

Human bocavirus: still more questions than answers

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The human bocavirus was first detected in 2005 and since then has been found in both respiratory secretions from patients with airway infections and in stool samples from patients with gastroenteritis. Meanwhile, four different genotypes have been identified that most likely derive from recombination events. Although the modified Koch's postulates have not yet been fulfilled completely, owing to the lack of an animal model or a simple cell culture system, there is increasing evidence that the human bocaviruses are serious participants in infectious diseases of the respiratory and the GI tracts. This article reviews the current status of the clinical features of human bocaviruses and provides an overview of the latest findings concerning the biology, phylogeny, epidemiology and diagnostic tools related to human bocaviruses. Furthermore, it discusses the potential pathogenicity of human bocavirus, as well as its persistence and reactivation in hosts.

Human bocavirus (HBoV), a human parvovirus, was first discovered in 2005 by Allander *et al.* using a molecular virus-screening technique based on random amplification, cloning, sequencing and phylogenetic analysis [1]. The virus was discovered in pools of nasopharyngeal aspirates of children with suspected acute respiratory tract infections [1] and later in stool samples from individuals with intestinal infections [2–5]. Therefore, the question arises as to whether HBoV gives rise to respiratory or to gastroenteric diseases, rather than being an innocent bystander. However, most studies still merely indicate the prevalence of HBoV associated with diseases. Indeed, the evidence for the virulence of HBoV continues to increase but, at the same time, its pathogenic role remains unanswered [6–10]. Mainly owing to a lack of adequate methods, such as efficient cell culture and animal models, HBoV cannot still be confirmed as a causative agent for diseases in the strict sense of Koch's modified postulates [11]. In addition, many epidemiological studies report a high number of coinfections, or report HBoV as the putative innocent copathogen, because copathogens were detected that fulfill Koch's postulates. The simultaneous occurrence of HBoV with other viruses makes it more complex to interpret its association with illness. One explanation discussed is the hypothesis assuming persistence and reactivation of HBoV based on, or influenced by, coinfections.

This article reviews the relevant studies that contain the latest findings on HBoV accessible up to March 2011.

Biology

Parvoviridae are small, nonenveloped viruses consisting of isometric nucleocapsids of 18–26 nm in diameter. The viruses hold single-stranded linear DNA of an average genome size of 5000 nucleotides with either negative-sense or positive-sense direction. The flanking termini are yet to be determined. Based on earlier observations, it is assumed that HBoV replication occurs via the rolling-hairpin mechanism, as described for other parvoviruses [12–15]; however, this hypothesis has not yet been verified by experimental data. Relevant to this assumption, Lüsebrink *et al.* recently detected head-to-tail DNA sequences of HBoV in clinical samples [16]. Since head-to-tail sequences contradict the rolling-hairpin replication [17], HBoV appears to have additional molecular features to other parvoviruses. Besides the possibility of being an artefact, dead-end product or a product of an as-yet unknown recombination event, the head-to-tail product would provide a rolling-circle replication in its classical sense. To confirm one or other of these hypotheses, further analyses of the genome structure and replication intermediates of HBoV are required.

Similar to all other members of the genus *Bocavirus*, the genome of HBoV contains three open reading frames (ORFs). The first ORF encodes a nonstructural protein (NS1). Its function in HBoV is unclear. However, NS1 is known as a multifunctional protein in other parvoviruses. In minute virus of canine (MVC) and in minute virus of mice, NS1 has an important role in viral DNA replication [18–20]. NS1 also

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- respiratory tract infection
- rolling-circle replication

participates in apoptosis, cell-cycle arrest and gene transactivation in B19 [21–24]. Whilst successfully characterizing the gene-expression profile of HBoV, Chen *et al.* were unable to use any of the HBoV1 proteins to induce apoptosis by transfection [25]; they were also unable to induce cell death in transfected cells by the expression of NS1 of MCV, which, in general, contrasts with findings for other parvoviruses (this appears to be a common feature for HBoV and MCV) [26,27]. However, Chen *et al.* have proven MVC to be able to induce mitochondrion-mediated apoptosis and cell-cycle arrest [26]. Nevertheless, an efficient HBoV infection system needs to be established to resolve the role of HBoV and its proteins with respect to pathogenesis.

