The Role of the Human Bocavirus (HBoV) in Respiratory Infections

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Introduction

The current classification of human bocaviruses is based on the latest recommendations of the International Committee for the Taxonomy of Viruses (ICTV) (https://talk.ictvonline.org/taxonomy/). The variant 1 of the human bocavirus (HBoV-1) that causes respiratory infections in primates and humans belongs to the family of Parvoviridae, subfamily Parvovirinae and genus *Bocaparvovirus* and was discovered originally in 2005 by Tobias Allander [1] and co-workers and represents together with the strains HBoV-3 and the gorilla bocavirus the species *Primate bocaparvovirus 1* [2].

The discovery of HBoV-1 was one among a series of virus discoveries that occurred during the first 15 years of this century. These discoveries were based on novel virus discovery systems using molecular approaches developed in order to reduce the considerable number of cases in which a clinical diagnosis of a respiratory infection could not be confirmed by the laboratory detection of a pathogen. Following the initial description of the virus, a huge number of clinical studies and case reports have been published which were supplemented by some basic research reports. In parallel, several related viruses have been newly identified, such as a swine bocavirus, a feline bocavirus and a novel canine parvovirus, of which of them share some biological features with HBoV [3, 4]. In 2016, an additional novel bocavirus variant occurring in chimpanzees was identified, which along with the gorilla virus gives rise to the assumption that a long co-evolution between primates and bocaviruses exists [5–7].

Unfortunately, HBoV research still relies on clinical studies and case reports with accompanying cell culture studies as the major source of information on HBoV

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pathophysiology, because to date no animal model has been identified. Preliminary data on the use of ferrets as a model for gene therapy with HBoV capsid-based vectors suggests that ferrets might be a possible model for future research on HBoV-host interactions and vaccinations [8].

HBoV Biology

The human bocavirus (HBoV) was initially discovered in clinical samples from the respiratory tract of children suffering from respiratory infections of unknown aetiologies [1]. To date, HBoV is the fourth most detected respiratory virus, but as there is still no animal model or a broadly convertible cell culture available, Koch's modified postulates have not been experimentally fulfilled yet [9], but a case study from the group of Maria Söderlund-Venermo, Klaus Hedman and Olli Ruuskanen has shown that human-to-human transmission is most likely [10]. This report describes an intra-family infection chain that was characterized by both symptomatic and asymptomatic infections/transmissions, subsequent reactivation of the virus and hints for latency of HBoV.

Nevertheless, HBoV is the second parvovirus known so far that is capable of infecting humans with the potential to cause clinical disease. Until HBoV was discovered, the parvovirus B19 was the sole human parvovirus, which is difficult to culture in in vitro cell cultures, likely because infection strongly depends on the optimal cell cycle phase [11–20]. This latter fact hampered the development of potent and specific antivirals; tenacity studies and the development of disinfectants active against human parvoviruses as surrogate pathogens with animal pathogenicity were used. The narrow parvoviral host tropism also hampered the development of cell culture systems that support the replication of human bocavirus.

The discovery of HBoV has resulted in several molecular findings that are of major interest regarding the pathophysiology of human parvovirus. Within a primary cell culture in which the human bocavirus was replicating, it was possible to identify the HBoV transcriptome including splicing variant of viral RNA [21]. This cell culture demonstrated for the first time a potential tool for the investigation of human parvovirus in its natural infectious setting, enabling investigations of the molecular biology of human parvoviruses in general and HBoV in particular. Unfortunately, the primary cell culture that enables HBoV growth in vitro is very expensive and requires a highly specialized laboratory. Moreover, this is an error-prone cell culture, which means the availability of this technology is limited to several laboratories worldwide, which in turn will delay further research. In search for a broadly convertible replication system, the group headed by Dr. Jianming Qiu from the University of Kansas Medical Center made a significant step forward: this group has established a plasmid-based replicon-like system that has identified additional RNA species that are transcribed during the HBoV replication cycle [22]. The system is based on plasmids that contain the complete published HBoV sequence but are flanked by ITR regions of the adeno-associated virus (AAV); the ITR regions

are terminal repeats containing palindromic sequences that form hairpin-like structures which in turn are required for the replication of parvoviruses according to the so-called rolling hairpin mechanisms of replication [23]. With this first replicon system, Chen et al. have shown that HBoV types 1 and 2 express a similar RNA pattern like other parvoviruses. In particular, they identified a spliced NS-1 transcript that was not recognized before and have shown that the NP-1 transcripts are expressed abundantly [22]. In this context it is worth to note that the viral NP1 protein, which is a small NS protein encoded by the middle open reading frame, is required for the expression of viral capsid proteins (VP1, VP2 and VP3), whereas the other NS proteins (NS1, NS2, NS3 and NS4) are not essential for the expression of VP proteins [24].

