Phylogenetic and Reverse Genetics Studies on Zoonotic Hepatitis E Virus

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What I cannot build, I do not understand.

(Richard Feynman)

Contents

1. Introduction

1.1 Hepatitis E virus – an overview

Hepatitis E virus (HEV) was first described in 1983 by Mikhail Surenovich Balayan [1], who, in a manner similar to Barry Marshall's daring "Attempt to fulfil Koch's postulates for pyloric Campylobacter" [2], ingested pooled stool extracts from soldiers stationed in Afghanistan who were suffering from symptoms of acute hepatitis of then undetermined, but suspected viral origin [3]. 36 days after inoculation, Balayan was hospitalized for the same symptoms [4]. In his feces, he identified viral particles by immune electron microscopy using sera from patients who had experienced non-B hepatitis but were seronegative for hepatitis A virus (HAV). He was further able to infect cynomolgus macaques (Macaca fascicularis) by intravenous inoculation with the virus containing stool extract upon which the monkeys developed hepatitis, excreted virus particles, and showed a specific serological response [1], thereby identifying the causative agent of, as the disease was called at the time, enterically transmitted non-A, non-B hepatitis (ET-NANBH) [4].

Before this, the existence of such a pathogen had been suspected due to several outbreaks of icteric jaundice with no evident connection to hepatitis B virus (HBV), HAV, or blood transfusions [5,6] (another source of viral hepatitis, caused mainly by hepatitis C virus (HCV) [7,8], which had been discovered a few years earlier). Balayan's work resulted not only in the discovery of the pathogen responsible for ET-NANBH, but also the serological tools required to detect it and thus made it possible to determine HEV as the causative agent of various outbreaks [4]. At present, it is known that HEV has been plaguing humanity long before its discovery. By retrospective serology analysis, HEV is known to have caused epidemics in India as far back as the outbreak of infectious hepatitis in Delhi 1955-1956 [9,10]. Based on the symptoms described in earlier cases, some authors consider HEV to be the most likely cause for icterus outbreaks during the 19th century [4], or even for similar episodes among medieval crusaders [11].

Today, HEV still causes epidemic outbreaks, particularly in developing countries with poor sanitation, where it is transmitted by the fecal-oral route due to contaminated water. These are estimated to result in 20 million infections and 70,000 deaths a year [12]. In contrast, epidemic outbreaks are not known to occur in industrialized countries, and so it was long thought that HEV is not endemic in industrialized countries and that hepatitis E only occurs as travel-associated disease. However, the number of diagnosed cases of autochthonous HEV in industrialized countries has been on the rise since the early 2000s and so far HEV "has been found in every single developed country in which it has been sought" [13]. In

Germany, the number of diagnosed cases increased dramatically within the last 10-15 years, although this increase is likely due to increased testing, rather than an actual increase in incidence [14]. It is estimated that around 400,000 seroconversions per year occur in Germany alone [15]. In industrialized countries, the virus typically follows a foodborne route of transmission, associated particularly with the consumption of undercooked or contaminated food, chiefly pork but also shellfish or game [16], resulting in sporadic cases of hepatitis E. Moreover, a trend for chronic hepatitis E has been detected, particularly in immunocompromised patients. By definition, hepatitis E is chronic if HEV replication persists for more than three months after infection [17]. Particularly at risk are patients with immunosuppressive therapy after organ transplantation, human immunodeficiency virus (HIV) infection, and hepatological malignancies [18]. Therapy options are limited [19]. The only available vaccine has yet to be licensed outside of China [20]. No drug is licensed for treatment of HEV [21]. Off-label use of antivirals such as ribavirin [22–25] or sofosbuvir [26,27] is inconsistent at best, with varying degrees of success.

One major hurdle for the development of treatment and prevention strategies is the lack of an efficient model system. While cynomolgus macaques have been established as an infection model in the very first description of HEV [1], infection experiments using primates are logistically challenging, as the required facilities are not ubiquitously available, and often ethically frowned upon [28]. Pigs are used as a model species for HEV infection, but differ in pathogenesis compared to humans [29]. Mice and rats are not susceptible to HEV, and therefore do not represent a reliable model [30]. In recent years, the rabbit has shown promise as an animal model for human HEV [31], but is not yet firmly established. However, the perhaps greatest difficulty lies in HEV's inability to grow in cell culture. With the exception of a few strains adapted to efficient growth in cell culture, isolation of HEV *in vitro* remains challenging [32]. Although some protocols have shown promise in recent years [33], the process is, at best, unreliable, challenging, and time-consuming. For this reason, many aspects of the HEV replication cycle and infection mechanisms remain elusive, even today, more than 40 years after its discovery by a self-sacrificing soviet scientist.

1.2 Epidemiology, Transmission, and Pathogenesis

1.2.1 Phylogeny of *Paslahepevirus balayani*

Hepatitis E Virus (species *Paslahepevirus balayani*; formerly: Orthohepevirus A [34]) is a member of the genus Paslahepevirus (formerly: Orthohepevirus) within the family Hepeviridae, subfamily Orthohepevirinae, a group of single-stranded, unsegmented, positive-sense RNA viruses infecting vertebrate hosts [35]. The species *Paslahepevirus balayani* is, subdivided into eight genotypes (referred to as HEV-1 to HEV-8) and 36 subtypes [36] (Figure 1).

The genotypes differ in host spectrum but share the same genetic structure. HEV-1 and HEV-2 infect humans and, at least in an experimental setting, non-human primates. In contrast, HEV-3 and HEV-4 are usually transmitted to humans zoonotically from swine (family Suidae) as the reservoir host and can also infect several other mammal species. HEV-5 and HEV-6 were detected in wild boars in Japan [37,38]. No human cases were identified so far, but their zoonotic potential is being discussed [36,39]. HEV-7 and HEV-8 infect dromedary and bactrian camels, respectively [40,41]. In the case of HEV-7, a human infection has been reported [42]. However, zoonotic HEV infections are typically caused by genotypes HEV-3 and HEV-4. In Europe, the majority of zoonotic cases are due to HEV-3, while HEV-4 is highly prevalent in China [43].

Transmission routes and epidemiology, as well as pathological manifestations of hepatitis E are diverse and depend on the geographic and cultural framework, in addition to viral genetics [44]. While HEV usually manifests as a mild or subclinical disease with low fatality rates (0.5% to 4%), complications, such as fulminant hepatitis, chronic manifestations, or increased mortality can occur, depending on various factors [45,46]. Furthermore, reports of extrahepatic manifestations of HEV infections [47] indicate that HEV replication is not restricted to liver tissue.

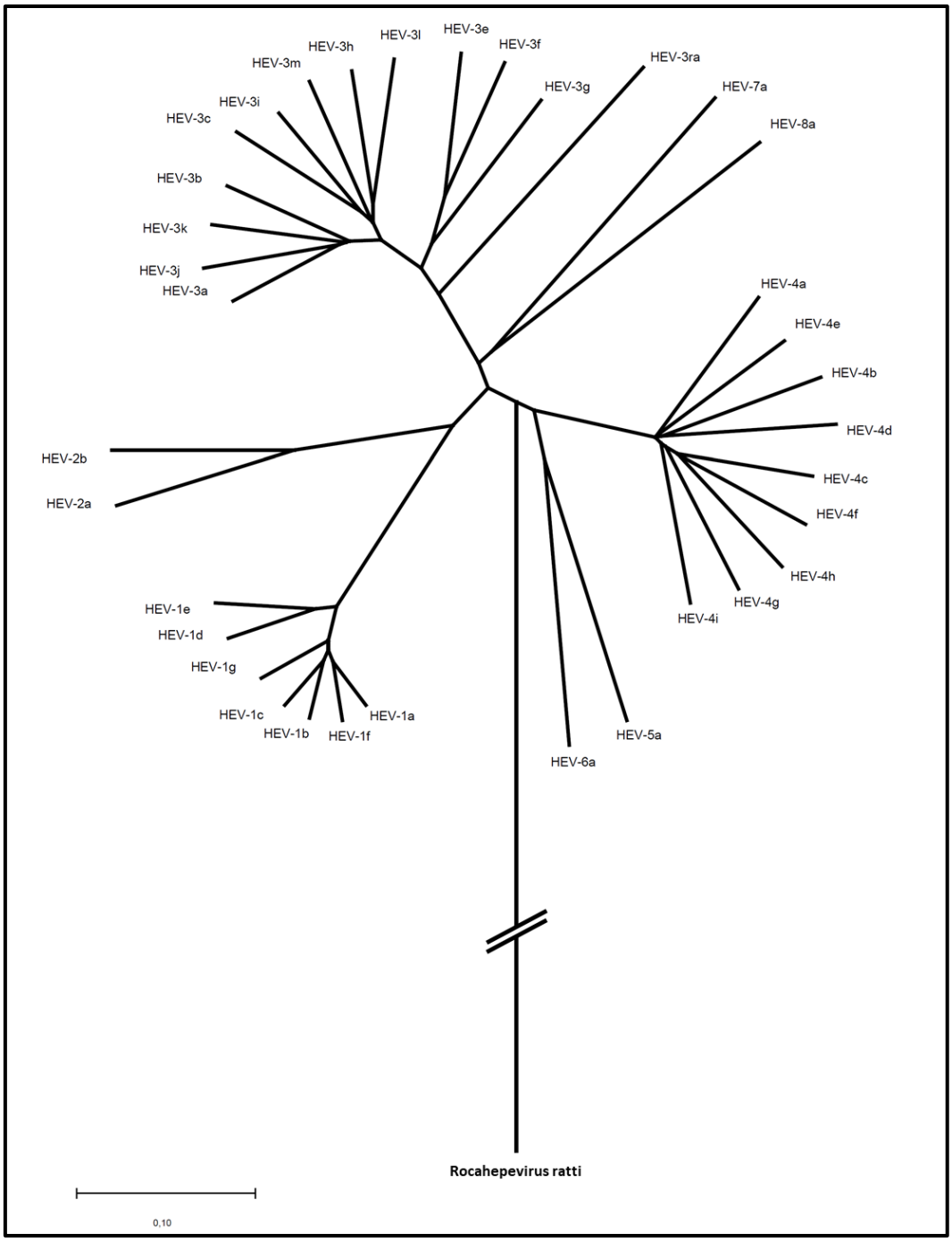

Figure 1: Neighbour-Joining tree of Paslahepevirus balayani indicating the major genotypes and subtypes based on the reference sequences proposed by Smith et al., [36]. The tree was constructed in MEGA 11 based on a multiple alignment of the full-length genomic sequences. HEV-3d was excluded because the reference sequences are only very short partial sequences. A rat hepatitis E virus sequence (species Rocahepevirus ratti; GenBank accession GU345042) was added as outgroup.

1.2.2 Distribution and pathogenesis of the major genotypes

HEV-1 causes recurring outbreaks of epidemic hepatitis E in developing countries of southern Asia and Africa. According to a study by Carratalà & Joost (2019), the regions at highest risk for spread of HEV epidemics are “Ganges Valley and Pakistan, the west coast of Saudi Arabia and subequatorial African countries” [48]. HEV-1 is transmitted by the fecal-oral route due to contaminated drinking water, especially in regions where sanitation and access to clean water are limited [49]. To a lesser degree, blood-borne transmission can occur [50]. In India, an emerging economic power where, despite great progress in recent years, many people live in poor hygienic conditions, epidemic outbreaks of hepatitis E are a recurring issue [51]. Several hepatitis outbreaks in the last 70 years are associated with HEV [10]. Invariably, these appear to be caused by HEV-1 in connection with sewage contamination of water [52]. Although HEV-4 is circulating within swine populations in India [53,54], zoonotic transmission to humans is negligible [52]. The largest recorded HEV-1 epidemic occurred in China in 1986-1988, with almost 120,000 cases [55]. In recent years, HEV-1 incidence has decreased in China [56], likely due to the unprecedented rate of development and modernization the country has undergone over the past few decades. Instead, reports of sporadic infections with HEV-3 and HEV-4 are on the rise [57]. A particular characteristic associated with many reports of HEV-1 is high mortality of pregnant women as well as fetal and neonatal complications [58]. In contrast, reports of chronic HEV-1 infections are very rare [59].

HEV-2 is, in many ways, similar to HEV-1, although fewer disease cases are known. Most reports of outbreaks stem from African countries, such as Namibia [60], Nigeria [61,62], Chad [63], or the Central African Republic [64]. However, the earliest report of HEV-2 is from an outbreak in Mexico in 1986 [65,66]. Transmission and pathogenic manifestations are similar to HEV-1, but fewer reports of deaths in pregnancy exist [58,67].

HEV-3 is the main cause of autochthonously acquired hepatitis E in industrialized countries [13]. Unlike HEV-1 and HEV-2, the main route of transmission to humans is not contaminated water but instead consumption of meat from infected animals, mainly pigs and wild boar, although deer, rabbits, and even shellfish are known sources of infection [68]. The virus can be inactivated by heating to >70°C for 20 minutes, but remains active in raw or undercooked food products [69]. Bloodborne transmission is another risk factor [70]. Waterborne transmission is generally not considered a major factor for human HEV infections in industrialized countries. However, some data from France suggest that drinking bottled water decreases the risk of infection [71], which implies that waterborne transmission

is also relevant. Generally, environmental transmission is of minor relevance compared to direct contact with or consumption of infected animals. An exception are infections with rabbit HEV, a subclade of HEV-3 (HEV-3ra), in which context environmental transmission is discussed as a potential source of infection [72]. Additionally, the transmission cycle among the reservoir host must be considered. Pigs and wild boars acquire HEV by direct contact with another infected animal, or due to environmental contamination [73]. In swine, the infection causes mild or subclinical symptoms but can damage the liver [74,75]. In some cases, acute hepatitis and jaundice can be observed [76] but other complications are not known to occur [29]. In humans, HEV-3 leads to a mild disease, typically subclinical and self-limiting with rare cases in which clinical symptoms of hepatitis manifest [56]. In some cases, chronic hepatitis E can develop [18]. This happens mainly in immunocompromised patients, particularly recipients of organ transplants [77], or with other forms of immunosuppressive therapy, e.g. for hematological malignancies [78], or in HIV patients [79]. However, reports of chronic hepatitis E in immunocompetent individuals do exist [80]. Chronic infection with HEV can lead to significant liver damage, acute-on-chronic liver failure, and death [81].

HEV-4 is similar in epidemiology and pathogenesis to HEV-3 [43]. It is also transmitted zoonotically, with pigs and wild boars as reservoir hosts. Other species, such as deer, are also known sources of infection. Most detected cases of HEV-4 come from eastern Asia, particularly China and Japan [82]. However, this genotype has also been detected in several countries in Europe, mainly France [83]. As with HEV-3, chronic infections and extrahepatic manifestations are known, although comparatively fewer cases are published in the context of HEV-4 [43].

Little is known about the remaining genotypes. HEV-5 and HEV-6 have only been detected in wild boars in Japan and are closely related [36]. Evidence of replication in human cell culture has been demonstrated [39], which hints at some degree of zoonotic potential. HEV-7 was first detected in dromedary camels [40] and its zoonotic character was recently proven by a case report of a chronic infection in a liver transplant recipient from the United Arab Emirates (UAE) [42]. HEV-8 is associated with bactrian camels [41], and although no human cases are known, trans-species transmission to cynomolgus macaques [84] and rabbits [85] was demonstrated. Therefore, it is suspected that HEV-8 also has the potential to infect human hosts.