The second ORF, which is unique to bocaviruses, encodes a second nonstructural protein (NP1). NP1 is known to play an essential role in DNA replication in MCV [28]. The third ORF encodes two structural capsid proteins, VP1 and VP2. Recent structural analyses of recombinant HBoV capsids, assembled from VP2, have disclosed a unique HBoV capsid surface topology, which still possesses a lot of common features with other vertebrate parvoviruses [29]. Despite high divergence on the sequence level, the structure of the major capsid protein of HBoV turned out to hold the same highly conserved capsid core as in all other parvoviruses. Furthermore, it has emerged that HBoV has the largest surface opening of the parvoviruses ascertained to date. Hence, regarding capsid topology, HBoV exhibits surface structural features closest to B19, the only parvovirus pathogenic to humans.

Classification

Since phylogenetic analysis has identified the novel virus as a parvovirus closely related to bovine parvovirus and MVC, the discovered virus was assigned into the family *Parvoviridae* within the genus *Bocavirus* (bovine–canine) and, consequently, named HBoV [1]. First and foremost, HBoV is frequently found in children younger than 2 years old with asthma exacerbation, bronchiolitis and wheezing [30–32].

Besides HBoV, one other new human parvovirus has been discovered recently, parvovirus 4 (PARV4). To date, PARV4 has not been associated with any illness and studies on the prevalence of the virus are rare. Based on its similarity, PARV4 is recommended to be allocated to the new genus *Hokovirus* [33].

Meanwhile, three additional HBoVs have been identified. HBoV1 has been primarily detected in respiratory samples, but is associated with

gastroenteric diseases [2,3,34]. Unlike HBoV1, the newer viruses (HBoV2 [35], HBoV3 [5] and HBoV4 [36]) have been found frequently in human stool samples. Amino acid analysis of the major structural protein VP2 of HBoV1 compared with those of the three other HBoV species has demonstrated a significantly closer relationship between HBoV2–4 [36]. Therefore, HBoV1 may have evolved from an enteropathogenic bocavirus after passing through the respiratory tract. Newer data suggest that HBoV3 was derived from HBoV1 and HBoV4 via recombination [37]. However, HBoV has been characterized recently by a rapid evolution, with a low level of polymorphisms probably ascribed to a relatively recent divergence [38].

Before the discovery of HBoV, parvovirus B19 of the genus *Erythrovirus* was the only member of *Parvoviridae* known to be pathogenic in humans. B19 infection appears to be associated with a variety of illnesses depending on the hematological and immunological state of the host [39,40]. B19 infections are associated with the fifth disease or erythema infectiosum, aplastic crisis, hydrops fetalis and arthropathy [41–43]. Based on their phylogenetic relationship, studies characterizing HBoV often refer to B19. However, the two viruses appear to be strongly divergent. For instance, unlike HBoV, B19 displays tropism for bone marrow and a lifelong persistence in heart tissue [44–46], whilst the question of which tissues and organs are the targets of HBoV and whether it can persist in these remains an unsolved riddle. Furthermore, in seroepidemiological studies with recombinant virus-like particles, HBoV and B19 have turned out to be antigenically distinct [47,48].

Zoonotic origin of HBoV

Approximately 75% of emerging pathogens are of zoonotic origin, and these viruses in particular have a high zoonotic potential and have been transmitted to humans by animal contact [49]. As members of *Parvoviridae* include significant pathogens in humans and animals, it is important to examine zoonotic events owing to host tropism, as well as evolutionary development. It might also contribute to an improved molecular and functional understanding of the still poorly characterized genus *Bocavirus*.

Recently, a new bocavirus species in gorillas has been found [50]. Phylogenetically, it is most closely related to HBoV and, similarly, provides two different NS proteins, which might be a feature restricted to primate bocaviruses.