Although the hairpin-like structures of HBoV were not described when the first genomic analyses were performed, it has been postulated that the HBoV genome also is flanked by such structures and that HBoV replicates its genome by the rolling hairpin mechanism, although this assumption is exclusively based on phylogenetic analogous conclusion rather than on experimental evidence. In theory, the rolling hairpin replication results in progeny genomes that occur in equal amounts of both polarities, whilst packaging of viral genomes is dependent on additional factors [25–31]. For almost four decades, it is postulated that all parvoviruses replicate according to this mechanism, although this replication model is solely based on experimental data obtained by the research on rodent parvoviruses. The model is characterized by a terminal hairpin-dependent self-priming initiation of the viral genome replication and concatemeric replication intermediates of head-to-head or tail-to-tail replication intermediates. Based on an early publication of the postulated model in 1976 in Nature, this replication model became a dogma in the field of parvovirology and was deemed to be true for all parvoviruses. Interestingly it was impossible to identify both genome polarities in clinical samples containing HBoV-infected cells [32]. Thereby, NASBA analyses revealed that all HBoV strains package negative-strand genome, whilst only a minority also packages the plus strand; this observation is compatible with another replication mechanism known as rolling circle replication. In order to test the hypothesis if rolling circle replication may occur in HBoV infection and in order to decipher the unknown terminal hairpins, a couple of systematic PCR-based analyses were performed [33].

This approach has identified DNA sequences that contain head-to-tail genome fragments linked by a newly identified linker stretch that has a partial by high homology to the minute virus of canine (MVC) ITR and to the ITR of bovine parvovirus. Most recently it was shown that these sequences most likely represent the missing terminal hairpin-like structures [33, 34]. Despite identifying the terminal sequences in both clinical samples and cell cultures, a lack of self-priming activity of HBoV genomes as well as the lack of intermediates typical for rolling hairpin replication has been noted. Instead the samples contained head-to-tail structures. Additional groups have published similar observations, all questioning the dogma of parvovirus replication [35–38]. It is thereby important to know that the head-to-tail episomal form of HBoV differs from formerly described circular parvoviral

episomes that have been shown to consist circular-closed genome dimers of head-to-head and tail-to-tail orientation [39].

Although the role of the linker sequence and the head-to-tail junction remains unclear, these findings were surprising as they support the hypothesis that HBoV replicates differently from non-human parvoviruses by possibly initiating a rolling circle mechanisms, at least as an alternative route of replication.

Based on the newly identified sequences, the structure of the putative terminal repeats of the HBoV genome was predicted in silico [34]. In addition, the Kansas group has developed a true full-length vector clone of HBoV which can be transfected to HEK-293 cells and produced a "recombinant wild-type" human bocavirus that in turn is infectious for differentiated CuFi-8 cells [40]. CuFi-8 cells are derived from a patient with cystic fibrosis and can be grown as monolayer cultures that can be differentiated into a polarized respiratory epithelial structure by changing the culturing media. This polarized respiratory epithelial structure in turn supports HBoV replication [40]. It is worth noting that CuFi-8 cells experience a serious cytopathic effect that is able to destroy the cellular glycocalyx structures (Fig. 1) and is accompanied by a loss of cilia [41]. This novel cell culture moreover supports the hypothesis that HBoV is a serious pathogen as it induced a remarkable cytopathic effect in the polarized CuFi-8 cell line which in turn is compatible with the assumption that the clinical symptoms of an HBoV infection are caused by tissue damages related to viral replication. Thereby, this infection model harbours a surprising feature that is a further hint for an alternative replication of the human bocavirus: if the full-length HBoV plasmid containing the hairpin sequences is transfected into HEK293 cells, infectious progeny virions are produced although based on the rolling hairpin model this process should be impossible, as the free (!) hairpin sequences are believed to be essential for the replication. In contrast, replication is possible in the plasmid although they are flanked by the vector's backbone sequence and no helper plasmids are required as known for the dependoviruses. This simple observation strongly contradicts the model of rolling hairpin replication but in turn favours other replication models known for circular DNA, as, for exam-
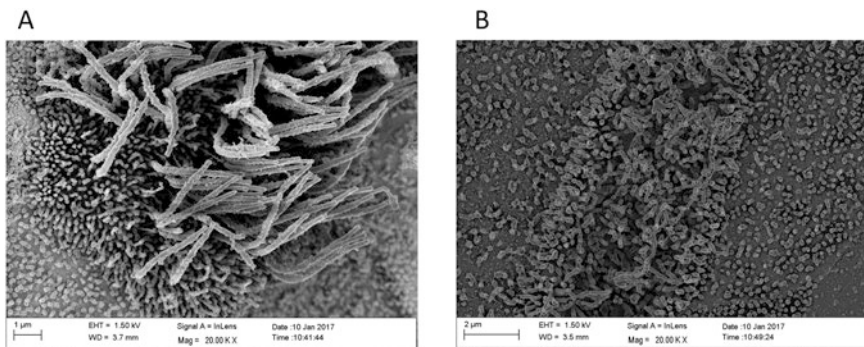