1.2.3 Hepatitis E in Germany

In Germany, hepatitis E was for many years thought of as a purely travel-associated disease typically acquired during trips to endemic areas [86]. Due to increased awareness of the disease, the number of diagnosed cases has seen a sharp increase in recent years,

mostly in patients with no travel history. As a consequence, autochthonous HEV infection in Germany became acutely relevant. It is now assumed that the increased number of detected HEV infections is, in fact, due to increased testing rather than due to rising numbers of infections, as the seroprevalence appears to be unchanging [14]. Serological analysis shows that almost a sixth of Germans had HEV-specific antibodies. Prevalence increases with age and reaches >25% in cohorts above 60 years of age [87]. Most autochthonously acquired HEV infections in Germany are caused by HEV-3, occasionally HEV-4, while the rare cases of HEV-1 in Germany are typically found in patients with travel history to India, Pakistan, or Bangladesh [88]. Autochthonous transmission is usually linked to consumption of raw or undercooked pork [89]. Other routes of transmission include, for example, shellfish, contact with animals, blood transfusions or organ transplants [90]. Organ transplant patients in particular are at an increased risk for chronic HEV infections. Due to immunosuppressive drugs that prevent graft rejection, HEV clearance can be impaired [77]. The extent of chronic hepatitis E in Germany is not yet fully understood but is presently being actively researched. As the vast majority of autochthonously acquired HEV cases in Germany are of zoonotic origin, it would be remiss not to consider the epidemiology within reservoir host populations. Pigs and wild boars in Germany show high prevalence of HEV infection. High seroprevalence (>50%) is reported in farmed pigs, depending on the animal's age and herd origin [91]. High prevalence was also detected in wild boars [92]. Put together, this indicates a significant risk of infection not only due to consumption of meat from pigs or wild boars but also from exposure to the animals, particularly for workers in pig rearing or processing facilities and hunters processing game. Apart from swine, evidence for HEV infection has been demonstrated in several other species, such as deer [92], and rabbits [93,94]. Especially in rabbits, a high proportion of HEV-infected or serologically positive animals was detected in various populations in Germany [93,94]. Strikingly, HEV positive rabbits are usually infected with a specific subtype within the clade of HEV-3, designated as rabbit HEV (HEV-3ra), [36]. Recently, Klink and colleagues have reported an infection with rabbit HEV in a transplant patient from Germany [95]. Similar cases are known from the neighboring countries of France [72] and Switzerland [96], which highlights the risk of zoonotic transmission of rabbit HEV and the relevance rabbits hold as as a source of human HEV infection.

1.3 Virus Structure, Genome Organization and Replication

The HEV genome is a single-stranded (+)-sense RNA molecule approximately 7.2kb in length, and contains at least three protein-encoding open reading frames (ORF) [97] (Figure 2 A). The longest of these, ORF1, is translated directly from the genomic RNA and encodes the nonstructural protein (NSP), also referred to as the viral replicase. ORF2 and ORF3 are

located downstream of ORF1. They encode the capsid protein (ORF2), and a small multifunctional protein (ORF3), respectively [98]. Unlike ORF1, ORF2 and ORF3 are expressed from a bicistronic subgenomic RNA [99] (Figure 2 B). Additionally, some HEV genomes contain an additional open reading frame (ORF4) which overlaps with ORF 1 and is likely translated under control of an internal ribosomal entry site (IRES) [100]. The genomic RNA and the subgenomic RNA are capped and polyadenylated [99,101], which is similar to eukaryotic messenger RNA (mRNA) [102]. Secondary structures within the 5' and 3' termini as well as within the junction region between ORF1 and ORF2/3 serve as recognition sites for the RNA-directed RNA polymerase (RdRp) during genome replication and synthesis of the subgenomic RNA [103] (Figure 2 B).

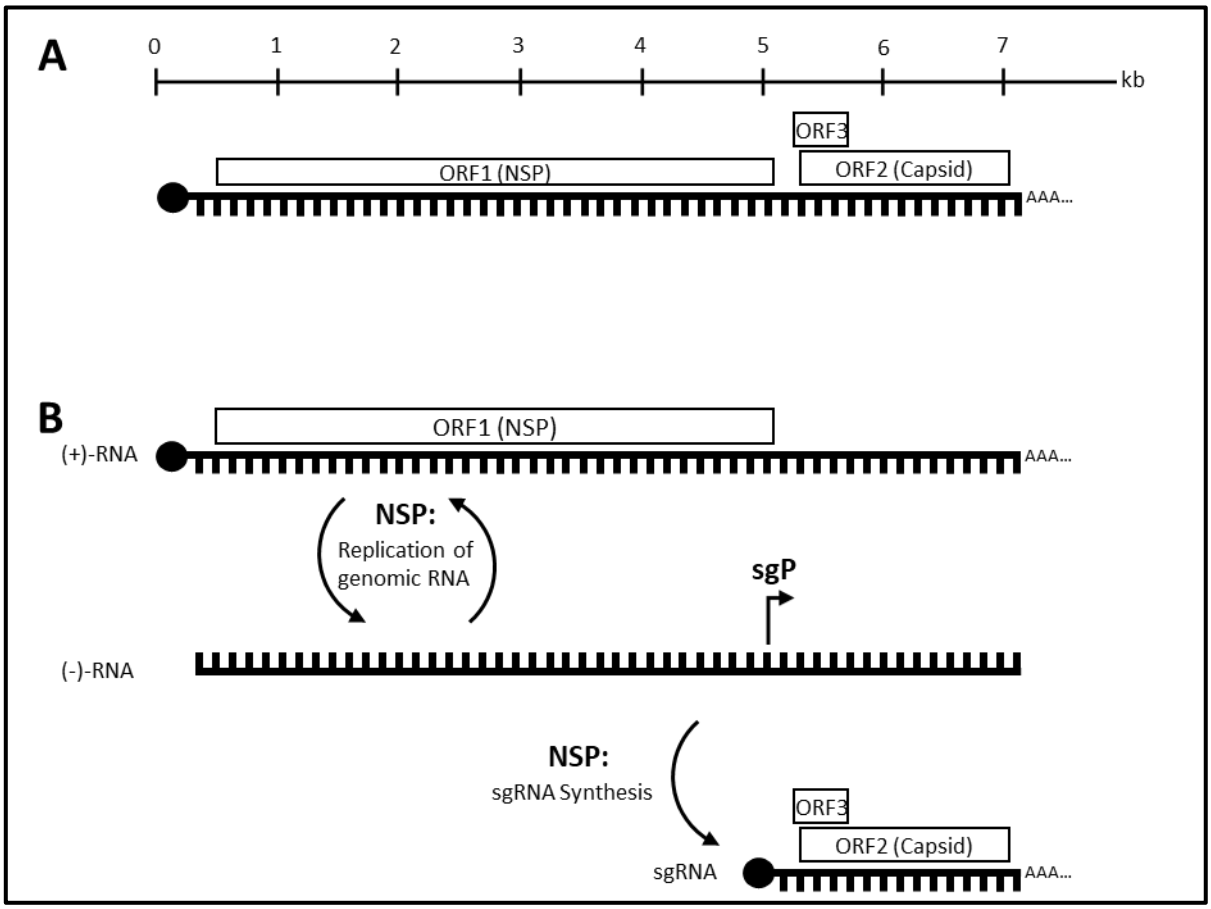

Figure 2: (A) Genome organization of HEV. The approximately 7.2kb (+)-RNA genome is capped (•) and polyadenylated (AAA…). It contains three ORFs, which encode the nonstructural protein (NSP) (ORF1), capsid (ORF2), and a small multifunctional protein (ORF3). (B) The NSP is translated directly from the viral genomic RNA. Subsequently, a (-)-sense RNA is synthesized, which serves as template for (+)-sense genomic RNA and contains the subgenomic promoter (sgP) that serves as starting point for the synthesis of the subgenomic RNA (sgRNA) from which ORF2 and ORF3 are translated.

1.3.1 The ORF1-encoded NSP

The NSP encoded by ORF1 contains approximately 1700 amino acid (aa) residues and is required for the replication of the genomic RNA. Furthermore, it transcribes the approximately 2.2kb bicistronic subgenomic RNA, which encodes the capsid protein (ORF2), and a small multifunctional accessory protein (ORF3) and modulates the host cell immune response [104]. The NSP is a polyprotein that consists of several domains with distinct activities that are required for the viral life cycle. It is still debated if and how the polyprotein is processed [104]. It shares significant similarities with nonstructural proteins of other RNA viruses, in particular with beet necrotic yellow vein virus, rubella virus, and members of the family of *Alphatetraviridae* [105]. In accordance with these structural similarities, several functional domains of the NSP have been proposed. In particular, Koonin et al. showed already in 1992 based on bioinformatic analysis of the HEV NSP aa sequence that the ORF 1 encoded protein contains such conserved regions [106]. Based on this analysis, they postulated a linear domain structure with seven functional domains: Methyltransferase (MT), “Y-domain” of unknown function (Y), papain-like protease (PCP), proline-rich hinge domain, also known as the hypervariable region [97] (HVR), “X-domain” of unknown function (X), RNA helicase (Hel), and RdRp. This model was largely confirmed by other authors in the years since. Some of the domains were characterized in-depth, while knowledge on others is lacking. For instance, the X-domain was identified as an ADP-ribose-1'-monophosphatase, also known as macro domain [107], while the exact function of the Y-domain remains unknown to this day [108].

In addition to bioinformatic analysis, several groups have done biochemical characterization of the putative subdomains, largely confirming the predictions of Koonin and colleagues. Magden et al. expressed various N-terminal fragments of the ORF 1 protein to identify the MT domain [109]. They were able to show that both methyltransferase and guanylyltransferase activities essential for capping were present, however, only when a fragment of 979 aa was assayed. Similar experiments with shorter fragments (470 aa and 527 aa) did not demonstrate any activity [109]. The 979 aa fragment contains the MT region predicted by Koonin et al. However, its length is greater than the predicted approximately 250 aa. In fact, it spans more than half the total length of the NSP and includes all of the predicted domains with the exception of the helicase and RdRp. Bioinformatic analysis by Kelly and colleagues suggests that the predicted MT is not the entire domain but one part of a larger MT domain together with the predicted Y-domain [105]. The function of the Y domain is unknown. However, mutational analysis has proven that it is essential for HEV replication [110]. The cysteine protease domain was initially disputed. Despite the predicted protease domain within the NSP of HEV, several experiments failed to show any evidence of protease

activity. Ropp et al. specifically conducted mutagenesis experiments substituting hypothetical active residues of the protease domain and found no effect on protease activity or lack thereof [111]. However, thirteen years later, Paliwal et al. were able to successfully express and purify a fragment of the HEV NSP and showed cleavage of both the NSP as well as the capsid protein [112]. The fragment in question corresponds almost exactly to the predicted protease domain. The protease is followed by a region of 120 aa with no significant structural homology according to Koonin et al. [106]. In contrast, Kelly et al. performed a similar analysis some 20 years later [105] and predicted a conserved domain of unknown function. Interestingly, a protein containing this region and a part of the protease domain has been expressed and crystallized, showing a zinc-binding protein, although its biological significance is undetermined [113]. A proline-rich hinge region is located approximately 120 aa downstream of the predicted protease domain. This region contains considerable sequence variability, and is for this reason also referred to as the HVR [97]. While no clear enzymatic activity was reported, it is known that insertions within this region can increase the replicative ability of HEV strains in cell culture [114,115]. Next is the 'X' domain, also known as macro domain. Structural analysis has shown that this domain functions as an ADP-ribose-1'-monophosphatase [107,116]. Additionally, the protein has a high affinity for poly(ADP-ribose) and poly(A) [116], and interacts with the viral MT and ORF3-encoded protein [117]. The helicase domain is located directly downstream of the X domain. It possesses NTPase and RNA duplex-unwinding activities, which are likely involved in RNA capping and in the initiation of RNA transcription by the RdRp [118]. The RdRp is located at the C-terminal end of the NSP and, with a length of almost 500 aa, is the largest of the domains predicted by Koonin et al. The RdRp binds specifically HEV RNA, in particular the polyadenylated 3'-end, the conserved 5'-terminal region, and the subgenomic promoter [103]. It is capable of initiating *de novo* negative strand RNA synthesis, using the positive strand as template without oligonucleotide primer and can also extend the template by a loop-back mechanism [103]. The RdRp domain is critical for replication of the HEV genome and synthesis of the subgenomic RNA encoding the capsid protein (ORF 2), and the ORF3 multifunctional protein but can be disabled almost completely by substituting the aspartates (D) in the conserved RdRp 'GDD' catalytic motif with alanines (A) [119].

1.3.2 The ORF2-encoded Structural Protein

The capsid, or structural protein, is encoded by ORF2. Its primary role is packaging the viral genome in order to transport it between hosts during the infection cycle. It does so by binding the 5' end of the viral genomic RNA [120]. The virus particles are icosahedral with T=3 symmetry and have a diameter of approximately 32nm [121]. The capsid protein consists of approximately 660 aa residues and can be subdivided into three domains. Being

morphologically similar to calicivirus capsids, these domains are sometimes referred to as the inner S (‘shell’) domain, and the outer P1 and P2 (‘protruding’) domains [122]. However, Xing et al. argue that unlike in caliciviruses, where P1 and P2 are subdomains of one larger ‘P’ domain, the outer HEV capsid domains are more distinct. The capsid structure is therefore more commonly described by the S (‘shell’), M (‘middle’), and P (‘protruding’) domains instead [123]. Additionally, the N-terminal 111 aa are referred to by Xing et al. as the N-domain, and are critical for interaction with the viral RNA genome [123]. The P domain likely contains the receptor binding site required for host cell membrane penetration, whereas the S and M domains interact with each other to construct the capsid scaffold. Recombinantly expressed in insect cells, the protein can self-assemble into T=1 icosahedral virus-like particles (VLP) approximately 27nm in diameter, or into T=3 icosahedral particles with 42nm diameter [124]. The T=1 particles do not contain RNA, while the T=3 particles do. This suggests an RNA-dependent assembly pathway for the T=3 particles [123]. However, neither of the VLPs accurately represents the size of the native virus particle. This is likely caused by different posttranslational modifications of the protein in an artificial expression system when compared to infection in the actual host, or due to usage of a truncated expression construct. Kapur et al. reported 35nm particles upon recombinant expression of the full-length capsid protein in *E. coli* BL21 [125]. Later, the same group demonstrated that these particles can be used to introduce mRNA into HuH-7 and A549 cells and as a delivery vehicle for vaccine RNA constructs in mice, so long as the 5’ end of the HEV genome and therefore the binding site for the N-domain was included within the construct [126]. *In vivo*, glycosylation of the capsid protein is essential for formation of infectious virions. Furthermore, there exist three forms of the capsid protein in the supernatant of infected cells: The capsid-associated form of the ORF2-encoded protein, a truncated version of the protein, and the secreted form of the capsid protein [127]. Interestingly, despite the apparent necessity of glycosylation for production of infectious virus particles, the virion-associated form of the ORF2-encoded protein is not glycosylated, only the truncated and secreted forms are [128]. In fact, most of the capsid protein produced does not end up in a virus particle. It is hypothesized that this is an adaptation to the host immune system, and that the secreted and cleaved forms of the ORF2-encoded protein serve as a decoy for the immune system [127].

1.3.3 The ORF3-encoded Multifunctional Protein

The protein encoded by ORF3 is a small protein of 113-114 aa residues and is essential for infection *in vivo* [129] but not in cell culture [130]. Several functions of this protein have been described. Its function is dependent on phosphorylation and palmitoylation [131,132]. The phosphorylated form of the ORF3-encoded protein interacts with the non-glycosylated form of the capsid protein [131], and mediates virion release from infected host cells [133] via

the endosomal sorting complexes required for transport (ESCRT) pathway by interacting with tumor susceptibility gene 101 (TSG101) [134]. In the process, it appropriates the host membrane, which is used to mask the virions circulating in the bloodstream from detection by the immune system [135]. Furthermore, the ORF3-encoded protein interacts with the cytoskeleton and interferes with microtubule dynamics [136]. It also influences elements of cellular signaling pathways, for instance extracellular signal-regulated kinase (ERK) activation by binding and inhibiting mitogen-activated protein kinase (MAPK) phosphatase Pyst1 [137]. Additionally, the protein has an effect on the expression of liver-specific genes [138] and may affect blood coagulation [139]. Finally, it functions as a viroporin, a virus-encoded ion channel, which is required for virion release from infected cells [140].

1.3.4 The HEV Life Cycle

HEV virions utilize two different forms to infect the host cell (Figure 3). The quasi-enveloped particle, found in the bloodstream, and the non-enveloped form, found in feces [135,141]. The non-enveloped particle consists of the viral RNA encapsidated with the capsid protein. The quasi-enveloped form is additionally associated with host cell membrane and ORF3-encoded protein [142]. This enveloped form of the virus particle is likely a result of viral egress from the host cell, which is mediated by the ORF3 protein [143]. The cell-derived membrane protects the circulating virus particles from detection by the immune system and neutralization by antibodies [144]. *In vivo*, the membrane is likely stripped during passage through the bile ducts before the virus is excreted via the gut [133]. Distinct infection mechanisms exist for each of the two forms [145]. The non-enveloped virions attach to the cell surface by binding to heparan sulfate proteoglycans and enter by utilizing a so far unknown receptor [146]. The quasi-enveloped particles enter the cell by a dynamin/clathrin-mediated endocytosis mechanism [147]. This mechanism is less efficient than uptake of non-enveloped particles and reliant on endosomal trafficking and acidification for successful infection [145]. After virion uptake by the host cell, the viral RNA genome is released into the cytoplasm where translation of ORF1 is initiated in a cap-dependent manner by the eukaryotic initiation factor 4F (eIF4F) complex [148].

