a nearly continuous basis.

Extending this home-based sampling model to human herpesvirus 6B (HHV-6B), Zerr et al were able to follow 277 children from birth to 2 years of age by enlisting family members to collect weekly saliva specimens and maintain daily health logs [28]. In addition to revealing a broader range of symptoms and effects due to primary HHV-6 infection, the study further demonstrated the utility and practicality of home-based specimen and data collection. Specimens and data from this study provided the foundation for the new study by Martin et al. Keys to success for such studies include identifying the right specimen to collect, training study participants to collect and ship them properly, ensuring logistical convenience for study participants, performing batch analysis of specimens, and capturing detailed health diaries relevant to the agent being studied.

## LONGITUDINAL STUDIES OF HBoV-1

In one of the earliest cohort studies of HBoV, nasal swabs and symptom diaries were collected by medical staff during monthly in-home visits for 1 year, beginning at birth [11]. HBoV was detected more frequently in children older than 6 months, and reinfections were observed. Prolonged shedding occurred in children with and those without acute respiratory tract infections, and nearly half (47%) of HBoV-positive specimens were simultaneously positive for another respiratory virus. Importantly, HBoV-1 DNA was detected in 8%–9% of respiratory tract specimens from children with and those without respiratory illness, making it clear that single positive specimens do not prove viral involvement in an episode of respiratory illness and amplifying the question of whether the virus has a pathogenic role in the disease.

In 2010, Martin et al reported a study that involved symptom-based longitudinal sampling of children attending day care who were experiencing respiratory illnesses [14]. Nasal swabs were collected at enrollment, at onset of each new respiratory

illness, and weekly during respiratory episodes until children no longer tested positive for any of the respiratory viruses studied and the illness was resolving. Of the 8 respiratory viruses assayed, only human rhinoviruses were detected more frequently than HBoV-1. HBoV-1 was detected at similar frequencies in enrollment specimens and during acute events, and 72% of acute events with specimens that tested positive for HBoV-1 also yielded specimens that positive for at least 1 other respiratory virus. Long periods of shedding were detected. There was no specific association between detection of HBoV-1 and respiratory illness, but coughing was more likely to persist for >7 days when HBoV-1 was present, and high HBoV-1 loads were associated with visits to a healthcare provider. As pointed out in an accompanying editorial [25, p 1613], "careful, prospective, controlled epidemiologic studies" are needed to determine the pathogenic potential of HBoV.

In 2012, Meriluoto et al measured IgM levels and IgG avidity in serum in children from age 3 months to for an average of 8 years, at intervals that averaged about 3 months for earlier time points and 6 months at later times [8]. Although the spacing of sampling intervals did not necessarily align neatly with acute clinical events, primary seroconversions (but not secondary events) were associated with upper respiratory tract infections and with acute otitis media.

To address questions about the presence of HBoV-1 in asymptomatic children and the relationship of the virus to illness, in their current study, Martin et al [1] made use of the specimens and data from the study by Zerr et al described above [28]. They identified 67 primary infections and found that shedding typically extended for >1 month, sometimes for >1 year. Importantly, they found that primary HBoV-1 infections are the likely cause of respiratory illness that is typically mild but can motivate individuals to visit healthcare providers. Some children experienced multiple rounds of infections with different allelic variants

of the virus, contributing to long-term asymptomatic shedding that probably contributes to transmission. This also showed that single rounds of infection do not confer sterilizing immunity in many children. The results of this and the study by Meriluoto et al suggest that associations between HBoV and illness might have been missed in the study by von Linstow et al and the earlier study by Martin et al, owing to the wider sampling intervals.

Collectively, these studies go a long way to validate the likely etiologic role of HBoV-1 in acute respiratory disease. Unfortunately, definitive rapid diagnosis of HBoV-1 clinical events remains elusive. As expressed by Martin et al [1], "detection of HBoV-1 at a single time point is not sufficient to diagnose an incident HBoV-1 infection and should be interpreted with care." Once is not enough.

Finally, the mild respiratory illnesses associated with HBoV-1 thus far might tempt some to label HBoV-1 a virus of little consequence. Studies of HHV-6B are instructive. When studied longitudinally in a birth cohort, HHV-6B was associated with a variety of mild symptoms and some physician visits, but none of the children required hospitalization [28]. Nonetheless, approximately 20% of emergency department visits for febrile young children are due to primary HHV-6B infections, only some of which have the characteristics of classic roseola [29]. The full pathogenic potential of HBoV-1 remains to be determined.