Fig. 1 Loss of cilia from glycocalyx of HBoV-infected cells in comparison to mock-infected cells

ple, the rolling circle replication, which in the natural infection would produce head-to-tail concatemers. However, it has to be mentioned that despite these conflicting data, the minimal essential origin of replication was identified in the right-end hairpin sequence [42]. Thereby, unlike other parvoviruses, the HBoV-NS1 protein did not specifically bind to the oriR in vitro, indicating that other viral and/or cellular components or oligomerization of NS1 is required for NS1 binding to the oriR. Of note, NP-1 and other viral nonstructural proteins (NS1–4) co-localized with the viral replication centres [42]. During the viral replication cycle, it appears that the expression of viral capsid proteins is regulated by polyadenylation mechanisms of the viral RNA transcripts [43]. It was shown that in addition to a distal polyadenylation signal named (pA)d, a further distal polyadenylation site named (pA)d2 is present in the right-end hairpin sequence, which does not contain the typical hexanucleotide polyadenylation motif. Moreover, the viral replication is strongly dependent on a newly identified small non-coding RNA named BocaSR within the 3′ non-coding region (nt 5199–5338) [44]. This RNA is transcribed by the RNA polymerase III from an intragenic promotor at amounts similar to the RNAs of the nonstructural genes. BocaSR accumulates in the replication centres within the nucleus and is suspected to directly influence the viral DNA replication.

Furthermore, clinical observations give rise to the hypothesis that the HBoV replication can be triggered or influenced by human herpesviruses such as HHV-6, CMV and herpes simplex virus. In this context it is noteworthy that herpesviruses, especially HSV, are capable of initiating a rolling circle replication mechanism of replication in trans as shown for SV40, which has a circular double-stranded genome [45].

Thereby herpesviruses may either act as a trigger that arrests the host cell at transition from G1- to S-phase of the cell cycle, or they could directly interact with the HBoV DNA supporting the replication by the herpesviral replication enzymes. The latter appears likely, as head-to-tail intermediates are a feature of the rolling circle replication that may be initiated by a couple of viruses including the human herpesviruses type 1 and type 6 [45–52]. These viruses (e.g. the adeno-associated virus, AAV) in turn are able to act as helper viruses for the parvoviral subclass dependoviruses that require those helper viruses for their replication [48–52]. Recently, a clinical case was observed in which the HBoV infection appeared to depend on a co-infection and co-replication of human herpesvirus type 6. In this case the HBoV infection persisted because of an immune disease but was terminated by antiviral therapy with cidofovir which is directed against HHV6 [53]. This was the key observation leading to the assumption that HBoV is either sensitive to cidofovir or that a possible rolling circle HBoV replication is triggered by HHV6, which in turn would explain the high frequency of co-infections observed in case of HBoV [52, 54, 55].