After translation of ORF1, the RdRp domain of the NSP then starts replicating the virus genomic RNA (gRNA) by synthesizing a negative-sense gRNA intermediate ((-)gRNA).The (-)gRNA serves as a template for synthesis of the positive sense (+)gRNA and the subgenomic RNA (sgRNA), which encodes ORF2 and ORF3 [149]. While this process is typically thought to take place at the endoplasmic reticulum (ER) membrane, recombinant HEV strains containing insertions with nuclear localization sequences within the hypervariable region of the nonstructural protein were reported [150]. These insertions impart growth advantages in cell culture, though nuclear localization alone is not sufficient to explain

this effect [150]. In order to copy the viral genome and to transcribe the subgenomic RNA, the replication process first produces negative-sense HEV RNA via a double-stranded RNA intermediate [151], which is recognizable to the host cell as a signal of viral replication [152]. This recognition is mediated by cytosolic pattern recognition receptors, such as retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA-5), and various Toll-like receptors (TLRs) [152]. As response to recognition of viral replication, the interferon (IFN) signaling pathways are activated, which exacts an inhibitory effect on virus growth [153]. In adaptation to this, HEV has developed the ability to inhibit interferon signaling on several levels. In fact, each of the HEV-encoded proteins has been shown to interact with the IFN pathway. The NSP of ORF1 down-regulates the IFN-β response. In particular, the MT and PCP domains appear to be responsible for this activity [154]. Part of this effect is caused by the deubiquitination activity associated with these domains, which efficiently hydrolyzes the product of IFN stimulated gene 15 (ISG15), a key factor in the IFN pathway [155]. Additionally, a 5'-methylguanosine cap structure is added to the genomic and subgenomic RNAs, presumably by the methyltransferase domain within the ORF1-encoded NSP [99,101,109]. This enhances translation efficiency and reduces recognition by RIG-I [154,156]. Similarly, the ORF2-encoded capsid protein suppresses RIG-I and TLR-induced IFN-β signaling. This activity is independent on whether the protein is glycosylated or dimerized [157]. In contrast, the ORF3 protein shows no influence on the activity of the IFN-β signaling. Instead, it can counteract the IFN-α response by specifically binding of signal transducer and activator of transcription 1 (STAT1) and inhibiting IFN-α induced phosphorylation [158]. Viral proteins and RNA colocalize within the cell and form replication complexes associated with host cell membranes. The formation of these complexes is mediated by interactions between different viral proteins and genomic as well as subgenomic RNA [159]. Similar replication complexes are typical for RNA viruses and enhance viral replication mainly by concentrating required viral and host factors [160]. Additionally, in the case of HEV, interaction between genomic RNA and capsid protein is sufficient for spontaneous assembly of non-enveloped virus particles [123]. It is therefore assumed that the replication complexes also play a role in viral assembly. The virions are secreted from the host cell via the ESCRT pathway. The ORF3-encoded protein is known to play a crucial role in this process by interacting with TSG101 [143]. In the process, the virions acquire the "quasi-envelope" consisting of host membrane from the exosomal pathway [142]. Subsequently, the particles are transported to multivesicular bodies (MVBs) and secreted from the host cell [161]. Details of this process are still unknown due to the lack of an efficient cell culture model system for HEV.

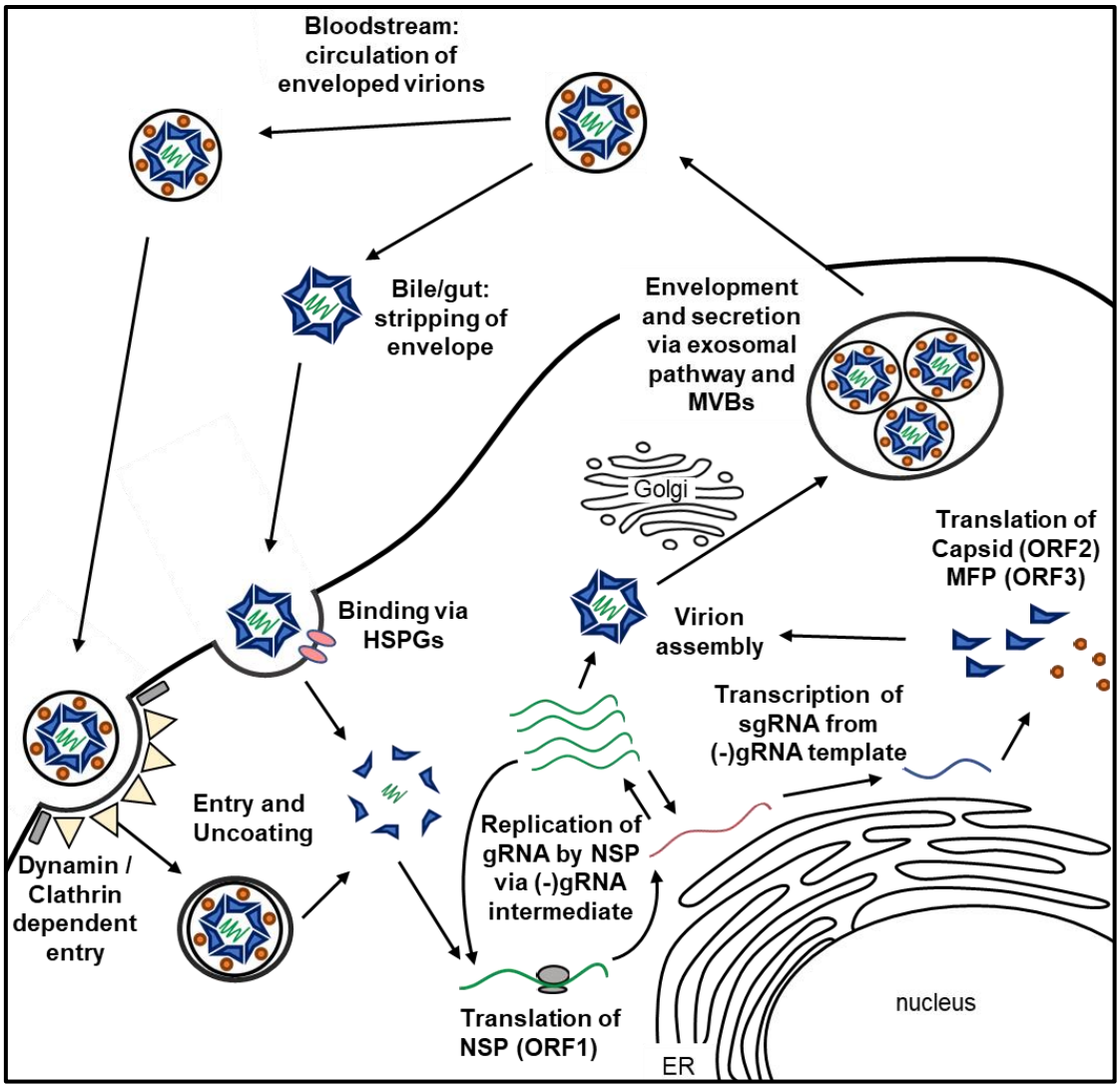

Figure 3: HEV Life cycle. Enveloped and non-enveloped virions enter the host cell by distinct mechanisms. The enveloped form relies on clathrin-mediated endocytosis [145], while the non-enveloped virions attach to the cell surface by heparan sulfate proteoglycans (HSPGs) [146]. It is assumed that upon uncoating the ORF1 of the viral genome is translated directly from the genomic RNA. The resulting nonstructural protein (NSP) begins replicating the genomic RNA (gRNA) via a negative-sense gRNA intermediate ((-)gRNA) and transcribing the subgenomic RNA (sgRNA), from which the structural proteins are translated [162]. Newly synthesized genomic RNA and ORF2- and ORF3-encoded proteins interact to form infectious virus particles, which are exported from the cell via the exosomal pathway involving the trans-Golgi network and multivesicular bodies (MVB) [161]. ER: Endoplasmic reticulum.

1.4 Model systems for HEV

In order to study the molecular biology and transmission of HEV in a laboratory setting, a suitable model system is required. This is quite the conundrum, as many standard model systems do not support HEV replication. Mice, for instance, are not naturally susceptible to *Paslahepevirus balayani*, and isolation of HEV in cell culture has so far only been successful for a few specific combinations of virus strains and host cell lines. As a consequence, the search for an ideal model system continues to this day.

1.4.1 Primates and Swine, the standard animal models of HEV research

Two primate models were essential for the identification of HEV: Cynomolgus macaques (*M. fascicularis*) and human volunteers (*Homo sapiens*) [1]. While the human volunteer model has actually been applied in at least one further instance [163], it has not gained hold in mainstream HEV research. On the other hand, non-human primate models, such as cynomolgus macaques, have been tremendously helpful in figuring out key aspects of the infection process. Aside from cynomolgus macaques, other non-human primate model species include chimpanzees (*Pan troglodytes*), owl monkeys (*Aotus trivirgatus*), patas monkeys (*Erythrocebus patas*), pig-tailed macaques (*Macaca nemestrina*), rhesus macaques (Macaca *mulatta*), and squirrel monkeys (*Saimiri sciureus*)(reviewed by Corneillie et al. [29]).

The main advantage of primate models is their phylogenetic proximity to *Homo sapiens*. As a consequence, the symptoms observed in the animals upon HEV infection, such as elevated liver enzymes, liver damage, viremia, and fecal virus shedding, are similar to the symptoms suffered by HEV infected humans [29]. Chronic infections are rare but have been reported [164]. Furthermore, non-human primates are susceptible to infection by several genotypes of HEV, though to which level is dependent on primate species and virus genotype [165]. Beside experimental infection in a laboratory setting, natural HEV infection has been documented in non-human primates. Evidence of this has been published from an outdoor monkey breeding facility at the university of Kyoto, where an outbreak among Japanese monkeys (*Macaca fuscata*) and rhesus monkeys (*M. mulatta*) occurred in the years 2004-2006 [164]. The virus responsible for this outbreak was identified as a strain of the HEV-3 clade. Intriguingly, the authors identified one monkey (*M. fuscata*) which had been persistently infected from 2006-2009 and continued shedding virus even in 2009, a rare observation of chronic HEV in a non-human primate, although no specific reasons for this persistence of the infection were deducible. It is unknown how the virus was introduced into the colony in the first place [164]. Another report from China describes the circulation of two separate HEV-4 strains in a rhesus monkey farm [166]. Additionally, serological evidence indicates HEV infection in several other primate species. In a study of primates in different German zoos, HEV-specific antibodies were detected in a bonobo (*Pan paniscus*), a drill (*Mandrillus leucophaeus*), a lar gibbon (*Hylobates lar*), and seven gorillas (*Gorilla gorilla gorilla*) [167]. However, despite their unique suitability as a model for human HEV infection, primate models come with several critical drawbacks. From a purely practical perspective, primates have comparatively long gestation periods and generation times. Consequently, control of their genetic backgrounds is limited. Furthermore, primate studies are limited in the number of animals due to small litter sizes, which can impede generation of statistically significant results [168]. This is to some degree due to special ethical considerations which

must be taken in the context of primate experiments, which drives up the cost of animal keeping compared to other species [28]. Finally, while humans are genetically more similar to other primates than to non-primate species, differences do exist which can detrimentally influence experimental results [168]. These factors make it desirable to identify a model system which is easier to handle than primates.

Swine (family Suidae) represent another important model system. Pigs and wild boars (*Sus scrofa*) are suitable in many ways for modeling HEV infections. For one, as the natural reservoir host of HEV-3 and HEV-4, they play a pivotal role in the evolution and spread of HEV [43]. Moreover, wild boars are also associated with HEV-5 and HEV-6 [37,38]. Additionally, while far from the least demanding model species, pigs are easier to handle compared to primates. In particular, experiments with pigs are generally considered more ethically acceptable compared to primate models [169]. The first evidence of HEV infection in pigs came from Balayan et al. in 1990 [170], who demonstrated productive infection upon inoculation with material from a patient with acute hepatitis E. However, the real breakthrough to identifying the pig as a potential host for HEV came in 1997, with the first description of “Swine HEV” in the USA [171], now simply classified as HEV-3. The data showed that HEV is highly prevalent among farmed pigs in the US. Soon after, a closely related strain was identified in a human patient [172]. Subsequent trans-species transmission experiments confirmed that pigs are susceptible to infection with the human-derived virus samples [76]. Similar results came from Taiwan, where closely related HEV sequences were discovered in a pig and a human, but only distantly related to the American sequences [173]. The sequences were later assigned to HEV-4 [36]. Reports of trans-species transmission of HEV-3 between humans and swine emerged from many other countries, including Germany [174]. In the following years, the pig has become a standard model in HEV research. Today, it is known that pigs represent a highly sensitive infection model [175]. They can be readily infected with HEV-3 and HEV-4. More specifically, pigs were experimentally infected with HEV from wild boars [74], human HEV [76], and with rabbit HEV (HEV-3ra) [176]. The animals show mild to moderate symptoms of hepatitis and fecal virus shedding [74]. However, the pig as a model for human hepatitis E comes with several limitations. For one, it is unsuitable for modeling of chronic manifestations of HEV infection. Although reports of chronic HEV infection in swine do exist [175,177], the course of the disease is usually self-limiting, which is a challenge for research on chronic hepatitis E in a controlled experimental setting. Notably, some success in this regard has been reported in pigs treated with immunosuppressants, which is an interesting parallel to chronic hepatitis E in organ transplant recipients [178]. Another aspect not observed in the pig model are the complications of HEV infection during pregnancy. Experimental infection of pregnant pigs

resulted in mild hepatitis symptoms, but no effect on the offspring, and no fulminant hepatitis or increased fatality rates [179]. This may be related to the fact that pigs are not susceptible to HEV-1 or HEV-2 [180], the genotypes mainly associated with poor feto-maternal outcomes in humans [58].

1.4.2 Rabbits, an emerging model of HEV infection

Rabbits (*Oryctolagus cuniculus*) were relatively recently discovered as host species for HEV and come with their own “brand” of HEV [181]. Rabbit HEV, or HEV-3ra, is a subtype of HEV-3, infectious to various other species, including humans, and thus zoonotic [182]. However, rabbit HEV is uniquely adapted to the rabbit, although it is not entirely understood why or how. Part of the reason may be a conserved insertion of 90/93 nucleotides with unknown function within the rabbit HEV genome, found across many HEV genomic sequences from rabbits [183]. Rabbit HEV has been detected in rabbits in Asia [181], Australia [184], Europe [93], and North America [176]. Additionally, several human cases of acute and chronic infections with HEV-3ra were reported, mainly from France and Switzerland [72,96], and recently, Germany [95]. Often, HEV-3ra infections occur in immunocompromised patients, although infections without underlying conditions are also possible [185]. In rabbits, the severity of the disease is dose dependent but usually ranges from subclinical to mild symptoms [186]. However, rabbits can exhibit similar symptoms and complications as those observed in humans. Persistence of virus replication and chronic hepatitis E has been observed in experimentally infected specific pathogen-free (SPF) rabbits [187]. Furthermore, infection of pregnant rabbits with HEV results in high feto-maternal mortality rates and vertical transmission, which is similar to HEV-1 infections in humans [188]. This makes rabbits and rabbit HEV a promising model for human HEV infections. Other than HEV-3ra, the rabbit shows limited susceptibility to other HEV genotypes. One study showed seroconversion upon infection with HEV-1 but no detectable viremia. The same study demonstrated seroconversion and viremia in one out of eight rabbits inoculated with HEV-4 [186]. A similar experiment was conducted a few years later with one strain of HEV-1 and three different strains of HEV-4. Here, the rabbits inoculated with HEV-1 showed no signs of infection. Interestingly, infection with HEV-4 resulted in different outcomes depending on the virus strain. Out of the three, only one strain elicited seroconversion and viremia in all animals (five out of five) [31]. Similarly, HEV-3 infection is dependent on the inoculum. For instance, experimental infection of rabbits with wild boar-derived HEV-3b was successful, resulting in viral shedding and seroconversion [30]. However, in another study, rabbits showed no signs of infection upon inoculation with a human-derived HEV-3b strain [189]. Apart from this, a recent study showed that rabbits are susceptible to HEV-8 but not HEV-5 or HEV-7 [85]. Put together, this makes rabbits a

promising model for pathogenicity and vaccination studies, especially considering how rabbits are easier to handle compared to pigs or primates. However more work will be necessary before the rabbit can be firmly established as model system for HEV. Critically, it will be necessary to determine why some strains can infect rabbits, while other, closely related strains, cannot.

