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CHAPTER

RESPIRATORY INFECTIONS OF THE HUMAN BOCAVIRUS

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1 INTRODUCTION

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, genus *Bocaparvovirus*, was discovered originally in 2005 by Tobias Allander[1] and his team 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 in the first decade of our millennium that was based on novel virus discovery systems developed in order to reduce the considerable number of cases in which a clinical diagnosis of a respiratory infection could not be confirmed by 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. Unfortunately, HBoV research still relies on clinical studies and case reports with accompanying cell culture studies as the major source of information on HBoV biology, as so far no animal model has been identified.

2 HBoV BIOLOGY

The human bocavirus (HBoV) was initially discovered in clinical samples from the respiratory tract of children suffering from respiratory infections of unknown etiology.[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 yet been completely fulfilled.[3]

Nevertheless, HBoV is the second parvovirus that is capable of infecting humans with the potential to cause clinical disease. Until HBoV was discovered, parvovirus B19 was the sole human parvovirus, which can hardly be cultured in in vitro cell cultures, likely because it strongly depends on the optimal cell cycle phase.[4–13] 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 discovery of HBoV led to a couple of molecular findings that are of major general interest for the human parvovirus biology and clinics. Within a primary cell culture that productively replicated the human bocavirus, it was possible

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to identify the HBoV transcriptome, including the splicing variant of viral RNA.[14] This cell culture displayed for the first time a tool for the investigation of a human parvovirus in its natural infectious surrounding enabling follow up studies on 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, requires a highly specialized laboratory, and is an error-prone cell culture, thus the availability of this technology is rather limited to a couple of laboratories worldwide, which in turn is a stumbling block for further research. In search for a broadly convertible replication system the group of Jianming Qiu from Kansas made a significant step forward: by establishing a plasmid based replicon-like system the group identified additional RNA species that are transcribed during the HBoV replication cycle.[15] 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.[16]

Although the hairpin-like structures of HBoV had yet not been described at that time, it was postulated that also the HBoV genome 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, while packaging of viral genomes is dependent on additional factors.[17–23] For almost four decades, it was 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.[24] Thereby, NASBA analyses revealed that all HBoV strains package negative strand genome while only a minority also package the plus strand; this observation is compatible with another replication mechanism, known as rolling circle replication. A couple of systematic PCR-based analyses were performed to test the hypothesis if rolling circle replication may occur in HBoV infection and to decipher the unknown terminal hairpins.[25]

This approach identified DNA sequences that contained head-to-tail genome fragments linked by a newly identified linker stretch that had 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,[25,26] and it is likely that the virus was originally transmitted as a zoonosis (Fig. 5.1). Despite identifying the terminal sequences in clinical samples and also in cell cultures, not only a lack of self-priming activity of HBoV-genomes but also the lack of intermediates typical for rolling hairpin replication were observed. Instead, the samples contained head-to-tail structures. Several groups published similar observations, all tackling the dogma of parvovirus replication.[27–30] It is therefore 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 of circular closed genome dimers of head-to-head and tail-to-tail orientation.[31]