In 2011, two severe cases of respiratory failure in adults associated with HBoV infection and herpesvirus co-infection, with a history of lung fibrosis likely related to the presence of chronic HBoV infection [56], strongly suggest that the head-to-tail structures could have been episomal reservoirs enabling the virus' persistence as postulated by Kapoor and co-workers [35]. It may be speculated as to whether the persistence of HBoV episomes in the lung of the patients is analogous to a HBV

infection, in which episomal cccDNA persists in the infected cell until the cell is targeted by the immune response or subjected to apoptosis and in which this chronic state frequently produces a mild inflammation that is subclinical but could induce fibrosis over time. The persistence of HBoV episomes in the lung could have led to mild chronic inflammation eventually resulting in fibrosis of the lung, which would not be easily compensated as in the liver. In the context of a putative chronic HBoV infection or a persistence of HBoV at a subclinical level, it thus appears possible that HBoV could directly or indirectly, by interactions with the immune system, contribute to chronic lung disease such as idiopathic lung fibrosis.

Another, recently detected novel feature of HBoV is the expression of more non-structural proteins that concluded from our previous knowledge on parvovirus replication studies. Shen et al. have shown that besides NS1 three novel proteins named NS2, NS3 and NS4 are expressed during the viral replication, of which NS2 is believed to have a crucial role during the viral life cycle [57].

Moreover it is important to mention that the HBoV replication cycle is independent of the cell cycle phase. As early as in 2010, it was shown in A549 cells that the expression of HBoV-1 proteins, unlike the parvovirus B19 infection, does not induce cell cycle arrest and apoptosis [22]. In contrast, two recent studies have shown that the DNA damage repair system is involved in HBoV-1 replication [58, 59]. Thereby the hallmarks of the DDR response, the phosphorylation of H2AX and RPA32, are activated accompanied by the activation of all three PI3KKs. In addition, the polymerases Pol-η and Pol-κ, both being part of the DNA repair system, are recruited to the viral replication sites, thus providing additional evidence that parvovirus DNA replication has to occur in cell cycle-arrested cells.

Epidemiology

Like all respiratory pathogens (except SARS and MERS coronavirus) causing respiratory infections, HBoV-1 is distributed worldwide and has been detected in patients from several regions of each continent [60–112]. However, unlike most other viruses that are known to peak seasonally in autumn and winter, HBoV infection peaks do not seem to be restricted to these seasons.

Although the route of transmission was not yet systematically investigated, it is widely accepted that the transmission of HBoV most likely occurs by smear or droplet infections or aerosols and nasal or oral uptake as described for the majority of "common cold viruses." The transmission route passes through airway excretions but could also be via the gastrointestinal route, as HBoV is shedded also by stool (Figs. 2 and 3).

The HBoV seroprevalence is high and reached 95% and more in children up to the age of 5 years [113, 114]. This seroprevalence remains high in most adults [76, 82] but decreases from 96% to 59% in European adults if antibodies against HBoV strains two to four were depleted. Thus in 41% of patients, no long-term immunity could be generated, supporting the assumption that the virus is able to persist and
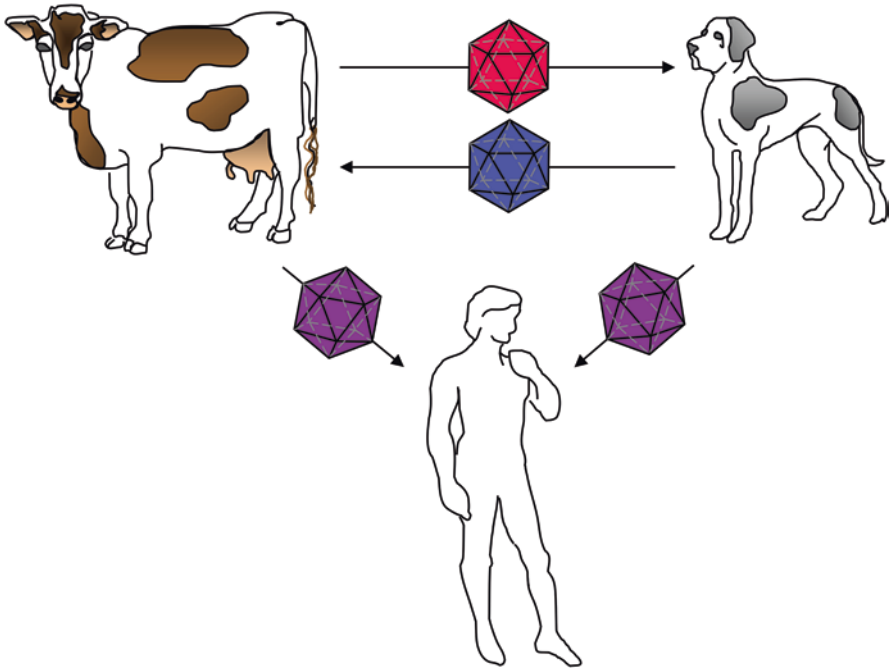
Fig. 2 Overview of the putative zoonotic transmission of animal bocaviruses to the human population. Based on sequence analyses, especially of the terminal sequences, a zoonotic event is likely, as HBoV-1 contains genome structures highly conserved from the *Canine minute virus* and the *Bovine parvovirus*