1.4.3 Modeling HEV infection in other species

Although mice (*Mus musculus*) are not susceptible to infection with human HEV [30], there has been success in establishing a chimeric mouse model with humanized liver, which can be productively infected with HEV [190]. Unfortunately, generating chimeric mice is quite laborious and thus it remains to be seen whether this model will become a standard tool in HEV research. Similarly, rats are not a reliable model, as infection experiments do not deliver consistent results [30]. In principle, rats (*rattus* spp.) are infectible with ratHEV (species *Rocahepevirus ratti,* genus *Rocahepevirus*) [191], which, like *Paslahepevirus balayani* belongs to the subfamily of *Orthohepevirinae*. Reports of human infection with ratHEVhave recently confirmed the zoonotic nature of this virus[192], which further demonstrates the relevance of ratHEV as a model system. However, the rat infection model is not very robust and reliable as the infection has so far only been shown in athymic nude rats [193], severely immunocompromised animals. Further optimization may, in the future, lead to a reliable rat model. Similarly, ferrets (*Mustela furo*) and ferret HEV (species *Rocahepevirus ratti)* are under consideration [194]. A recent publication demonstrates the Mongolian gerbil (*Meriones unguiculatus*) as a viable model for infection with HEV-3 and HEV-4, providing a small rodent model. However, for a reliable infection, intraperitoneal injection of high-titer virus stock or intrahepatic injection of HEV RNA is required [195]. Beyond mammals, the chicken model in conjunction with avian HEV (species *Avihepevirus magniiecur*) may be taken into consideration, although it is limited by genetic differencesas well as different manifestations of the disease [196]. Finally, Cutthroat trout virus (CTV; species *Piscihepevirus heenan*) has been proposed as a possible model of HEV replication, mainly due to the availability of an efficient CTV cell culture system [197,198].

1.4.4 HEV in cell culture

Cell culture is a key model system for biology in general and virology in particular. It allows for reproducible, quick, and inexpensive characterization of key aspects of the viral reproduction process. Unfortunately, propagation of HEV in established cell culture systems is very inefficient and highly dependent on the combination of HEV strain and host cell line. However, as a robust cell culture system would be an invaluable tool in HEV research,

several attempts at cultivating the virus *in vitro* have been made over the years, to varying degrees of success.

Early reports of HEV isolation from cell culture reach as far back as 1987, when Pillot et al. were able to successfully infect human hepatoma cell line PLC/PRF/5 and detected viral particles and antigen in the cell culture [199]. The PLC/PRF/5 system has since then shown promise as a host cell line for HEV. Productive infection has been demonstrated for strains of most genotypes, i.e. HEV-1 [119], HEV-3 [200], including rabbit HEV [201], HEV-4 [202], HEV-5 [39], HEV-7 [203], and HEV-8 [204]. Additionally, the cells are permissive for ratHEV [205] and ferret HEV [206]. Notably, Takahashi et al. showed that HEV from patient samples could be propagated in PLC/PRF/5 cells only when inoculated with a rather high viral load of at least 3.5×10^5 copies per milliliter inoculate [144]. This was later confirmed by Schemmerer and colleagues, who additionally demonstrated that the correct density of the cell culture prior to inoculation plays a crucial role in achieving high viral titers [33].

In addition to PLC/PRF/5, the hepatoma cell lines HepG2 and HuH-7 and their derivatives are permissive HEV host cells. Both systems are established model for other hepatotropic viruses, particularly HBV (HepG2) and HCV (HuH-7) [207]. In the case of HuH-7, a subclone was identified which allows for relatively efficient transfection and infection with HEV [130]. Furthermore, the HuH-7 Lunet BLR subline supports replication levels comparable to PLC/PRF/5 [33]. Similarly, HepG2 can be infected with different strains of HEV and supports their replication. The derived cell line HepG2/C3A was used for the isolation of the Kernow-C1/p6 strain, which replicates readily in various cell lines from different mammalian species [208]. This strain was initially isolated from a chronically infected patient and was capable of establishing productive infection of cultured cells. In this case, HepG2/C3A cells were more permissive in comparison to other cell lines, such as PLC/PRF/5, HuH-7 derivative HuH-7.5, or the epithelial lung carcinoma cell line A549 [208].

Reports of HEV replication in non-hepatic cell lines reach back to 1992, when Huang et al. showed the productive infection in a human embryonal lung cell line (2BS) using fecal suspension of a patient who suffered from acute hepatitis and from whom several fecal samples were taken over a time of two months. The authors noted that the infection was possible only with this particular cell line and only with a fecal suspension sample taken during the incubation period [209]. The same strain was later shown to infect A549 cells [210]. A549 cells have subsequently become one of the standard models for HEV research along with the aforementioned hepatoma cell lines. A549 cells are permissive for HEV-1, HEV-3, HEV-4, and HEV-5 strains. They support infection and HEV replication at levels similar to HepG2/C3A [211] or PLC/PRF/5 [144]. Notably, A549 cells were used to isolate

HEV strain 47832c [212], another cell culture adapted model virus. This isolate was, in turn, used to identify A549/D3, a highly permissive subclone of A549 [213].

There is a number of reports of productive HEV replication in further cell lines, such as HepaRG [214], CaCo-2 [119], baby hamster kidney cells (BHK-21) [215], primary human hepatocytes (PHH) [211], and induced pluripotent stem cells (iPSC) [216]. However, none of these systems have been widely adapted as they tend to be inefficient, difficult to handle, or both. In many cases, HEV cell culture systems rely on adapted strains which may not accurately represent field strains of HEV. In particular, cell culture isolates often contain point mutations or insertions which enhance HEV growth in cell culture conditions [217]. Insertions within the hypervariable region of ORF1 have been identified in several model strains of HEV, such as Kernow-C1/p6 or 47832c and are a prerequisite for the ability of these strains to replicate in cell culture [114,115]. However, the exact mechanism enabling efficient growth of HEV *in vitro* is currently unknown. Further work will be necessary to determine which viral and host factors are involved.

1.5 Reverse genetics and replicon systems

Reverse genetics can be defined as “an approach wherein a nucleic acid is modified at predetermined positions *in vitro* and the effects of these interventions are scored *in vitro* or *in vivo*” [218]. This constitutes a complement to classical, or *forward*, genetics, where a trait or phenotype is identified first, followed by investigation of the underlying genotype [219]. In the context of RNA viruses, the term “reverse genetics” is these days used more broadly to refer to “the creation of a virus from a full-length cDNA copy of the viral genome” [220]. Reverse genetics systems (RGS) and cDNA clones are an indispensable tool for working with RNA viruses because they greatly simplify manipulation of the viral genome. Initially demonstrated with the RNA bacteriophage Qβ in 1978 [221], the approach had been applied to poliovirus by 1981 [222]. In the years since, the technique has been applied to many other viruses and refined by incorporation of emerging technologies, such as DNA synthesis [223]. The first cDNA clone of HEV (pSGI-HEV(I); genotype HEV-1) was published in 2000 by Panda and colleagues [224]. Upon *in vitro* transcription and transfection of HepG2 cells, the authors detected viral proteins and antisense RNA, and demonstrated that the cell culture supernatant was infectious to rhesus macaques. Surprisingly, infectious virus was rescued despite using RNA without a 5’ cap structure [224]. In contrast, Emerson et al. showed just a year later with a different clone (Sar55; genotype HEV-1) that capping is quite important in order to establish productive infection when the RNA is injected intrahepatically into chimpanzees [225]. Similarly, HEV-3 cDNA clones were constructed from swine HEV sequences. The RNAs generated from these clones were replication competent in cell

culture and infectious *in vivo* upon intrahepatic inoculation of pigs [226]. cDNA clones of other HEV-3 strains were published subsequently, particularly of established cell culture adapted strains, such as JE03-1760F [227], Kernow-C1/p6 [114], and 47832mc [228]. Similarly, cDNA clones were generated of HEV-4 [229], HEV-5 [39], HEV-7 [203], and HEV-8 [204]. Over the years, cDNA clones have been instrumental in decoding the viral genome. For example, intragenotypic chimeric clones have given some insight into the viral factors that determine host specificity [230]. However, one of the greatest advantages is the ability to omit the inefficient infection process by transfecting cells with HEV RNA. In this way, higher viral titers can be recovered compared to conventional infection assays, which simplifies the investigation of the viral life cycle [211].

The ability to initiate virus replication without having to rely on effective infection opens up the possibility of working with noninfectious subgenomic replicons. A replicon was defined by Jacob, Brenner, and Cuzin as “a unit capable of independent replication” [231]. Although this definition was originally formulated in the context of DNA replication in bacteria, the concept also applies to self-replicating RNA sequences. A replicon must carry two components, the replicator and the initiator, wherein the *initiator* is a gene encoding a protein which can, actively or passively, initiate replication by acting on a specific DNA or RNA sequence, the *replicator* [231]. Accordingly, the HEV genome can be seen as a replicon, which encodes the nonstructural protein or replicase (initiator), that interacts with the 3’-termini of the sense and antisense genomic RNA (replicator). Additionally, the HEV genome encodes the structural proteins of ORF2 and ORF3, which are not required for replication and can therefore be knocked out, or deleted, thus resulting in a *subgenomic* replicon (Figure 4). In the context of RNA viruses, the term “replicon” is often used to refer specifically to a subgenomic, non-infectious replicon [232,233] in contrast to infectious virus.

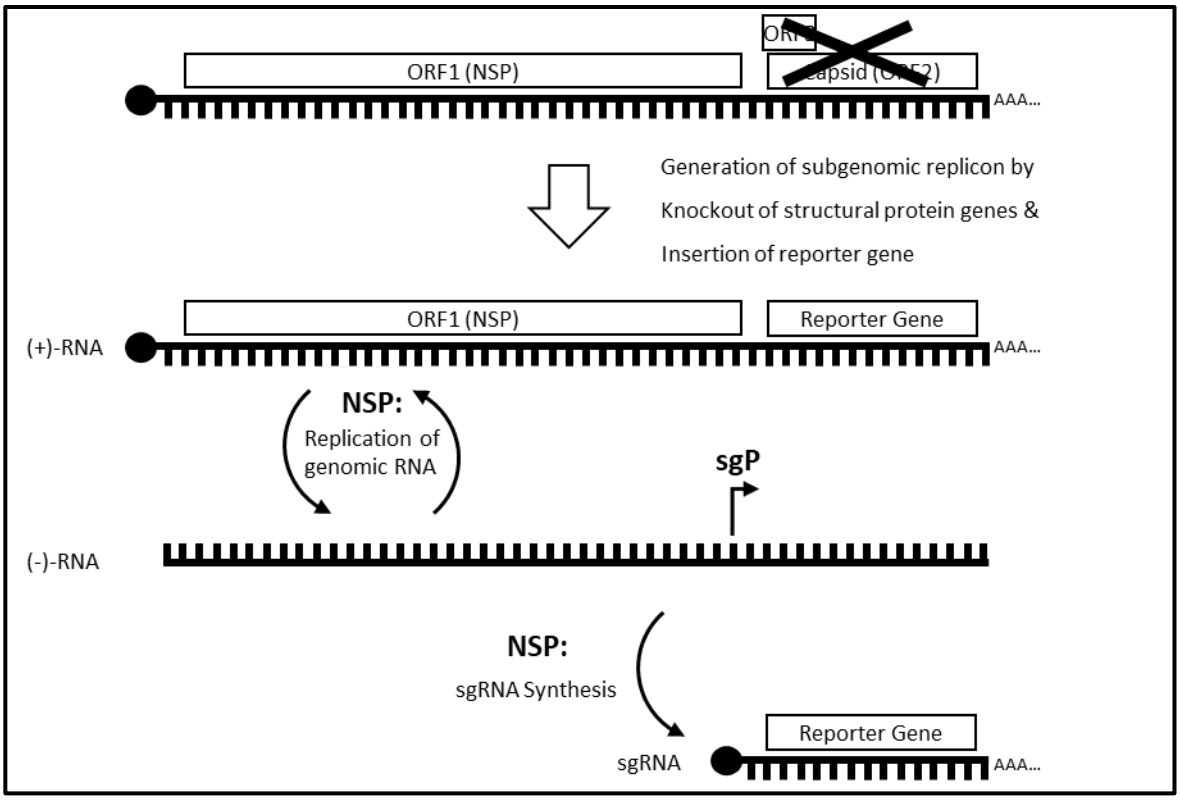

Figure 4: Schematic representation of a subgenomic HEV replicon. The structural protein genes (ORF2 & ORF3) in the viral genome are replaced by an appropriate reporter, such as a fluorescent protein, luciferase, or antibiotic resistance gene. During viral replication, the subgenomic RNA (sgRNA), which now contains the reporter coding sequence rather than the structural protein genes, is transcribed by the nonstructural protein (NSP) under control of the subgenomic promoter (sgP) and expressed by the host cell.

Subgenomic replicons are nowadays a valuable tool in virology and beyond. For instance, an HCV replicon was the key to an HCV cell culture model, which eventually made it possible to identify antiviral drug candidates, several of which proved successful in clinical trials. As result, the formerly untreatable chronic hepatitis C can now be treated in 90% of the cases [234]. Furthermore, replicons can also be used as powerful vectors for protein expression in eukaryotic cells [235], or as self-amplifying RNA vaccines [236]. In the case of HEV, replicons were used in the evaluation of several key parameters of viral replication. Examples include the transcription and translation of the subgenomic RNA [99], as well as identification of the subgenomic promoter [237], insights into the role of RNA secondary structures within the HEV genome [238], characterization of the structure and function of viral proteins [130,159], or screening for antiviral drug candidates [239]. In summary, replicons have contributed greatly to our knowledge of HEV and are a promising model system for future research. However, RGS and replicon systems as cell culture models for HEV have limitations. For one, by cloning a single viral sequence, the natural virus diversity within a quasispecies [240] is reduced to a single, clonal, virus genome. Furthermore, they are

dependent on a model virus that is adapted to growth in cell culture in the first place. Conversely, it is almost impossible to examine non-adapted strains due to the lack of output. High genetic diversity among HEV strains makes it difficult to predict which mutations are the cause of such adaptation. A good example for this is a recent publication by Zhang et al., in which three clones of different rabbit HEV strains were constructed and tested in the same conditions. One showed high levels of replication in cell culture, whereas the remaining two did not replicate under the same conditions. It was not possible to deduct a reason for this divergence [241]. In conclusion, despite considerable progress in elucidating of HEV molecular virology, much of the viral life cycle is still in the dark. Further work is needed to decipher the diverse factors involved in the replication, infection, and pathogenesis of HEV.

2 Objectives

Zoonotic transmission of HEV has been recognized in recent years as a leading cause of human hepatitis E. Consequently, HEV is now increasingly being discussed in the context of One Health. By understanding the processes of infection, replication, pathogenicity, and transmission in the animal host, we can better understand the course of the infection in humans and vice versa. In Germany, the known animal reservoirs are pigs and wild boars, as well as rabbits. Thus, along with humans, these animals are our main concern for understanding the epidemiology, pathogenesis, and transmission of HEV in Germany.

As part of the German One Health Initiative (GOHI), the focus of this work lies in the phylogenetic and molecular characterization of potentially zoonotic strains of HEV from these species. This is achieved by

- Investigation of the epidemiology of HEV in the known reservoir hosts of zoonotic HEV, pigs and wild boars, in north-eastern Germany.
- Demonstrating evidence of a hitherto unknown HEV subtype in a rabbit, which adds further support to the role of rabbits as reservoir species and animal model of HEV infection.
- Establishment of a modular RGS with HEV reporter replicons to investigate the influence of discrete subgenomic fragments on virus replication in cell culture.

3 Publications

(I) Co-circulation of different hepatitis E virus genotype 3 sub-types in pigs and wild boar in north-east Germany, 2019

Priemer, G.; Cierniak, F.; Wolf, C.; Ulrich, R. G.; Groschup, M. H.; Eiden, M.