## Note

## References

1. Martin ET, Kuypers J, McRoberts JP, Englund JA, Zerr DM. Human bocavirus-1 primary infection and shedding in infants. J Infect Dis **2015**; 212:516–24.
2. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A **2005**; 102:12891–6.
3. Kapoor A, Simmonds P, Slikas E, et al. Human bocaviruses are highly diverse, dispersed, recombination prone, and prevalent in enteric infections. J Infect Dis **2010**; 201: 1633–43.
4. Paloniemi M, Lappalainen S, Salminen M, et al. Human bocaviruses are commonly found in stools of hospitalized children without causal association to acute gastroenteritis. Eur J Pediatr **2014**; 173:1051–7.
5. Kantola K, Sadeghi M, Antikainen J, et al. Real-time quantitative PCR detection of four human bocaviruses. J Clin Microbiol **2010**; 48:4044–50.
6. Kantola K, Hedman L, Arthur J, et al. Seroepidemiology of human bocaviruses 1–4. J Infect Dis **2011**; 204:1403–12.
7. Nascimento-Carvalho CM, Cardoso MR, Meriluoto M, et al. Human bocavirus infection diagnosed serologically among children admitted to hospital with community-acquired pneumonia in a tropical region. J Med Virol **2012**; 84:253–8.
8. Meriluoto M, Hedman L, Tanner L, et al. Association of human bocavirus 1 infection with respiratory disease in childhood follow-up study, Finland. Emerg Infect Dis **2012**; 18:264–71.
9. Bonvicini F, Manaresi E, Gentilomi GA, et al. Evidence of human bocavirus viremia in healthy blood donors. Diagn Microbiol Infect Dis **2011**; 71:460–2.
10. Jula A, Waris M, Kantola K, et al. Primary and secondary human bocavirus 1 infections in a family, Finland. Emerg Infect Dis **2013**; 19:1328–31.
11. von Linstow ML, Hogh M, Hogh B. Clinical and epidemiologic characteristics of human bocavirus in Danish infants: results from a prospective birth cohort study. Pediatr Infect Dis J **2008**; 27:897–902.
12. Fry AM, Lu X, Chittaganpitch M, et al. Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J Infect Dis **2007**; 195:1038–45.
13. Longtin J, Bastien M, Gilca R, et al. Human bocavirus infections in hospitalized children and adults. Emerg Infect Dis **2008**; 14:217–21.
14. Martin ET, Fairchok MP, Kuypers J, et al. Frequent and prolonged shedding of bocavirus in young children attending daycare. J Infect Dis **2010**; 201:1625–32.
15. Martin ET, Taylor J, Kuypers J, et al. Detection of bocavirus in saliva of children with and without respiratory illness. J Clin Microbiol **2009**; 47:4131–2.
16. Ruohola A, Meurman O, Nikkari S, et al. Microbiology of acute otitis media in children with tympanostomy tubes: prevalences of bacteria and viruses. Clin Infect Dis **2006**; 43:1417–22.
17. Rezes S, Soderlund-Venermo M, Roivainen M, et al. Human bocavirus and rhino-enteroviruses

in childhood otitis media with effusion. J Clin Virol **2009**; 46:234–7.

18. Beder LB, Hotomi M, Ogami M, et al. Clinical and microbiological impact of human bocavirus on children with acute otitis media. Eur J Pediatr **2009**; 168:1365–72.

19. Nokso-Koivisto J, Pyles RB, Miller AL, Jennings K, Loeffelholz MJ, Chonmaitree T. Role of human bocavirus in upper respiratory tract infections and acute otitis media. J Pediatr Infect Dis Soc **2014**; 3: 98–103.

20. Sadeghi M, Kantola K, Finnegan DP, et al. Possible involvement of human bocavirus 1 in the death of a middle-aged immunosuppressed patient. J Clin Microbiol **2013**; 51: 3461–3.

21. Schildgen O, Muller A, Allander T, et al. Human bocavirus: passenger or pathogen in acute respiratory tract infections? Clin Microbiol Rev **2008**; 21:291–304.

22. Jartti T, Hedman K, Jartti L, Ruuskanen O, Allander T, Soderlund-Venermo M. Human bocavirus-the first 5 years. Rev Med Virol **2012**; 22:46–64.

23. Schildgen O. Human bocavirus: lessons learned to date. Pathogens **2013**; 2:1–12.

24. Fredricks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. Clin Microbiol Rev **1996**; 9:18–33.

25. Williams JV. Deja vu all over again: Koch's postulates and virology in the 21st century. J Infect Dis **2010**; 201:1611–4.

26. Tata S, Johnston C, Huang ML, et al. Overlapping reactivations of herpes simplex virus type 2 in the genital and perianal mucosa. J Infect Dis **2010**; 201:499–504.

27. Johnston C, Zhu J, Jing L, et al. Virologic and immunologic evidence of multifocal genital herpes simplex virus 2 infection. J Virol **2014**; 88:4921–31.

28. Zerr DM, Meier AS, Selke SS, et al. A population-based study of primary human herpesvirus 6 infection. N Engl J Med **2005**; 352: 768–76.

29. Hall CB, Long CE, Schnabel KC, et al. Human herpesvirus-6 infection in children. A prospective study of complications and reactivation. N Engl J Med **1994**; 331: 432–8.