could also reinfect elderly patients [115]. Surprisingly, HBoV-1 DNA can also be detected in blood and blood products from healthy Chinese blood donors with a lower seropositivity compared to the above-mentioned cohorts [116].

In recent months a few studies have been published that demonstrated that human bocaviruses are also stable in the environment. As an example, Iaconelli et al. have shown the frequent detection of HBoV in urban sewages, an observation confirmed by a study from Egypt [117].

Clinical Features

HBoV-1 respiratory infection is clinically indistinguishable from other respiratory infections and can only be diagnosed using molecular assays. The spectrum of HBoV infections ranges from asymptomatic [67, 118, 119] to mild upper respiratory infections [67, 120–122] up to serious and life-threatening lower respiratory tract infections [70, 109, 123–133] in all age groups [70, 71, 109, 119, 123–136]. The immune response against HBoV starts with an IgM response and is followed by
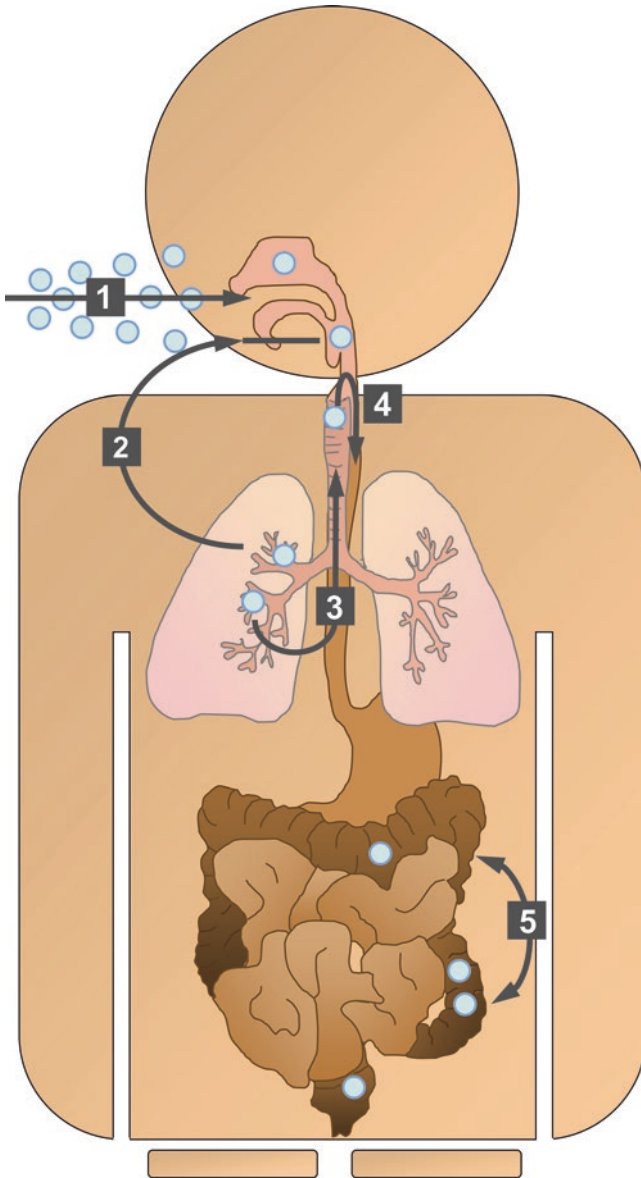
Fig. 3 Schematic overview of the HBoV life cycle. (1) Entry through the nasopharyngeal space, (2) infection of the lung, (3) and (4) swallowing of the expectorated infectious secretion, and (5) infection of the gastrointestinal tract. Additionally, the virus spreads via the bloodstream and causes classical viremia (not indicated)

the formation of IgG [113, 114], but no lifelong immunity is generated in at least 40% of patients due to the original antigenic sin [i.e. Hoskins effect] [76, 82, 137].