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Co-Circulation of Different Hepatitis E Virus Genotype 3 Subtypes in Pigs and Wild Boar in North-East Germany, 2019

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Abstract: Hepatitis E is a major cause of acute liver disease in humans worldwide. The infection is caused by hepatitis E virus (HEV) which is transmitted in Europe to humans primarily through zoonotic foodborne transmission from domestic pigs, wild boar, rabbits, and deer. HEV belongs to the family *Hepeviridae*, and possesses a positive-sense, single stranded RNA genome. This agent usually causes an acute self-limited infection in humans, but in people with low immunity, e.g., immunosuppressive therapy or underlying liver diseases, the infection can evolve to chronicity and is able to induce a variety of extrahepatic manifestations. Pig and wild boar have been identified as the primary animal reservoir in Europe, and consumption of raw and undercooked pork is known to pose a potential risk of foodborne HEV infection. In this study, we analysed pig and wild boar liver, faeces, and muscle samples collected in 2019 in Mecklenburg-Western Pomerania, north-east Germany. A total of 393 animals of both species were investigated using quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR), conventional nested RT-PCR and sequence analysis of amplification products. In 33 animals, HEV RNA was detected in liver and/or faeces. In one individual, viral RNA was detected in muscle tissue. Sequence analysis of a partial open reading frame 1 region demonstrated a broad variety of genotype 3 (HEV-3) subtypes. In conclusion, the study demonstrates a high, but varying prevalence of HEV RNA in swine populations in Mecklenburg-Western Pomerania. The associated risk of foodborne HEV infection needs the establishment of sustainable surveillance and treatment strategies at the interface between humans, animals, and the environment within a One Health framework.

Keywords: *Hepeviridae*; genotype; HEV-3; subtype; reservoir; transmission; One Health

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1. Introduction

Hepatitis E virus (HEV) belongs to the *Hepeviridae* family and is the most common cause of acute viral hepatitis throughout the world [1]. In total, 20 million HEV infections occur each year, with over three million acute cases and 44,000 hepatitis E-related deaths [2]. Hepeviruses are subdivided into the subfamilies *Orthohepevirinae* and *Parahepevirinae* with fish-infecting hepeviruses. Most human pathogenic genotypes are grouped within the species *Orthohepevirus balayani* [3]. These are the exclusively human-associated genotypes HEV-1 and HEV-2 as well as the zoonotic genotypes HEV-3/HEV-4, which circulate between animal reservoirs and humans. In contrast to HEV-3, which is endemic in Europe, there are only few reports of HEV-4 in Europe so far. This includes infection of pigs in

Belgium [4] and human cases in Germany in 2007 [5], Italy in 2011 [6] and France in 2014 [7]. Recently, the camel-associated genotype HEV-7 has been detected in a human patient [8].

Genera *Avihepevirus* comprises avian, and *Chirohepevirus* bat-associated hepevirus strains. Genus *Rocahepevirus* comprises rodent and mustelid borne hepeviruses; rat HEV (species *Orthohepevirus ratti*)-related infections of patients have been described recently [9–11], demonstrating the zoonotic potential of this hepevirus.

HEV is highly endemic in regions in Africa, Asia, or the Middle East and causes large epidemics of acute hepatitis in these regions, mainly due to poor sanitation and contaminated drinking water. One major route of transmission in developed countries is zoonotic [12]. In Europe, which is almost exclusively dominated by genotype HEV-3, the consumption of contaminated and undercooked food, especially pork and meat products, is the most common cause of infection [13]. Transfer of blood, blood products, and organ transplantations are additional routes of transmission and infection [14].

The HEV genome is a single-stranded positive-sense RNA genome of approximately 7.2 kb. The open reading frame 1 (ORF1) at the 5′ end of the genome encodes the nonstructural polyprotein. ORF2 encodes the capsid protein and is located at the 3′ end of the viral genome. ORF3 encodes a small multifunctional protein and overlaps with the 5′ end of ORF2 [15]. This overlapping region is highly conserved and can be used for molecular detection of HEV RNA [16,17]. Additionally, the 5′ end of the viral genome acts as binding site for the viral RNA-directed RNA polymerase (RdRP), is also highly conserved, and can therefore be used as a target for molecular detection as well [17].

HEV-3 infections of humans through ingestion of contaminated, undercooked animal products have been thoroughly investigated and provide evidence for a broad spectrum of animal species including swine, deer, rabbit, and camel as source of infection [13]. In Europe, this mainly comprises wild boars and pigs, but also rabbits and deer [18]. In Germany, rabbits show high detection rates with a rabbit-specific subtype (HEV-3ra) displaying a RNA prevalence of 17 to 25% [19–21]. Isolated human cases of infection, especially in France with this genotype, demonstrate the zoonotic potential of this virus variant [22]. In contrast, the German roe and red deer populations exhibit only low detection rates of both HEV-specific antibodies and RNA [23], which suggests a rather minor importance of these animal species as virus reservoirs for human infections in Germany so far.

In our study, liver and muscle tissue samples and faeces from 318 pigs and 75 wild boars were collected during 2019 by the State Office for Agriculture, Food Safety and Fisheries Mecklenburg-Western Pomerania (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, LALLF) and provided for HEV diagnostic investigations. Viral RNA was detected in 27 farmed pigs and 6 wild boars, including one muscle sample, which demonstrates a continuing high prevalence of HEV in farmed pigs and wild boar in the north-east of Germany.

2. Materials and Methods

2.1. Collection of Wild Boar and Pig Samples

Wild boar and pig liver and muscle samples were collected over the entire year 2019 by the LALLF and monthly shipped to FLI for further analysis. Muscle tissue originates from lateral femoral musculature (musculus biceps femoris). Accompanying faeces samples were collected between August and December 2019 (see Supplementary Table S1).

2.2. RNA Isolation

RNA extraction from liver and muscle tissue samples was performed with the Qiagen RNeasy Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Liver and muscle tissues were freshly prepared during dissection and immediately frozen. For RNA extraction, MS2 bacteriophages were added to the tissue and faeces samples. RNA was then isolated with the RNeasy kit using guanidine thiocyanate and selective binding of RNA on a silica-based membrane, which removes most of the potential inhibitors, enables efficient RNA recovery and is especially recommended for liver and muscle tissues. From

faecal samples, a 10% suspension was made with 0.89% NaCl-solution. After vortexing and centrifugation (4400× *g*, 4 °C, 20 min), the supernatant was sterile filtrated using a sterile 0.22 µm MILLEX-GP Syringe Filter Unit (Millipore, Tullagreen, Ireland) and subjected to RNA isolation using the QIAamp viral RNA Kit (Qiagen, Hilden, Germany).

2.3. Quantitative Real-Time RT-PCR and Sequence Analysis

HEV RNA was detected with a quantitative real-time RT-PCR (RT-qPCR), which targets a sequence within a conserved overlapping ORF2/ORF3 region, and determined by cycle threshold (ct) values [17]. The following primers and probes were used: forward primer (5′-GTGCCGGCGGTGGTTTCTG-3′), reverse primer (5′-GCGAAGGGGTTGGTTGGATG -3′) and probe 5′-FAM-TGACMGGGTTGATTCTCAGCC-BHQ1-3′). As internal RNA extraction and RT-qPCR control, RNA bacteriophage MS2 particles were added to each sample to exclude false negative results in accordance with [24]. MS2 bacteriophage derived RNA was detected using primers MS2F (5′-CTCTGAGAGCGGCTCTATTGGT-3′), MS2R (5′-GTTCCCTACAACGAGCCTAAATTC-3′) and MS2 probe (5′-HEX-TCAGACACGCGGTC CGCTATAACGA-BHQ1-3′). The assays were carried out following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [25]. For phylogenetic analysis, partial sequences were amplified targeting an ORF1 region (nucleotide positions 127–376; numbering refers to FJ705359) using a nested RT-PCR protocol with first round primers HEV.ORF1_F1 (5′-CCCAYCAGTTYATWAAGGCTCCTGGC-3′) and HEV.ORF1_R1 (5′-TGCARDGARTANARRGCNAYNCCNGTCTC-3′) followed by second round primers HEV.ORF1_F2 (5′-AAYTCYGCCYTGGCGAATGCTGTGGTGGT-3′) and HEV.ORF1_R2 (5′-CCVCGRGTNG GRGCRGWRTACCA-3′). In brief, reverse transcription was carried out with Superscript® III Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the subsequent nested PCR with Maxima SYBR Green/Fluorescein qPCR Master Mix Kit (Thermo Fisher Scientific Inc., USA). Finally, a melting curve analysis was performed starting with a temperature gradient from 68 to 94 °C in steps of 0.2 °C. Positive samples were identified by melting peaks and amplicons were subsequently sequenced (Eurofins Genomics, Munich, Germany). Detailed protocol is found in a previous publication [17].

2.4. Phylogenetic Analysis

Reference sequences for HEV-3 subtypes were selected according to [26]. The sequences were aligned using, with Minimap2 [27], using HEV-1 (GenBank acc. no. M73218) as reference (full HEV-3 sequence set). Multiple alignments of amplicon sequences were made using MUSCLE in MEGA 11 [28,29] and subsequently manually inserted into the reference alignment using Ugene [30]. The phylogenetic analysis of the amplicon region was performed with MEGA 11 using the Maximum Likelihood method and General Time Reversible (GTR) model. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories). The rate variation model allowed for some sites to be evolutionarily invariable. Tree visualisation was done in MEGA and in R [31] using Rstudio [32] with packages dplyr [33], ggplot2 [34], ggtree [35–37], and treeio [38].

3. Results

In 2019, samples from 317 pigs and 76 wild boars were collected by routine sampling in the framework of a disease monitoring program by the LALLF and transferred to FLI. Liver, muscle, and faecal samples were submitted from 179 animals, liver, and muscle tissue from 212 animals, from one individual-only faeces, and from one individual-only muscle tissue. A compilation of collected samples is displayed in Supplementary Table S1. All samples were subjected to RNA isolation followed by HEV-specific RT-qPCR. To avoid false negative results due to RT-PCR inhibitors within the tissue or faeces samples a silica bead-based RNA extraction method of freshly prepared samples was performed to which a bacteriophage MS2 based RNA extraction control was added. A multiplex RT-qPCR was

performed detecting HEV RNA, in the FAM channel and the MS2-RNA in the HEX channel, which provides the validity of the RT-PCR reaction.

In total, in 33 individuals,27 farmed pigs and 6 wild boars, viral RNA was detected either in liver (n = 31) and/or accompanying faeces (n = 14) (Table 1). The monthly number of analysed individuals varied between 23 and 44. The number of HEV RNA-positive animals varied between 0 and 6, resulting in monthly prevalences of 0 to 14.6% (mean 8.14%; 95% confidence interval, CI, 5.66–10.62%) over the year (Figure 1). In one case, in addition to liver, viral RNA could be detected in a muscle sample exhibiting a low ct value of 20.1. RT-PCR detection of HEV RNA followed a nested SYBR Green RT-PCR protocol followed by sequencing of generated amplicons at Eurofins (Munich, Germany). For 31 out of 33 animals, a partial sequence from the ORF1 region of the genome could be recovered which was used for phylogenetic analysis with MEGA 11 (Figure 2).

Table 1. Summary of positive animals, including ct values from liver, faeces, and muscle samples as well as subtype annotation of the HEV sequence.

No.	Sample ID	Species	Tissue (ct-Value)			Genotype	Subtype	Accession Number
			Liver	Faeces	Muscle			
1	MWP2019-9	wb	32.7	n.a.	neg	HEV-3	3i-like	ON240936
2	MWP2019-14	pig	21.3	n.a.	neg	HEV-3	3c	ON240935
3	MWP2019-22	wb	22.86	n.a.	neg	HEV-3	3i-like	ON240934
4	MWP2019-23	pig	26.52	n.a.	neg	HEV-3	3f	ON240933
5	MWP2019-24	pig	24.14	n.a.	neg	HEV-3	3f	ON240932
6	MWP2019-33	pig	32.36	n.a.	neg	no sequence	-	-
7	MWP2019-35	pig	19.23	n.a.	neg	HEV-3	3c	ON240931
8	MWP2019-53	pig	27	n.a.	neg	HEV-3	3f	ON240930
9	MWP2019-97	pig	26.2	n.a.	neg	HEV-3	3a	ON240929
10	MWP2019-100	pig	22.2	n.a.	neg	HEV-3	3a	ON240928
11	MWP2019-104	pig	33.1	n.a.	neg	HEV-3	3a	ON240927
12	MWP2019-113	pig	32.8	n.a.	neg	HEV-3	3f	ON240926
13	MWP2019-117	wb	28.1	n.a.	neg	HEV-3	3c	ON240925
14	MWP2019-170	pig	23.1	n.a.	20.7	HEV-3	3f	ON240924
15	MWP2019-190	pig	27.4	n.a.	neg	HEV-3	3c	ON240923
16	MWP2019-208	pig	23.5	n.a.	neg	HEV-3	3f	ON240922
17	MWP2019-209	pig	32.4	n.a.	neg	HEV-3	3f	ON240921
18	MWP2019-242	pig	30.4	29.19	neg	HEV-3	3f	ON240949
19	MWP2019-255	pig	31.34	38.20	neg	HEV-3	3k	ON240948
20	MWP2019-256	pig	34.62	34.19	neg	HEV-3	3k	ON240947
21	MWP2019-257	pig	36.65	40.71	neg	HEV-3	3k	ON240946
22	MWP2019-276	pig	21.26	20.26	neg	HEV-3	3e	ON240945
23	MWP2019-277	pig	n.a.	34.68	neg	no sequence	-	-
24	MWP2019-288	pig	22.86	24.56	neg	HEV-3	3e	ON240943
25	MWP2019-292	wb	23.95	27	neg	HEV-3	3i-like	ON240942
26	MWP2019-314	pig	22.8	22.69	neg	HEV-3	3c	ON240950
27	MWP2019-315	pig	22.1	22.39	neg	HEV-3	3i-like	ON240941
28	MWP2019-316	pig	23.29	21.19	neg	HEV-3	3c	ON240940
29	MWP2019-317	pig	20.18	22.1	neg	HEV-3	3c	ON240939
30	MWP2019-354	pig	23.56	24.19	neg	HEV-3	3a	ON240938
31	MWP2019-366	pig	28.05	n.a	neg	HEV-3	3k	ON240937
32	MWP2019-385	wb	n.a.	23.51	neg	HEV-3	3c	ON240944
33	MWP2019-386	wb	16.3	n.a.	neg	HEV-3	3c	ON240944

wb, wild boar; n.a., no sample available; neg, negative.

The examination revealed for each sequence an affiliation to HEV-3 genotype, but a high variety of HEV-3 subtypes. In total, six different subtypes were identified in pig and wild boar including subtypes 3a (n = 4 pigs/0 wild boar), 3c (n = 6 pigs/3 wild boars), 3e (n = 2 pigs/0 wild boar), 3f (n = 8 pigs/0 wild boar), and 3k (n = 4 pigs/0 wild boar).

One set of four (1 pig/3 wild boars) sequences (3i-like) cluster with wild boar strains (MF959764, KP294371) that have not been assigned to a subtype by Smith et al. [26], but were assigned to subtype 3i in a more detailed analysis of HEV-3 [39]. A detailed phylogenetic tree based on recovered partial sequences is deposited as Supplementary Figure S1. In general, when both liver and faecal samples were HEV RNA-positive, the ct value (as proxy for viral load) was approximately the same (n = 11). The two exceptions iare pig MWP2019-255 with ct values of 31.34 (liver) vs. 38.2 (faeces).and pig MWP2019-257 with ct values of 36.65 (liver) vs. 40.71 (faeces).