Furthermore, novel porcine bocaviruses have been characterized [51]. Surprisingly, the *NS1* gene of PBoV has demonstrated a higher similarity to the homogeneous gene in HBoV than to all other known bocaviruses [52].

Epidemiology

Human bocaviruses have a worldwide distribution and patients test positive predominantly during the winter [53]. The occurrence of HBoV as the sole pathogen in a clinical course appears to represent the minority of clinical cases; thus, HBoV is frequently accompanied by at least one other pathogen. Although HBoV has been detected significantly more often in the absence of other pathogens [1], in most studies mono-infections were rare, whereas double infections dominated [54,55]. Up until now, the rate of HBoV coinfections with other viruses, whether due to respiratory or gastrointestinal infections, has been significantly higher than that of other viruses [3,56]. When associated with respiratory infections, HBoV appears predominantly with respiratory syncytial virus [57–59]. However, a significant appearance of HBoV with other respiratory viruses has not yet been determined.

The prevalence of HBoV in respiratory tract samples ranges between 1.5 and 19% [53,60]. In stool specimens, HBoV prevalence varies between 0.9 and 17.2% [5,61]. Based on recent transmission studies demonstrating hospital-acquired HBoV infections in children, HBoV screening for nosocomial childhood infections would be reasonable [62,63]. Acute infections of HBoV mainly seem to occur in children, whereas they are rare in adults, and nearly 100% of adults have immunity [64,65]. However, not only are the number of studies low, but, in addition, most studies refer to symptomatic patients. Therefore, further studies that systematically address the seroprevalence in healthy and infected patient cohorts are required to fill in the gaps of the seroprevalence puzzle of HBoV.

With respect to seroepidemiology, analyses have demonstrated that IgG seropositivity for HBoV in individuals increases with age (i.e., >85% are seropositive by an age of over 4 years, rising to almost 100% by an age of 7 years) [47,66]. Lindner *et al.* detected HBoV-specific IgM in 1% of adult healthy blood donors in Germany [67].

The clinical picture associated with HBoV infection owing to respiratory illness is similar to other respiratory virus infections. The most common symptoms are fever, rhinorrhea, cough and wheezing [53]. Common gastrointestinal

symptoms associated with HBoV in stool samples are nausea, vomiting and diarrhea [68]. Studies investigating the effects of drugs on HBoV are rare. For instance, Jartti and colleagues did not find any efficacy of corticosteroids in acute wheezing associated with HBoV infection [69]. So far, there is no specific clinical treatment or medication for patients diagnosed with HBoV.

Diagnostics

Newly discovered respiratory viruses, such as HBoV, are often less suitable for being detected via traditional culture or antigen diagnostics (otherwise they would have most likely been detected in the past, when cell culture was a routine diagnostic screening method in most virology departments). Therefore, tools to identify multiple clinically relevant respiratory viruses, as well as those unidentifiable by conventional methods, are necessary.

For many years, the diagnostic tools to determine respiratory virus infections have been very limited. Antigen detection performed in hospitals on patients with acute respiratory tract infection has identified a low percentage of the causative pathogens. At the same time, the number of potential viral pathogens has increased. Therefore, a rapid, simultaneous and sensitive method has become necessary. Initially, conventional PCR followed by real-time PCR were the main tools to detect HBoV [70]. Meanwhile, semiquantitative multiplex reverse transcription PCR (RT-PCR) systems, with a panel of up to 16 respiratory viruses, are already used in hospitals, with a detection rate of the potential etiological agent of up to 95% [71,72].

The multiplex real-time RT-PCR assay with fluorescence resonance energy transfer hybridization has emerged as a highly sensitive and specific quantitative method [71]. Furthermore, identification of respiratory viruses by RT-PCR combined with electrospray ionization mass spectrometry has been demonstrated [73].