The general HBoV-1 infections appear to start in the upper airways; in 2014 Proenca-Modena and co-workers demonstrated that hypertrophic adenoid is a major infection site with 25.3% of tested tissues positive for viral RNA and DNA, followed by nasopharyngeal secretions (10.5%), tonsils (7.2%) and peripheral blood (1.5%) [138]. Thereby it is worth to note that tonsils are suspected to be a major site of persistence as hypothesized by Clement and colleagues [139]. Subsequently the virus most likely initiates a downstream infection caused by swallowing of virus-containing secretions, which then enter the gastrointestinal tract where active viral replication occurs and is accompanied by a true viremia. Persistence of HBoV in the respiratory tract has been confirmed by a novel pyrosequencing approach by Wagner and co-workers, who observed primary infections and recurrence in a large cohort of paediatric patients [140].

HBoV-1 is able to infect the central nervous system and induces clinical symptoms of encephalitis or necrotizing encephalopathies [96, 98, 141]. HBoV-1 has been identified as a putative cause of idiopathic lung fibrosis [56] supported by the fact that a set of profibrotic cytokines were upregulated during HBoV infection in adults and their HBoV-dependent upregulation was confirmed in cell culture [142], whereas HBoV does not induce a clear Th1 or Th2 response [143]. The HBoV-dependent regulated cytokines furthermore include a subset of cytokines which are known to be involved in several cancer-associated pathways, supporting the hypothesis that HBoV may be associated with chronic diseases or even cancerogenesis [144–146]. Although this hypothesis requires further prospective studies, HBoV DNA was detected in lung and colorectal tumours. Detection of HBoV DNA, eventually associated with persistence, has been described in addition to detection in normal lung tissue [119] and in lung and colorectal tumours [146, 147]. HBoV-1 has been detected in other tissues such as tonsils [35, 139, 148, 149] and myocardium and may affect additional tissues that have not yet been tested for HBoV positivity.

Lung fibrosis, especially idiopathic lung fibrosis (IPF), is characterized by a Th2-type dominated immune response in the affected tissue (reviewed by [150–152]). The Th2 response in the lung is accompanied by increased expression levels of IL-4, IL-5, IL-10 and IL-13 and is followed by increased levels of CCL17 (TARC), CCL5 (RANTES) and others. Moreover, fibrosis is related to expression of TNF and IL-8; it is worth noting that the neutralization of TARC leads to a reduction of fibrosis in the animal model [151, 153]. In addition, an elevation of the TARC/IP-10 ratio is also characteristic for fibrosis and was previously discussed as a marker for IPF [154].

Moreover, a unique case has been described in which the infection/reactivation of HBoV occurred between two episodes of BAL sampling; the fibrosis-associated cytokines were expressed in association with the HBoV infection but not before, supporting the previously mentioned data. This data leads to the conclusion that HBoV colonization/chronic infection may be at least one trigger that could stimulate airway remodelling. However, it could be argued that not only the resident airway epithelial cells are involved in the in vivo immune response, but also additional patient-specific factors will contribute to altered profibrotic cytokine profiles. In

order to address this problem, experiments in an air-liquid interface culture of human airway epithelial cells were performed. These experiments confirmed that profibrotic cytokines were expressed by the infected cell cultures but were minimally or not at all expressed in mock-infected cells; the identified cytokines belong to the initial immune response following HBoV infection [123].

According to the literature, the two HBoV proteins VP2 and NP1 seem to influence the regulation of the interferon-beta pathway, but the data appear to be controversial as VP2 upregulates the pathway [155], whilst NP1 inhibits the IFN-beta production when overexpressed [156]. In addition, in an experimental setting with overexpression conditions, it has been shown that HBoV NS1 and Ns1–70 proteins inhibit the TNF-α-mediated activation of NF-κB by targeting p65 [157].