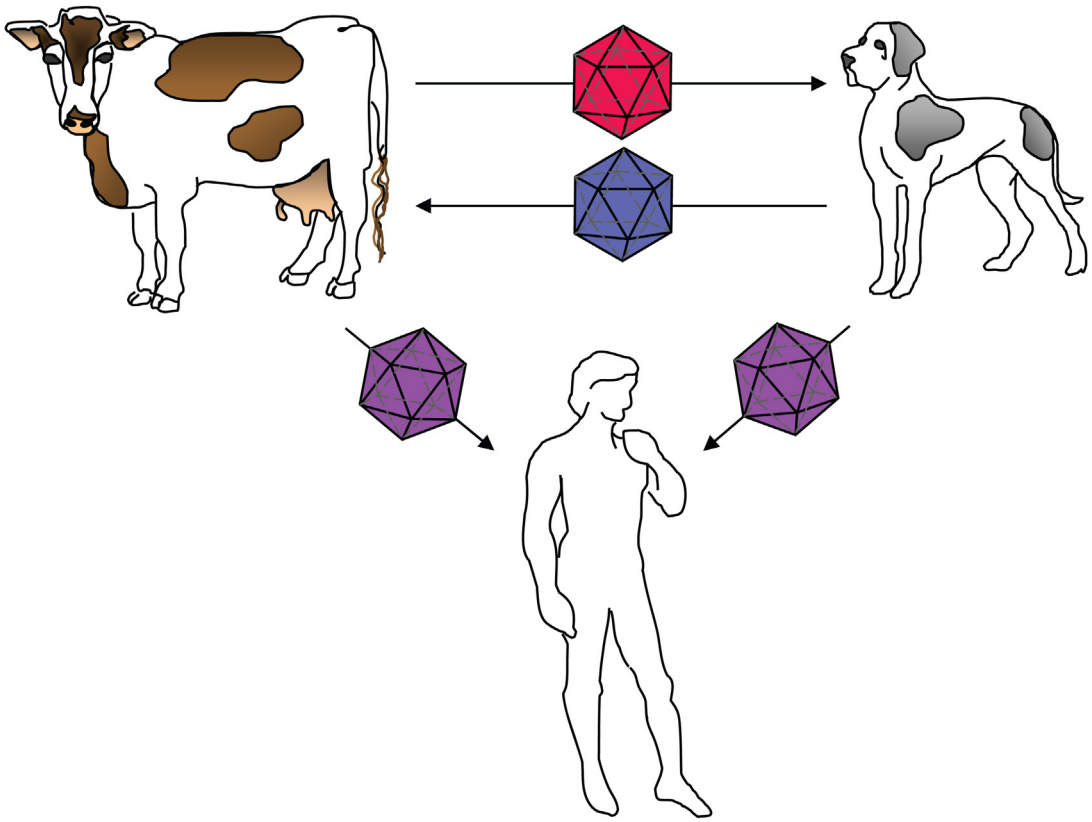

FIGURE 5.1 Overview of the Putative Zoonotic Transmission of Animal Bocaviruses to the Human Population

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.

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 nonhuman parvoviruses by possibly initiating a rolling circle mechanism, 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 were predicted in silico.[26] Beyond that, the Kansas group developed a true full-length vector clone of HBoV which can be transfected to HEK-293 cells and produces a "recombinant wild type" human bocavirus that in turn is infectious for differentiated CuFi-8 cells.[32] CuFi-8 cells are derived from a patient with cystic fibrosis and can be grown as monolayer cultures that by change of the culturing media can be differentiated into a polarized respiratory epithelial structure that in turn supports HBoV replication.[32] This novel cell culture gives raise to the hypothesis that HBoV is a true serious pathogen because it induced a remarkable cytopathic effect in the polarized CuFi-8 cell line, which in turn is compatible with the assumption that clinical symptoms of an HBoV infection are caused by tissue

damages due to the viral replication. Thereby, this infection model harbors 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, then infectious progeny virions are produced, although based on the rolling hairpin model this process must be impossible because the free (!) hairpin sequences are believed to be essential for the replication. In contrast, in the plasmid they are flanked by the vector's backbone sequence, replication is possible although 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, for example the rolling circle replication, which in the natural infection would produce head-to-tail concatemers.

Furthermore, clinical observations give raise 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.[33]

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 because 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.[33–40] These viruses (eg, AAV) in turn are able to act as helper viruses for the parvoviral subclass dependoviruses, that require those helper viruses for their replication.[36–40] Recently, a clinical case was observed in which the HBoV infection appeared to depend on coinfection and coreplication 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.[41] 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 coinfections observed in case of HBoV.[40,42,43]

In 2011, two severe cases of respiratory failure in adults associated with HBoV infection, herpesvirus coinfection, and a history of lung fibrosis likely dedicated to the presence of chronic HBoV infection[44] indicate that the head-to-tail structures could have been episomal reservoirs enabling the virus' persistence as postulated by Kapoor and coworkers.[27] It may be speculated whether the persistence of HBoV episomes in the lung of the patients in analogy 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 goes ahead with a mild inflammation that is subclinical but finally could induce fibrosis, could have led to mild chronic inflammation eventually resulting in fibrosis of the lung, which could 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 nonstructural proteins than 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.[45]

3 EPIDEMIOLOGY

Like all respiratory pathogens (except SARS- and MERS-coronavirus) that cause respiratory infections, HBoV-1 is distributed worldwide and has been detected in patients from several regions of each continent.[46–98] 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 has not yet been 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 happen via the gastrointestinal route, as HBoV is also shedded by stool (Fig. 5.2).