Owing to the lack of an animal model, for better characterization of HBoV infection, molecular diagnostics of the anti-HBoV immune reaction became more important. Those molecular techniques were the trigger for the assumption that HBoV infection is putatively associated with pathogenic changes by HBoV, creating the clinical picture of respiratory tract infections and gastroenteritis [74–76]. HBoV/IgG avidity enzyme immunoassays have been proven to be adequate for distinguishing primary infection or immunoreactivation of HBoV, respectively [77]. Similarly,

immunofluorescence assays for the detection of IgG antibodies against structural proteins of HBoV VP1 and VP2 in human sera have been tested [78] and are a potentially promising tool for diagnostics.

To improve the diagnosis of acute viral respiratory infection, Sumino *et al.* searched for biomarkers [79]. They analyzed 17 respiratory viruses, including HBoV, and found a significantly higher level of the mediator IFN- γ -inducible protein 10 (IP-10) in bronchoalveolar lavage fluids of patients with respiratory viruses [79]. Inflammatory mediator profiles according to specific viruses may be useful to differentiate patients with respiratory virus infection. Virus-specific biomarkers correlating with the severity of viral infection will constitute a milestone in diagnostics. Using T-cell responses, Kumar *et al.* demonstrated strong HBoV-specific T-helper cell reactivity [80]. In the same study, similar levels of IFN- γ , IL-10 and IL-13 responses in B19 and in HBoV-seropositive subjects have been found. However, HBoV virus-specific response patterns demonstrated a significant interdependence between cytokine and proliferation response, whereas with B19 the cytokine response turned out to be more divergent [80].

Pathogenesis

Numerous studies show HBoV to be strongly associated with upper and lower respiratory tract diseases [53,81]. However, at the same time, Martin *et al.* could not find any significant difference of HBoV prevalence between asymptomatic and ill patients [82]. The report of Christensen *et al.* supports the hypothesis that HBoV is causative for respiratory tract infections by detecting viremia and high viral load to be associated with respiratory tract infections only [83]. However, they also found HBoV to be common in healthy children as well. The findings are also controversial regarding HBoV prevalence in gastroenteric infections. In several studies, HBoV has been detected in association with gastrointestinal illness [5,61,84]; however, HBoV has also been detected repeatedly in stool samples of asymptomatic patients [85,86].

Reported cases of infection with HBoV in the presence of severe immunodeficiency (three of which were associated with the transplantation of allogenic hematopoietic stem cells) have been suspected to lead to a high level of HBoV replication [87]. The aforementioned findings give rise to a hypothesis of reactivation of persistent HBoV infection during

immunodeficiency. For instance, a high viral load was found in an 18-year-old boy, while recent seroepidemic data suggested that HBoV antibodies are present in 90% of children at the age of 6 years [46,66]. Summarizing the results indicates that HBoV infections during immunodeficiency lead to persistent infection and prolonged viral shedding. This may supply additional support for the detection of HBoV1 and HBoV2 DNA or anti-HBoV antibodies in children with no symptoms of respiratory tract infections [37,88].

Persistence & reactivation of HBoV in the host

Owing to the lack of an animal model and the lack of proof of Koch's modified postulates, some open questions remain concerning HBoV. Surprisingly, an otherwise healthy control group tested positive for HBoV after elective surgery in the ear, nose and throat section (43%) [89]. Reasons for this phenomenon have not yet been deciphered, but several explanations may apply. First, inflammation of tonsillar tissues may facilitate bacterial superinfection, prompting surgical intervention or, second, inflammation of lymphoid tissue by recruitment of immune cells may trigger permissiveness to HBoV and, thus, lead to viral shedding in healthy people [90]. The assumption of persistence and reactivation would also explain the very high prevalence of coinfections with other viruses; in analogy, it was demonstrated that adult mouse kidneys became permissive to acute polyoma infection and reactivated persistent infection in response to cellular damage and regeneration [91]. It appears possible that HBoV is reactivated owing to covirus-induced cellular damage, which may result in stimulation of reactivation and replication of HBoV. Alternatively, HBoV may establish latent or persistent infection of mucosal lymphocytes (or contribute to tonsillar hyperplasia in young children) [90]. However, it still has to be investigated whether coinfections and elective surgery may have similar effects on HBoV regarding its reactivation from a persistent phase. An animal model must be established to obtain clear evidence for the pathogenicity of HBoV. Furthermore, in 2009, Dijkman *et al.* established a cell culture system for HBoV infection on pseudostratified human airway epithelial cells [92]. Unfortunately, its low efficiency combined with high costs make the system rather unattractive for more extensive investigations into HBoV.