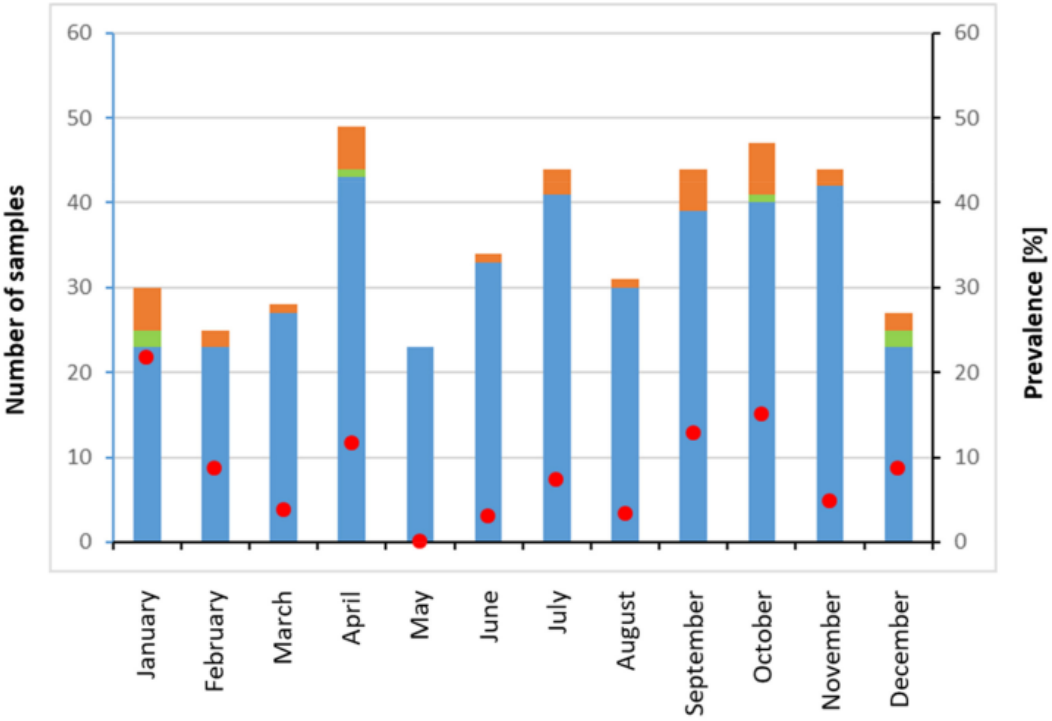

Figure 1. Number of pigs and wild boars collected per month (blue and green column, respectively) with the respective number of HEV RNA-positive liver (orange bar) and corresponding prevalence rates (red dots). Animals were sampled in 2019 in Mecklenburg-Western Pomerania, Germany.

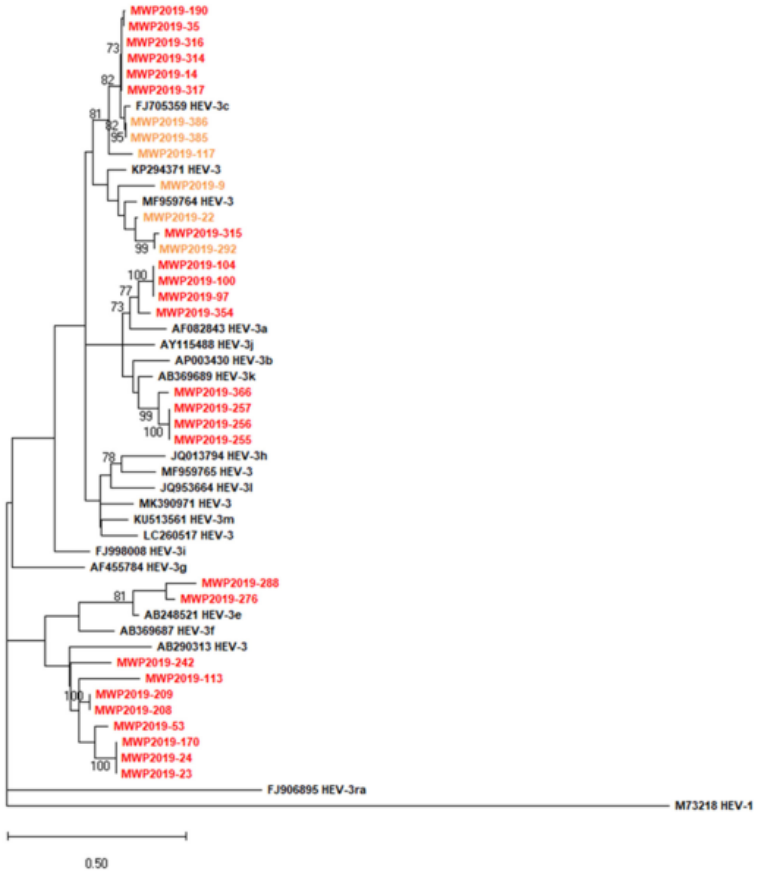

Figure 2. Phylogenetic relationship of HEV sequences from domestic pig and wild boar livers or faeces (MWP2019-385). The phylogenetic tree is based on the 250 nucleotide ORF1 region of HEV (nucleotide positions 127–376 of reference sequence FJ705359). The tree is drawn to scale, as the evolutionary distances used to derive the phylogenetic tree. The sequences retrieved from the NCBI GenBank are given with accession numbers. The HEV sequences obtained in this study from pigs are in red, sequences obtained from wild boar are in orange. Reconstruction of phylogenetic tree using Maximum Likelihood method with 500 bootstrap iterations. Bootstrap values >70 are annotated.

4. Discussion

In total, 393 animals were investigated for HEV RNA, yielding a prevalence of 8.5% in domestic pigs and 7.9% in wild boars. The results confirmed the circulation of HEV-3 in pigs and wild boar in the federal state of Mecklenburg-Western Pomerania located in north-east Germany on the Baltic Sea coast. The results are in line with previous findings in the same region from 1996/1997 and 2005/2006 where viral RNA was detected in wild boar blood samples at prevalences of approximately 3.4% and 5.2%, and in addition, in wild boar livers from the Greifswald region with a prevalence of 10.4%, respectively [17].

Several studies in Germany assigned domestic pigs and wild boars as main HEV reservoirs, which is reflected by high seroprevalence rates in farmed pigs, ranging from 42.7% and 49.8%, up to 100% [40–42] and 33%, up to 41% in wild boars [43,44]. High rates in pigs were also found throughout Europe with seroprevalences from 20.4% in Spain [45],

45.1% in Italy [46], 60% in France [47], 70% in The Netherlands [48], and 92.8% in the United Kingdom [49]. These high prevalences indicate the significant risk for developing HEV infection after consumption of pork. This route of transmission has been directly confirmed in case and outbreak investigations, in which the same virus strain was detected both in the patient and in the consumed food. In France, an outbreak of hepatitis E was described in seven people infected by eating traditional sausage ("Figatellu") containing raw pork portions [50]. A similar case has also been reported in Switzerland, which occurred after consumption of a different type of sausage containing raw pork liver [51]. Another report also confirmed the presence of infectious virus in pork liver sausage from southern France [52]. For Germany, several studies have been conducted that identified the consumption of offal and wild boar meat [5], ready-to-eat pork products [53] and sausages [54] as the highest risk factor for hepatitis E disease. Especially liver and liver sausages are main infectious food sources, which is reflected by high viral RNA prevalences ranging from 5.2% in blood [17], over 18% in liver up to 56.3% in bile samples of wild boars [55] as well as 13.5% in pig derived liver samples [56] and 22.0% in liver sausages from Germany [57]. Similar observations have been made throughout Europe [13].

HEV strains from 31 out of 33 HEV positive animals could be genotyped and exhibited a great variety of HEV-3 subtypes including 3a, 3c, 3e, 3f, and 3k. Most of the subtypes (3a, 3c, 3e, 3f) have been regularly found in pigs from Germany and were also detected in humans in Germany [17], which indicates that pigs are a probable source of human infections. Regarding subtype 3k, there is only one notification in Europe from pigs from Slovenia [17] and otherwise reports from human strains in Japan [58]. Additionally, sequences of four samples, designated as HEV-3i-like, cluster with MF959764 and KP294371, sequences which were not assigned to a subtype proposed by Smith et al. [26]. However, a more recent analysis groups both sequences with subtype HEV-3i [39], Notably, KP294371 was detected in a wild boar in Mecklenburg-Western Pomerania in 2010 [59,60]. In each case, the faecal samples that accompanied the positive liver samples were also positive and recovered sequences were in virtually all cases highly similar or identical (Figure S1). For further conclusions on the transmission and circulation of HEV strains between humans, pigs, and wild boars in Germany, a higher number of German porcine-derived HEV sequences would be supportive.

The detection of HEV RNA with high load in a muscle tissue from one pig underlines the public health risks associated with HEV as a foodborne pathogen. The analysed muscle derives from lateral femoral musculature, which is often supplied as high-quality meat, e.g., steaks. So far, HEV RNA presence in muscle of naturally infected pig was documented only in few studies: In one study from Spain, a HEV-positive diaphragm in 1 out of 225 slaughterhouse pigs was detected [61] and a second surveillance documented 1 and 2 positive lingual muscle samples collected in slaughterhouses from Czech Republic and Italy, respectively [62]. A second report from Italy detected 8 positive diaphragm muscles from 585 slaughtered pigs [63]. A positive diaphragm muscle (1/45) was also detected in slaughtered pigs from Spain [64]. No viral RNA could be observed in muscle tissue from slaughterhouse samples in Canada (Leblanc et al. [65]; number of samples: $n = 43$), France ([47], $n = 1134$) and Denmark ([66]; $n = 10$). In contrast, multiple findings were notified in muscle tissue samples of German wild boar and deer [67] and wild boar muscle tissues from Italy [68]. However, in such studies, attention must be given to the risk of cross-contamination of carcass surfaces during the dissection as notified by others [69,70]. In our study, however, the muscle sample had a very low ct value compared to the liver (ct value 20.7 vs. 23.1) and was prepared during dissection by an experienced pathologist for subsequent examination, which minimised the risk of contamination. The detected HEV strain in pig muscle belongs to subtype 3f which needs further attention because this subtype has been assigned to acute human HEV cases in France [71] and higher risk of hospitalisation in Belgium [72] as well as was involved in a hepatitis E outbreak in Italy 2019 [73].

In conclusion, this study highlights the need for the implementation of control measures including continuous surveillance and monitoring of HEV in domestic pigs and wild boars throughout Germany. Within the framework of a One Health concept, further epidemiology studies should consider the inclusion of human specimen and elucidate the interplay of HEV subtypes on acute hepatitis, hospitalisation, and chronic courses of HEV infections.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11070773/s1, Figure S1: Molecular phylogenetic analysis of 139 250-nucleotide-long partial ORF1 HEV-3 sequences corresponding to the region 127–376 of reference sequence FJ705359. Table S1: Summary of the data for all sampled domestic pig and wild boar.

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(II) A Putative Novel Hepatitis E Virus Genotype 3 Subtype Identified in Rabbit, Germany 2016

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A Putative Novel Hepatitis E Virus Genotype 3 Subtype Identified in Rabbit, Germany 2016

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Abstract: Hepatitis E is an emerging viral disease that is the leading cause of viral hepatitis in the world. The vast majority of hepatitis E cases in developed countries are caused by zoonotic genotypes 3 and 4 of hepatitis E virus (HEV) for which pig and wild boar and to lesser extent rabbits are the main reservoir. According to recent reports rabbits are a source of human HEV infection and highlight the risk of zoonotic foodborne transmission. Here we report the molecular analysis of a novel HEV strain identified in a rabbit during a countrywide surveillance of rabbits and hares in Germany, 2016. The analysis of the complete genome reveals characteristics of a putative novel recombinant subtype of the species *Orthohepevirus A* within the clade of genotype 3 but not closely related to any known subtypes. Importantly, the genome of this strain possesses a nucleotide exchange in the overlapping region of open reading frames ORF2 /ORF3 interfering with a broadly applied diagnostic real-time RT-PCR. In conclusion, a new type of HEV strain was identified in a German rabbit with atypical and novel sequence characteristics.

Keywords: hepatitis E virus; novel genotype; rabbit

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1. Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E, leading to waterborne epidemics in resource-poor countries and sporadic cases in industrialized countries [1]. HEV belongs to the family *Hepeviridae*, genus *Orthohepevirus*. HEV is a small virus with an RNA genome of positive polarity. The genome of approximately 7500 nucleotides contains three open reading frames (ORF), untranslated regions (UTR) at the 5′ and 3′ ends, and a polyA tract at the 3′ end. ORF1 encodes a nonstructural polyprotein, ORF2 encodes the capsid protein, and ORF3 encodes a small accessory protein, which acts as viroporin [2]. HEV particles were described as naked, non-enveloped virions that are shed into feces as well as membrane-associated quasi-enveloped forms that circulate in blood [3]. Typically, the course of disease is self-limiting and subclinical [4]. Due to the large number of infections, however, HEV remains a constant public health threat with an estimated 20 million cases of hepatitis E, including 3.4 million symptomatic cases and 70,000 deaths per year [5].

Hepeviruses are grouped within four *Orthohepevirus* species that were detected in birds (species *Orthohepevirus B*), rodents and carnivores (species *Orthohepevirus C*), bats (species *Orthohepevirus D*), and genus *Piscihepevirus* with a fish-associated strain [6]. The species *Orthohepevirus A* is further divided into eight major genotypes and subsequent subgenotypes [7] and is mainly found in humans, domestic pigs, wild boar, deer, and rabbits.

Genotypes 1 and 2 (HEV-1 and HEV-2) occur in Africa and Asia, are exclusively associated with humans, and are transmitted via the fecal–oral route mainly through consumption of contaminated water [8]. In contrast, autochthonous hepatitis E in developed countries is mainly caused by the zoonotic genotypes 3 (HEV-3) and 4 (HEV-4) [1]. HEV-3 strains typically trigger mild disease with rare cases of fulminant hepatitis. However, in immunocompromised individuals or patients with pre-existing conditions the infection can lead to prolonged or chronic forms and final liver failure [4]. Recent estimates for Europe show prevalences between 4.6% and 29.5% depending on the country surveyed (as reviewed by [9]). Pigs and wild boar are the main reservoirs for zoonotic HEV and transmission is generally assumed to occur by ingestion of food products from infected animals [10]. Rabbit HEV was first detected in farmed rex rabbits in China in 2009 [11], and subsequently in farmed and wild rabbits from several other countries, including USA, Germany, the Netherlands, and France [12–15]. It forms a distinct phylogenetic group within genotype HEV-3, designated HEV-3ra [7] and harbors a characteristic 90/93 nucleotide (nt) insertion within the coding region of ORF1 [14]. There is evidence for zoonotic potential due to reported rabbit HEV infections in humans, most likely affecting immunocompromised patients. Interestingly, direct contact with rabbits does not appear to be necessary for infection with rabbit HEV [16].

During surveillance of hare and rabbits throughout Germany in 2016, several rabbit-derived HEV sequences were recovered [12]. A partial HEV sequence of one individual rabbit indicated the presence of an atypical strain (rab81) outside the rabbit-associated subgenotype HEV-3ra. In this study, we determined the full-length genome of this strain followed by a comprehensive phylogenetic analysis and demonstrated that it represents a unique novel strain within the genotype HEV-3.

2. Materials and Methods

2.1. RNA Isolation

Frozen liver samples were thawed, and subsequently a small fragment (approximately 30 mg) was transferred to a 1.5 mL tube. Initially, the samples were homogenized in RLT buffer using a TissueLyser II (Qiagen, Hilden, Germany) and RNA extraction was performed with the Qiagen RNeasy Kit according to the manufacturer's instructions. Alternatively, the samples were homogenized in 750 µL of TriZol LS reagent followed by addition of chloroform and RNA extraction from the aqueous phase. The aqueous phase was mixed with an equal volume of 75% ethanol and loaded on a RNeasy column. Subsequent washing and elution were done according to the RNeasy Kit protocol.

2.2. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Initially, we used the SuperScript III One-Step RT-PCR Kit (ThermoFisher, Hennigsdorf, Germany) for reverse transcription (RT) with target-specific oligonucleotides and PCR according to the manufacturer. Alternatively, the SuperScript IV First-Strand Synthesis Kit (ThermoFisher) was used for RT with random hexamers followed by polymerase chain reaction (PCR) using Phusion DNA polymerase (ThermoFisher) with HEV-specific primers. PCR products were purified using the Qiagen PCR Purification Kit or were extracted after agarose gel electrophoresis using the Qiagen Gel Extraction Kit and subsequently sequenced using the dideoxy-chain-termination method (Sanger sequencing) by Eurofins Genomics (Ebersberg, Germany). Used primers are listed in Table S1.

2.3. Quantitative Real-Time RT-PCR (RT-qPCR)

The diagnostic RT-qPCR was carried out using a standard protocol with the QuantiTect Probe RT-PCR Kit (Qiagen) and a primer/probe concentration of 0.8 µM and 0.1 µM, respectively [17]. Primer and probe sequences are depicted in Table S1. The RT was carried out at 50 °C for 30 min. After denaturation/activation step at 95 °C for 15 min, DNA was amplified with 45 cycles at 95 °C (10 s), 55 °C (25 s), and 72 °C (25 s). The assay was

performed using the CFX96 Real-Time PCR Detection system according to established protocols [17].