The HBoV seroprevalence is high and reached 95%, and more in children up to the age of 5 years.[99,100] This seroprevalence remains high in most adults,[62,68] but decreases from 96% to 59% in European adults if antibodies against HBoV strains 2–4 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 could also reinfect elderly patients.[101] 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 afore mentioned cohorts.[102]

4 CLINICAL FEATURES

The HBoV-1 infection is clinically indistinguishable from other respiratory infections and can solely be proven by molecular assays. The spectrum of HBoV infections ranges from asymptomatic[53,103,104] to mild upper respiratory infections[53,105–107] up to serious and life threatening lower respiratory tract infections[56,95,108–118] in all age groups.[56,57,95,104,108–121] The immune response against HBoV starts with an IgM response followed by the formation of IgG,[99,100] but no life long immunity is generated in at least 40% of patients due to the original antigenic sin.[62,68,122]

HBoV-1 is able to infect the central nervous system[82,84] and it has been identified as a putative cause of idiopathic lung fibrosis[44] 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.[123,124] Whereas, HBoV does not induce a clear Th1 or Th2 response.[125] Furthermore, the HBoV dependent regulated cytokines 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 long term diseases or even cancerogenesis.[126–128] Although this hypothesis requires further prospective studies, HBoV DNA was detected in lung- and colorectal tumors. Detection of HBoV DNA, eventually combined with persistence, was described besides detection in normal lung tissue,[104] and in lung- and colorectal tumors,[128] in other tissues such as, tonsils,[27,129–131] and myocardium, and may affect further tissues that have not yet been tested for HBoV-positivity.

Lung fibrosis, especially the idiopathic lung fibrosis (IPF) is characterized by a Th2-type dominated immune response in the affected tissue (as reviewed by:[132–134]). 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—besides others—of CCL17 (TARC) and CCL5 (RANTES). Moreover, fibrosis is related to expression of TNF and IL-8, and it is worth mentioning that the neutralization of TARC leads to a

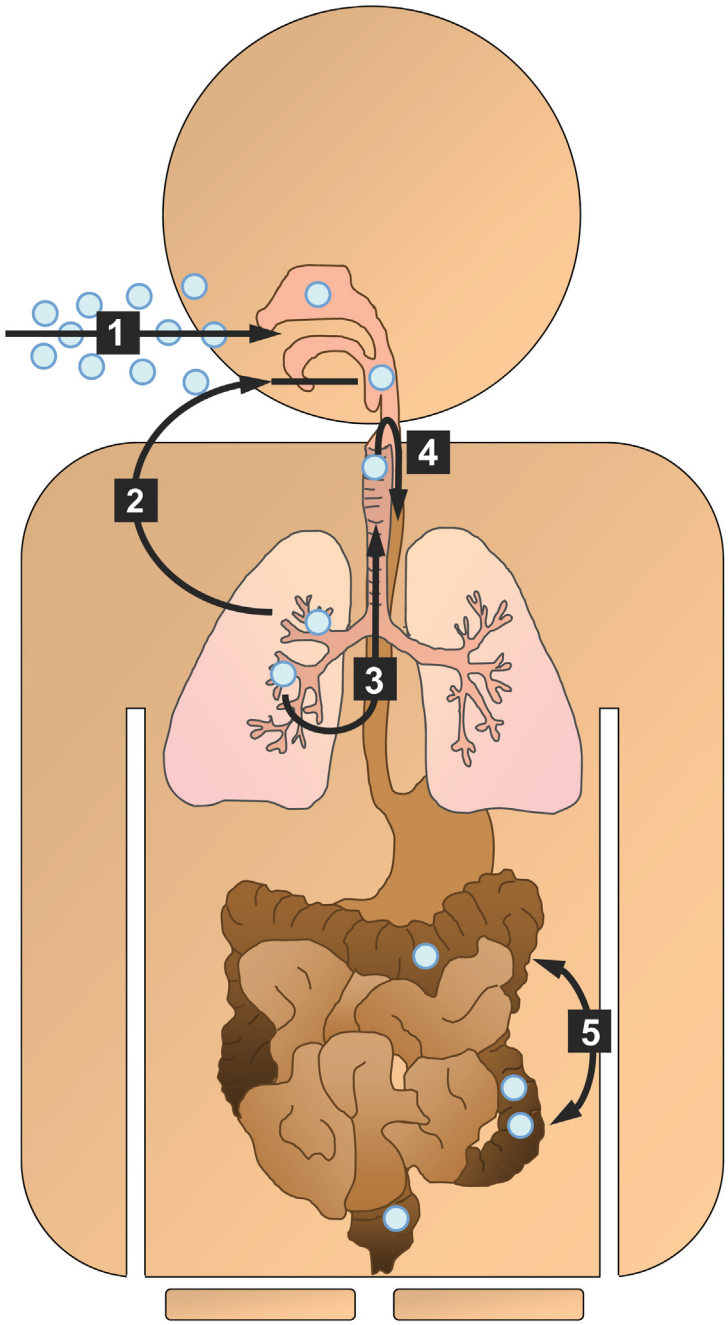

FIGURE 5.2 Schematic Overview of the HBoV Life Cycle

1, entry through the nasopharyngeal space; 2, infection of the lung; 3 and 4, swallowing of the expectorated infectious secretion; 5, infection of the gastrointestinal tract. Additionally, the virus spreads via the bloodstream and causes classical viraemia (not indicated).