Conclusion & future perspective

Variations in clinical emergence as well as in rate of coinfections led to misgivings of antibiotics due to pathogenesis of HBoVs. Almost all recent studies provide clues and increasing evidence for HBoVs as a causative agent in respiratory and/or gastrointestinal diseases. However, in the strict sense, Koch's modified postulates have not yet been fulfilled, and so the pathogenic role of HBoV is yet to be confirmed. The next chapters to be written in the story of HBoVs are the establishment of simple cell culture models and/or the establishment of an animal model. Such experimental systems are highly required in order to fulfill Koch's modified postulates, to analyze the replication

cycle in more detail and to develop specific therapies, especially since the conventional supportive therapy, prednisolone, has been shown to be ineffective in cases of HBoV infections [68].

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Executive summary**Background**

- Human bocaviruses (HBoVs) are associated with respiratory tract diseases and gastrointestinal illnesses in children and adults all over the world.
- A strongly varying prevalence of HBoVs, as well as a high rate of HBoV coinfections with other eligible pathogens, complicates the question of whether HBoV is a true pathogen rather than an innocent bystander.

Biology

- The HBoVs have most characteristics regarding genome, protein organization and topology in common with other parvoviruses. However, head-to-tail DNA intermediates in HBoV samples from patients point to an alternative or additional replication mechanism. Unlike the parvovirus-typical rolling-hairpin mechanism, HBoV could replicate in a rolling-circle manner.
- No cell death could be induced by the expression of HBoV1 proteins in transfected cells. HBoV shares this feature with minute virus of canine, which differentiates them from other parvoviruses.

Classification

- To date, four genotypes of human bocaviruses are known and have been designated HBoV1–4. It is assumed that HBoV1 evolved from enteric HBoV2–4.
- The HBoVs are characterized by a rapid evolution with a relatively recent divergence between the known genotypes.

Zoonotic origin of HBoV

- Recently discovered primate and porcine bocaviruses display high similarity to HBoV and have led to the hypothesis that HBoV could have a zoonotic origin.

Epidemiology

- Human bocaviruses are distributed worldwide and infections occur predominantly during the winter.
- Coinfections with other pathogens are significantly higher than for other viruses.
- The prevalence of HBoV in respiratory tract samples ranges between 1.5 and 19%, and in stool specimens, HBoV is distributed between 1.2 and 17.2%.
- Acute infections almost exclusively emerge in children, but nearly 100% of adults are seropositive.

Diagnostics

- Rapid, semiquantitative multiplex reverse transcription PCR systems or multiplex real-time PCR assays combined either with fluorescence resonance energy transfer hybridization or with electrospray ionization mass spectrometry are the latest tools to identify HBoV.
- Serological assays, such as enzyme immunoassays and immunofluorescence assays, supplement the HBoV diagnostics.

Pathogenesis

- While several studies show no significant difference in HBoV prevalence between ill and asymptomatic patients, others have demonstrated demonstrate a strong association with respiratory tract or enteric infections, respectively.
- Certain case reports indicate persistence and reactivation of HBoV in the host associated with immunodeficiency, mainly accompanied, or even elicited, by surgeries or coinfections.
- A cell culture system and animal model would be very helpful in simplifying the resolution of questions concerning the pathogenesis of HBoV.

Conclusion & future perspective

- While numerous studies indicate that HBoV causes respiratory and/or enteric diseases, the pathogenic role of HBoV is still unconfirmed.

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