2.4. Rapid Amplification of cDNA-Ends (RACE) with PCR

In order to amplify the 3′ and 5′ ends of the viral genome, we slightly modified the 3′- and 5′-RACE Systems (ThermoFisher). The modifications were as follows: SuperScript II reverse transcriptase from the kit was substituted with SuperScript IV reverse transcriptase. PCR amplification was performed using Phusion DNA polymerase. Inosine-containing primers from the kits were replaced by appropriate, inosine-free oligonucleotides. For 5′-RACE, TdT-tailing was first performed using dCTP according to the manufacturer's instructions. Additionally, a separate tailing reaction was done using dATP. Wherever necessary, DNA was purified using the Qiagen PCR Purification Kit for downstream applications.

2.5. Phylogenetic and Sequence Analysis

The novel genome was compared to the updated reference sequences proposed by Smith et al. [7]. Additionally, due to structural similarities, putative HEV-3/HEV-3ra recombinant MG783571 was included in the analysis. For phylogenetic and recombination analyses, the hypervariable region and the rabbit-specific insertion were excluded (between nucleotide positions 2145–2380 and 2834–2835 of MT920909, respectively).

The sequences were aligned using the ClustalW or MUSCLE packages in Geneious Prime v. 2021.0.1 (Bioinformatics Software for Sequence Data Analysis; Biomatters Ltd.; Auckland, New Zealand, 2020). Phylogenetic analysis was conducted in MEGA X (Molecular Evolutionary Genetics Analysis across computing platforms; [18]) using the maximum-likelihood (ML) method [19] with the general time-reversible (GTR) model with gamma distributed rate variation among sites (G) and invariable sites (I) for nucleotide and the Jones–Thornton–Taylor (JTT) model for amino acid sequences with default parameters and 500 bootstrap replicates. Phylogenetic analysis was carried out with complete genome sequences as well as partial sequences as indicated. In the case of amino acid sequences, the maximum-likelihood method and JTT matrix-based model [20] were used. Bootscan recombination analysis [21] was conducted using SimPlot v. 3.5.1 (Stuart C. Ray; Baltimore, MD, USA, 2003).

3. Results

3.1. Full Genome Sequencing and Determination of Genome Organization

Seven overlapping fragments were generated from RNA of an HEV positive rabbit (rabbit 081), using a primer set encompassing the whole virus genome (Table S1). These fragments were sequenced and assembled to a complete genome of 7214 nucleotides followed by a poly-A tail (accession number: MT920909). Sequence analysis and alignment with reference strains demonstrated that the genome exhibits the typical features of the *Orthohepevirus A* species group with three open reading frames (ORF1-ORF3), encoding a nonstructural polyprotein (ORF1, nucleotide positions 28–5130), capsid protein (ORF2, nucleotide positions 5165–7150), and the ORF3 protein (nucleotide positions 5154–5498) (Figure 1).

The 5′ UTR consists of 27 nucleotides, but it should be noted that the first three nucleotides (5′-UGG- . . . 3′) might be generated due to terminal transferase activity of the reverse transcriptase in the 5′-RACE reaction [22]. The 3′ UTR consists of 64 nucleotides followed by a poly-A tail.

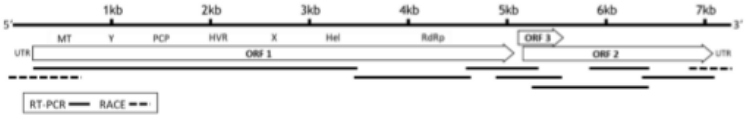

Figure 1. Genome organization and schematic representation of the amplicons used for dideoxy-chain termination (Sanger) sequencing of the complete HEV genome. The genome was determined by sequencing of seven overlapping fragments (solid line), generated by RT-PCR and rapid amplification of cDNA ends (RACE, dotted line). The open reading frame 1 (ORF1) codes for methyltransferase (MT), Y-domain (Y), papain-like cysteine protease (PCP), hypervariable region (HVR), X-domain, RNA helicase (Hel), RNA-dependent RNA polymerase (RdRp), ORF2 codes for capsid protein, and ORF3 for a small accessory protein. Untranslated regions (UTR) are located at the 5′ and 3′ends of the genome.

3.2. Sequence and Phylogenetic Analysis

Phylogenetic analysis of the novel genome together with the genotype HEV-3 reference sequences according to Smith et al. [7] revealed rab81 at the basal position of the majority of HEV-3 subtypes separate from all original rabbit HEV sequences (HEV-3ra) (Figure 2). Strain MG783571, a putative genotype HEV-3/HEV-3ra recombinant detected in a human plasma sample from France clustered among other HEV-3 subtype genomes.

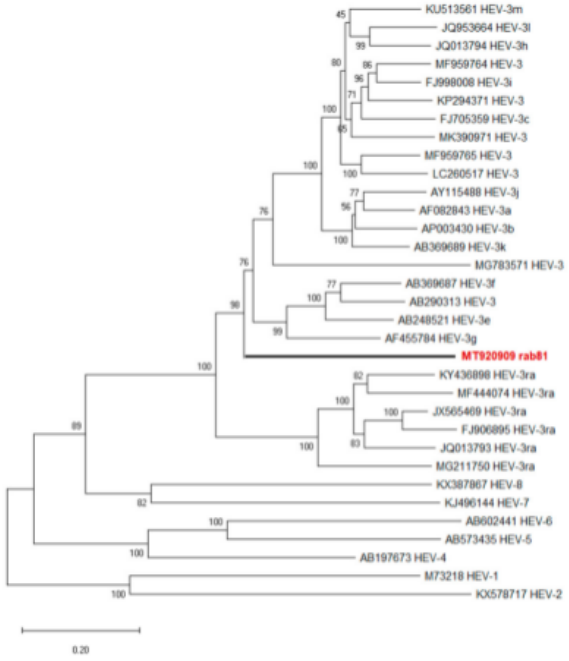

Figure 2. Phylogenetic reconstruction based on complete HEV genomes. Red boldface indicates the novel rabbit HEV strain rab81. The tree represents a maximum-likelihood phylogeny based on the GTR+G+I model with support values at nodes derived from 500 bootstrap repetitions.

Further phylogenetic analysis of the amino acid sequences showed ORF1-coded polyprotein of rab81 and MG783571 at the basal position of the non-HEV3a HEV-3 subtypes (Figure 3a), while the ORF2-encoded capsid protein sequences of rab81 and MG783571

clustered with all HEV-3ra sequences, but provided very little resolution within this clade (Figure 3b).

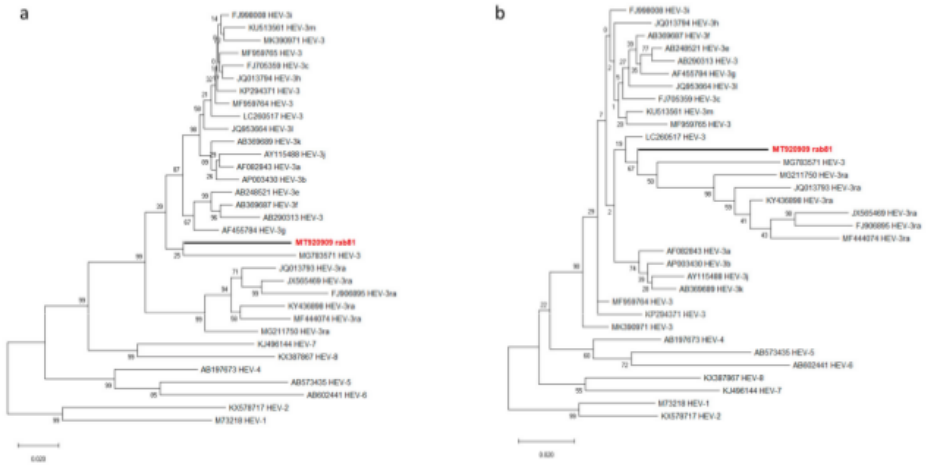

Figure 3. Phylogenetic trees based on amino acid sequences of ORF1-encoded polyprotein ORF1 (**a**) and ORF2-encoded capsid protein (**b**) of HEV. Red boldface indicates the novel rabbit HEV strain. The phylogenies were reconstructed using maximum-likelihood and the Jones–Thornton–Taylor matrix-based model with node support values given from 500 bootstrap replicates.

The novel genome rab81 showed overall a nucleotide sequence identity of 78.6–80% with HEV-3 subtypes, but only 75% with the reference strain of the HEV-3ra clade (Table 1). The lower similarity with HEV-3ra was consistent for ORF1, ORF2, and ORF3 at the level of the nucleotide sequences as well as on the amino acid sequences of the encoded proteins (Table 1). The HEV recombinant strain MG783571 exhibited amino acid sequence identity of 87.8% (ORF1), 91.8% (ORF2), and 88.6% (ORF3) to the novel rab81 strain (Table 1).

Table 1. Nucleotide (nt) and amino acid (aa) sequence identities (in %) between novel strain rab81 (MT920909), reference HEV strains within genotype HEV-3, and the putative recombinant strain MG783571.

HEV-3 Subtype	Accession Number	Complete Genome	ORF1		ORF2		ORF3	
		nt	nt	aa	nt	aa	nt	aa
HEV-3a	AF082843	78.6	77.4	89.8	81.6	93.0	90.4	87.7
HEV-3b	AP003430	78.6	77.5	90.3	81.6	92.9	90.6	89.5
HEV-3c	FJ705359	78.7	77.6	90.0	81.6	93.2	88.9	84.2
HEV-3e	AB248521	79.3	78.1	90.2	82.1	93.5	88.9	86.8
HEV-3f	AB369687	79.8	78.2	90.2	82.3	93.5	90.1	89.5
HEV-3g	AF455784	80.0	79.2	90.7	82.3	92.9	89.2	88.6
HEV-3h	JQ013794	78.6	77.3	90.4	81.9	93.2	91.5	89.5
HEV-3i	FJ998008	79.0	77.9	89.8	81.9	93.6	90.4	90.4
HEV-3j	AY115488	78.8	77.4	89.2	82.1	92.9	90.1	88.6
HEV-3k	AB369689	79.8	78.9	90.7	81.9	92.9	90.7	90.4
HEV-3l	JQ953664	79.2	77.9	90.2	82.0	93.2	90.4	90.4
HEV-3m	KU513561	78.8	77.3	89.4	82.1	94.3	91.2	88.6
HEV-3ra	FJ906895	75.3	73.4	84.8	79.8	89.7	83.8	80.7
HEV-3	AB290313	79.4	78.1	89.3	82.6	92.7	88.1	86.8
HEV-3	KP294371	79.4	78.2	89.6	82.4	92.4	87.8	86.8
HEV-3	LC260517	79.0	77.6	89.9	82.2	94.0	89.7	83.4
HEV-3	MF959764	79.1	78.1	89.9	81.7	93.5	89.3	86.0
HEV-3	MF959765	79.2	78.0	89.8	82.2	92.7	89.6	87.7
HEV-3	MK390971	79.1	77.7	89.8	82.6	92.6	89.3	86.8
HEV-3	MG783571	77.8	76.2	87.8	81.2	91.8	91.2	88.6

To determine whether the novel sequence showed evidence of recombination events in its evolutionary history, the HEV-rab81 genome was compared to the reference genomes of all defined HEV-3 subtypes [7] and the potentially recombinant strain MG783571 using Bootscan in SimPlot [21]. The similarity plot showed overall low nucleotide sequence similarity between rab81 and the HEV-3 subgenotype reference sequences along the entire genomes (Figure 4a).

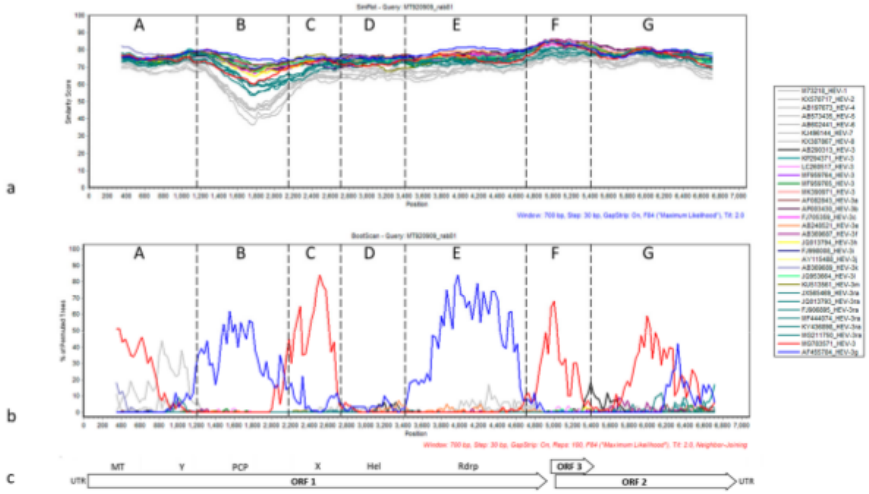

Figure 4. SimPlot analysis of rab81 (MT920909) with similarity plot (**a**) and the result of bootscan analysis (**b**) and a schematic representation of the HEV genome organization as reference (**c**). Note that the hypervariable region was excluded for this analysis. Gray lines refer to HEV genotypes HEV-1, HEV-2, and HEV-4–HEV-8. Teal lines indicate HEV-3ra sequences, blue subgenotype HEV-3g, and red recombinant strain MG783571. The remaining colors were assigned to distinct HEV-3 subgenotypes. The sections A–G were designated based on the peaks of the bootscan plot. See Figure S1 for detailed phylogenetic analyses of the sections.

The sequence of rab81 exhibits overall a higher degree of similarity with HEV-3 subtypes than with the remaining genotypes. This is particularly visible between nucleotide positions 1500–2000 (section B) in the similarity plot. This part of the genome contains the relatively diverse protease encoding region and is adjacent to the hypervariable region, which was excluded from the analysis due to a high number of insertions/deletions (indels). In fact, this region shows the highest similarity for non-rabbit HEV-3 subtypes, followed by HEV-3ra, and finally by the remaining HEV genotypes. A similar pattern can be observed in the highly conserved ORF2/ORF3 overlap region at about nucleotide position 5000 (section F), where the similarity to non-rabbit HEV-3 subtypes is higher compared to all other sequences. Bootscan analysis indicated a mosaic-like genome composition with six putative recombination breakpoints and alternating sections of rab81 clustering with HEV-3g (AF455784, blue line) and recombinant HEV-3 (MG783571, red line) sequences (Figure 4b). These sections are roughly equivalent to the conserved domains within HEV ORF1-encoded protein described by Koonin et al. [23]. Phylogenetic reconstructions based on the sequence sections between putative recombination breakpoints supported the closer local similarity of rab81 with the mentioned sequences although node support was generally low (Figure S1a–g).

The sequence of rab81 exhibited additional notable properties that differ from the typical pattern in HEV-3ra strains: First, the absence of the 90 nt insertion within the X-region of ORF1 (nucleotide positions 2776–2932), which is characteristic for other rabbit-derived HEV sequences (Figure 5a). This insertion is also absent in recombinant strain MG783571 and

otherwise only absent in non-rabbit HEV-3 strains. In addition, the hypervariable region (HVR) which is flanked by conserved N-terminal TSGFSS and C-terminal RRLL amino acid sequences (corresponding to nucleotide positions 2155–2388), contains a unique 82 amino acid stretch which does not match with any of the HEV-3ra HVR domains (Figure 5b). Finally, the capsid protein-encoding ORF2 sequence contains within the ORF2/ORF3 overlapping regions a unique proline codon insertion (nucleotide positions 5412–5414), which is also found in strain MG783571 (Figure 5c).