reduction of fibrosis in the animal model.[133,135] In addition, an elevation of the TARC/IP-10 ratio is also characteristic for fibrosis and was previously discussed as a marker for IPF.[136]

Moreover, a so far unique follow up case in which the infection/reactivation of HBoV occurred between two episodes of BAL sampling, and in which the fibrosis associated cytokines were expressed in association with the HBoV infection but not before, supports the previously obtained data. This leads to the conclusion, that HBoV colonisation/chronic infection may be at least one trigger that could stimulate airway remodelling. However, it could be argued that in vivo not only the resident airway epithelial cells are involved in the immune response but also additional patient specific factors will contribute to altered profibrotic cytokine profiles. To address this problem, experiments in an air-liquid-interface culture of human airway epithelial cells were performed. These experiments confirmed that the profibrotic cytokines were expressed by the infected cell cultures but were hardly or not at all expressed in mock-infected cells and they reveal that the identified cytokines belong to the initial immune response after 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 controversially as VP2 upregulates the pathway[137] while NP1 inhibits the IFN-beta production when overexpressed.[138]

5 COINFECTIONS AND PERSISTENCE

Simultaneously with the discovery of HBoV in 2005, multiplexing methods started to become an accepted diagnostic tool and as a consequence detection of multiple infections, especially in respiratory tract diseases, has become a common phenomenon.[53,139–143] Nowadays, multiple infections with up to six pathogens being simultaneously present in a single respiratory sample are frequent[53,139–144] and have misled some researchers to the statement that the human bocavirus, also occurring in asymptomatic patients, is a harmless bystander rather than a pathogen.[145,146] This hypothesis seems to be supported by the fact that for HBoV a formal fulfilment of Koch's modified postulates was not yet possible[147] because no animal model exists so far and also because volunteer transmission trials cannot be recommended based on our current knowledge.[127]

On the contrary, although there is a cohort of asymptomatic carriers,[53,104,139,146,148,149] several studies have shown that HBoV induces clinical symptoms.[50,77,111,112,139,150–156] The asymptomatic viral shedding is meanwhile believed to originate from a long term shedding after an acute infection or from persisting viruses,[26,27,81,122,157–160] most recently confirmed by a long term prospective cohort study.[53,161] 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.[53]

Moreover, HBoV induces a serious cytopathic effect in infected cell cultures, which is a typical feature of a pathogen.[14,32,45,157]

6 DIAGNOSTICS

Besides several published homebrew PCRs and real-time PCRs (as reviewed by[3]), numerous commercial assays, such as the Luminex RVP–Assay,[104,162] the Idaho FilmArray,[144,162] or the RespiFinder assay[104] have been developed and released to the market enabling the detection of HBoV from clinical samples. However, multiplexing solely allows us to detect the viral DNA in a respiratory sample

without providing the essential information if an active replicative infection underlies the currently clinical episode requiring laboratory testing.[53] As HBoV can be shedded for longer than 3 months after the acute symptomatic phase,[53] 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,[77,93,101,122,163–167] or by detection of spliced viral RNA transcripts that were shown to be present exclusively during the active phase of the replication.[168]

7 SUMMARY AND PERSPECTIVE

There is an increasing body of evidence showing that the human bocavirus is a serious pathogen that on the one hand is associated with acute respiratory infections, sometimes with life threatening complications, and on the other hand also could contribute to long term diseases of the airways resulting in lung carcinoma or lung fibrosis. Therefore, it remains crucial to analyze the long-term effects of HBoV infections to identify the mechanisms of HBoV persistence and to determine the host factors for asymptomatic infections, as well as to test the hypothesis that HBoV could trigger the development of lung cancer and fibrosis. In any cases, the proper diagnostics of HBoV require attention and need to be evaluated regarding its interaction with other respiratory viruses that may simultaneously be detected in clinical episodes.

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