Moreover, based on clinical observations of a longitudinal study, Martin and colleagues concluded that HBoV infections could possibly be divided into two distinct clinical subgroups, one with a short viremic phase and short viral shedding, most likely being the primary infection, and a second group with long-term shedding; the second group is likely to be co-infections with other pathogens or a reactivation of a persistent HBoV infection [158].

Coinfections and Persistence

Simultaneously with the discovery of HBoV in 2005, multiplexing PCR methods started to become an accepted diagnostic tool, and consequently detection of multiple infections, especially in respiratory tract diseases, has become a common phenomenon [67, 159–163]. Nowadays, multiple infections with up to six pathogens being simultaneously present in a single respiratory sample are frequent [67, 159–164] and may mislead some researcher to claim that the human bocavirus, also occurring in asymptomatic patients, is a harmless bystander rather than a pathogen [165, 166]. This hypothesis seems to be supported by the fact that a formal fulfilment of Koch's modified postulates was not yet possible for HBoV [167], as no animal model exists to date and volunteer transmission trials cannot be recommend based on our current knowledge of this virus [145].

In contrast, although there is a cohort of asymptomatic carriers [67, 119, 159, 166, 168, 169], several studies have shown that HBoV induces clinical respiratory symptoms [64, 91, 126, 127, 159, 170–176]. The asymptomatic viral shedding is meanwhile believed to originate from long-term shedding after an acute infection or from persistent viruses [34, 35, 95, 137, 177–180]. This has most recently been confirmed by a long-term prospective cohort study [67, 181]. Thereby it was shown that the rate of asymptomatic HBoV infections is similar to the rate of rhinovirus infections and no one would doubt that rhinoviruses are true pathogens [67]. Finally, HBoV is known to induce serious cytopathic effects in infected cell cultures, which is a typical feature of a pathogen [21, 40, 57, 177].

Diagnostics

In addition to several published home-brew PCRs and real-time PCRs (reviewed by [9]), numerous commercial assays, such as the Luminex RVP assay [119, 182], the Idaho FilmArray [164, 182] or the RespiFinder assay [119], have been developed and released to the market enabling the detection of HBoV from clinical samples. However, multiplexing solely allows detection of the viral DNA in a respiratory sample without providing the essential information as to whether an active replicative infection underlies the currently clinical episode requiring laboratory testing [67]. As HBoV can be shedded for longer than 3 months after the acute symptomatic phase [67], a proper diagnostics of human bocavirus requires the proof of active replication, which can be done either by detection of a viremia in the peripheral blood [91, 107, 115, 137, 183–187] or by detection of spliced viral RNA transcripts that were shown to be present exclusively during the active phase of the replication [188]. Recently, a novel rapid antigen test was developed which could be a major advance in HBoV diagnostics [189]. Further progress in this direction can be expected from novel approaches to test for human antibodies and bocviral antigens from all four subtypes based on yeast-derived virus-like particles [190].

Advanced Molecular Techniques in HBoV Research and Diagnostics

The discovery of HBoV has become possible due to the usage of a novel virus discovery strategy used by Allander and colleagues in 2005 [1]. These authors used a strategy of a virus screening library combined with a 96-well format high-throughput sequencing approach based on rolling circle amplification and sequencing. This technique was used subsequently also by other labs and has become a simple but work-intensive strategy to identify novel viruses and virus variants.

In addition, with the isolation and propagation of HBoV in three-dimensional air-liquid interface cell cultures, another novel method has set standards for the research on respiratory viruses in general and human bocavirus in particular [21]. This technique has meanwhile been refined, and several models are available [8, 40, 59, 177].

Summary and Perspective

There is an increasing body of evidence showing that the human bocavirus is a serious pathogen that is associated with acute respiratory infections, sometimes with life-threatening complications. In addition, there is evidence that the human bocavirus could contribute to long-term disease of the airways resulting in lung carcinoma

or lung fibrosis. It is therefore crucial to analyse the long-term effects of HBoV infections in order to identify the mechanisms of HBoV persistence as well as for determining host factors for asymptomatic infections and to test the hypothesis that HBoV could trigger the development of lung cancer and fibrosis. Novel studies have identified the antigenic epitopes on the viral surface and may enable the development of potent vaccines or antibody-based therapies [191].

In any cases, the proper diagnostics of HBoV require additional attention as does the need for HBoV to be evaluated in terms of its interaction with other respiratory viruses that may simultaneously be detected during clinical episodes.

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