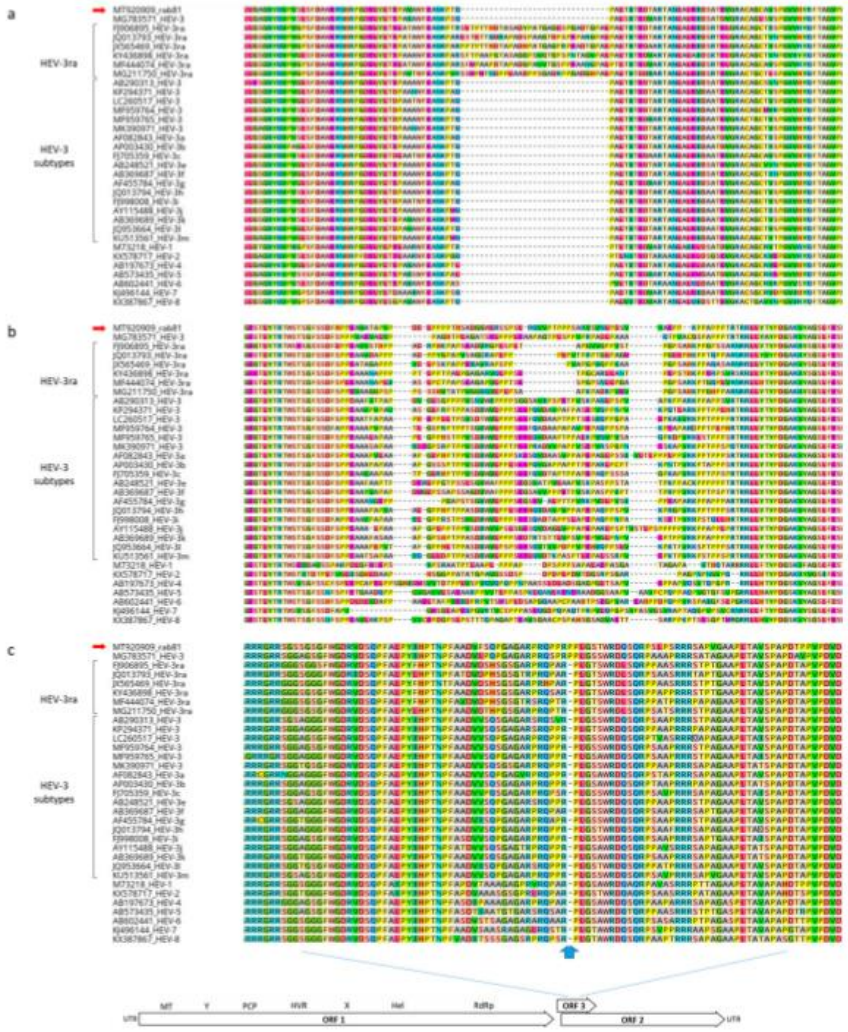

Figure 5. Amino acid sequence alignments of X-region (**a**) and hypervariable region of ORF1-encoded polyprotein (**b**), and capsid protein encoded by ORF2 within the ORF2/ORF3 overlap region (**c**). The X-region of ORF1 refers to nucleotide positions 2776–2932 of MT920909 (strain rab81). The hypervariable region is flanked by conserved TRTWS and RRLL amino acid sequences (refers to nucleotide positions 2146–2379 of MT920909, strain rab81). The position of the proline insertion within the ORF2-encoded capsid protein amino acid sequence refers to nucleotide positions 5408–5410.

Finally, a unique nucleotide exchange (G/A, position 5311) is found in a highly conserved ORF2/ORF3 overlapping region, which is the target region for widely used real-time RT-PCR [17,24]. Modification of the original probe sequence (5′-TGATTCTCAGCC CTT CGC-3′, [17]) to the rab81 adapted probe sequence (5′-TGATTCTC AACCCTTCGC-3′) leads to lowered threshold cycle (ct) values (on average 3 ct values) and thus substantially increased sensitivity as demonstrated for a plasmid template encoding the rab81 structure protein region (Figure S2, Table S1).

4. Discussion

We determined the complete genome sequence of an HEV strain detected in the liver of a rabbit [12]. Initial phylogenetic analysis based on a short partial sequence of the RdRp coding region assigned the strain to subtype HEV-3g [12]. However, analysis of the entire genome sequence demonstrated a generally low sequence similarity to previously described HEV-3 subtypes. In particular, it does not cluster within the HEV-3ra clade, but at the base of a phylogenetic branch with the remaining HEV-3 subtypes and constitutes a putative new subtype or recombinant strain.

The novel rab81 strain displays characteristics that are similar to that of a recently described genotype HEV-3 recombinant strain (MG783571), which was detected in a human plasma sample from France [25]. This strain also clusters phylogenetically outside HEV-3ra clade and at the base or among the remaining HEV-3 subtypes. The nucleotide sequence identity of the complete genomes of both strains is about 77.8%. Both strains harbor a unique proline codon insertion within ORF2 in the ORF2/ORF3 overlapping region, both lack the HEV-3ra typical 90 nucleotide insertion and exhibit a unique insertion within the HVR region. Further, MG783571 is proposed to carry a mosaic genome derived from rabbit HEV-3ra and other HEV-3 subgenotype sequences [25], and different parts of the genome showed different affinities in our section-wise phylogenies (Figure S1). Similarly, in our analysis, the comparison of the rab81 sequence with the HEV-3 subgenotype reference sequences revealed several possible recombination events. However, the bootstrap support values in these analyses are too low to decide conclusively whether the rab81 strain is a recombinant. Therefore, we conclude that rab81 may represent a novel subgenotype. Since the rab81 sequence contains a single nucleotide exchange in the probe binding region of commonly used RT-qPCR assays [17,24], a probe sequence should be adapted or alternatively broad-spectrum RT-PCR protocols should be applied [17,26].

Finally, these findings further stress the role of rabbits as sources for novel zoonotic HEV strains. The transmissibility of rabbit HEV strains has been already confirmed experimentally, where HEV strains isolated from rabbits were successfully inoculated to pigs [27] as well as cynomolgus macaques [28] with subsequent productive replication. Furthermore, in France and Switzerland, immunosuppressed patients who suffered from persistent infections were infected by HEV-3ra strains [14,16,29]. Since none of the patients was exposed directly to rabbits, the presumed origin of the infection seemed to be a foodborne infection probably due to consumption of meat and liver of infected animals or derived products. However, the detection of HEV-3ra infected rabbits in urban regions also shows that there is potential for indirect transmissions [30].

5. Conclusions

In summary the results, obtained from phylogenetic, SimPlot, bootscan, and amino acid sequence analyses revealed a putative novel rabbit-derived HEV-3 subgenotype. Further studies will have to evaluate the virulence and infectivity of the novel strain by using cell culture systems and inoculation in animal models.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v13061065/s1, Table S1: Primers used for HEV RNA quantification, genotyping, and genome sequencing, Figure S1: Molecular phylogenetic analysis of seven partial HEV sequences (a–g) as determined by SimPlot analysis, Figure S2: Nucleotide sequence alignment of quantitative real-time RT-PCR (RT-qPCR) probe target region (a), RT-qPCR runs (triplicate) at three dilution steps (b), and mean ct values yielded by original probe or adapted probe (c).

Author Contributions: Conceptualization, M.E. and M.H.G; methodology, F.C., F.v.A. and M.E.; software, F.C.; validation, M.E., G.H. and R.G.U.; investigation, F.C. and F.v.A.; resources, F.v.A.; data curation, F.C. and M.E.; writing—original draft preparation, F.C., R.G.U. and M.E.; writing—review and editing, F.C., G.H., R.G.U., M.H.G. and M.E.; visualization, F.C.; supervision, M.E., G.H., R.G.U. and M.H.G.; project administration, M.E.; funding acquisition, M.H.G. All authors have read and agreed to the published version of the manuscript.

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Table S1 : Primers used for HEV RNA quantification, genotyping, and genome sequencing

Name	Sequence	nucleotide position*	use	Reference
JVHEVR	AGGGGTTGGTTGGATGAA	5348 - 5331	qPCR	Jothikumar et al., 2006
JVHEVF	GGTGGTTTCTGGGGTGAC	5279 - 5926	qPCR	Jothikumar et al., 2006
JVHEV.P81	TGATTCTCAACCCTTCGC	5302 - 5319	qPCR	this work
JVHEV.P	TGATTCTCAGCCCTTCGC	5302 - 5319	qPCR	Jothikumar et al., 2006
HEV.R	GCGAAGGGGTTGGTTGGATG	5325 - 5333	RT-PCR/Sequencing	Vina-Rodriguez et al., 2015
HEV.RdRp_F2b	GTGCTCTGTTTGGCCCNTGGTTYMG	4280 - 4304	RT-PCR/Sequencing	Hammerschmidt et al., 2017
R5674	TGAGTGTTGGTGCCGTCCTG	5685 - 5666	Sequencing	this work
R5625	CATACAAGACAAGATTAGTGCC	5636 - 5615	RT-PCR/Sequencing	this work
F5086	GGACCTTACAAATTCTATTATACAG	5094 - 5118	Sequencing	this work
F5048	GGCAAGGCTCACTTTACCGA	5056 - 5075	RT-PCR/Sequencing	this work
AAP mod	GGCCACGCGTCGACTAGTACGGGGGGGGGGGGGGG	RACE adapter	Sequencing	ThermoFisher (adapted)
AP	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT	RACE adapter	Sequencing	ThermoFisher
AUAP	GGCCACGCGTCGACTAGTAC	RACE adapter	Sequencing	ThermoFisher
R6591	GAAAATGGTCTGATCGTACTC	6622 - 6602	RT-PCR/Sequencing	this work
R6578	GAGAGCCACAACACATCATT	6588 - 6569	Sequencing	this work
R5926	CCTGGTTACGATAATGGAGG	5936 - 5917	Sequencing	this work
R2429	CACAATCCGACTCGAATAAGG	2449 - 2429	Sequencing	this work
K081.RACE5'700	GACAACTACGCGACTTCCATC	684 - 664	Sequencing	this work
K081.RACE5'400	CGCGAGTTGGGGCAGAGTAC	400 - 381	Sequencing	this work
K081.RACE3'300	GGCCCTGTGTCCATTTCTGC	6938 - 6957	Sequencing	this work
F5906	CCTCCATTATCGTAACCAGG	5917 - 5936	RT-PCR/Sequencing	this work
F1610	CTTGAGGCCCTTTATAGTGC	1609 - 1628	Sequencing	this work
R6506	GTCCTGTTCATGTTGATTGTCG	6517 - 6496	RT-PCR/Sequencing	this work
R3157	CAGCAAATGCGGTGGTAATGAC	3165 - 3144	Sequencing	this work
F6485	CGACAATCAACATGAACAGGAC	6496 - 6517	RT-PCR/Sequencing	this work
F5305	CCTTCGCCCTCCCCTATATTC	5313 - 5333	RT-PCR/Sequencing	this work
F960	CATTCCACGCAGTCCCAGTTC	959 - 979	Sequencing	this work
R1042	CGAGCAACAAAAGGCCTGATC	1041 - 1021	Sequencing	this work
R725	CATCATGGTTATACCCTGCACTAG	724 - 701	Sequencing	this work
F2991	CTATCCAGCAGGGGGATGTTG	2999 - 3019	RT-PCR/Sequencing	this work
F705	GTGCAGGGTATAACCATGATG	704 - 724	Sequencing	this work
F32	GAGGCCCATCAGTTTATTAAGG	31 - 52	RT-PCR/Sequencing	this work
terR	CTCCCGGGTYTTRCCTACCYTC	7144 - 7123	RT-PCR/Sequencing	this work
4R	CACATAAAATGTTTTAGAATGC	6781 - 6760	Sequencing	this work
3R	CATAAGACCATTGAATCATCTC	4718 - 4697	RT-PCR/Sequencing	this work
3F	GAGATGATTCAATGGTCTTATG	4697 - 4718	Sequencing	this work
2R	GCCGTAGCAATAATAGTAGTC	3518 - 3498	RT-PCR/Sequencing	this work
2F	GACTACTATTATTGCTACGGC	3498 -3518	Sequencing	this work

***numbering according to MT920909**

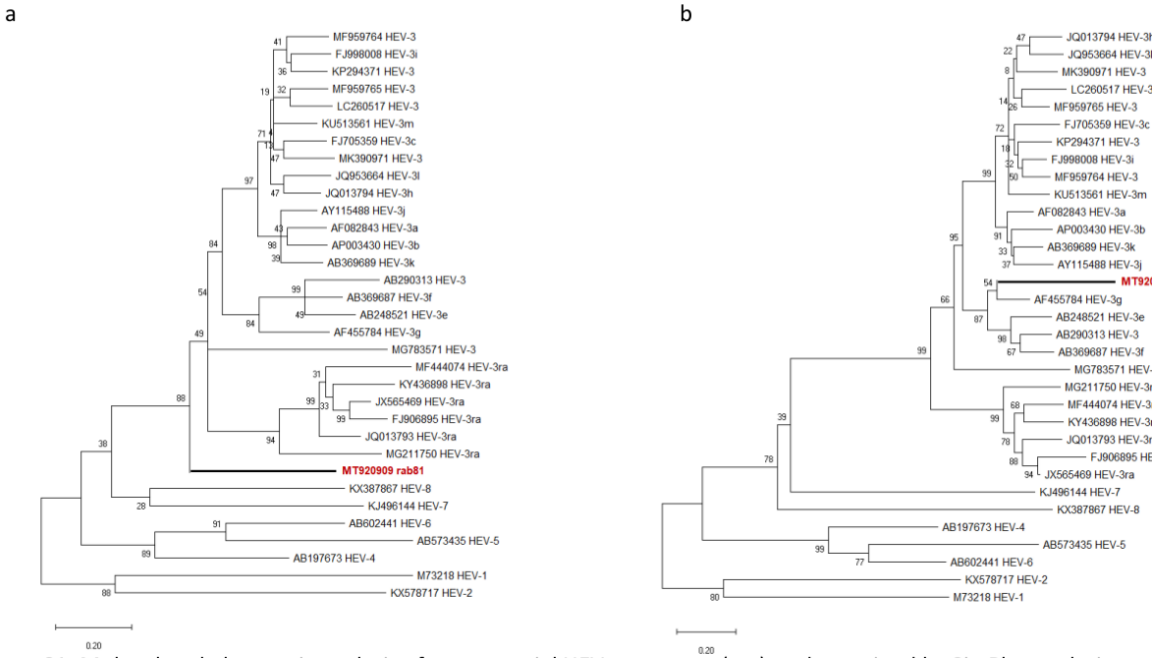
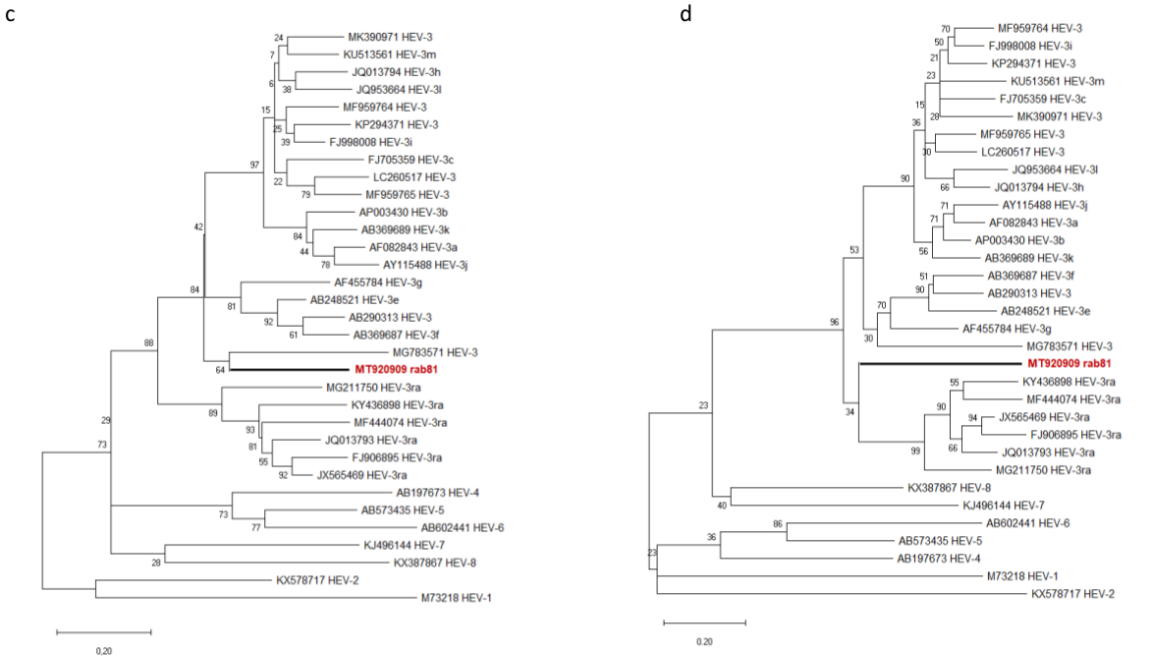
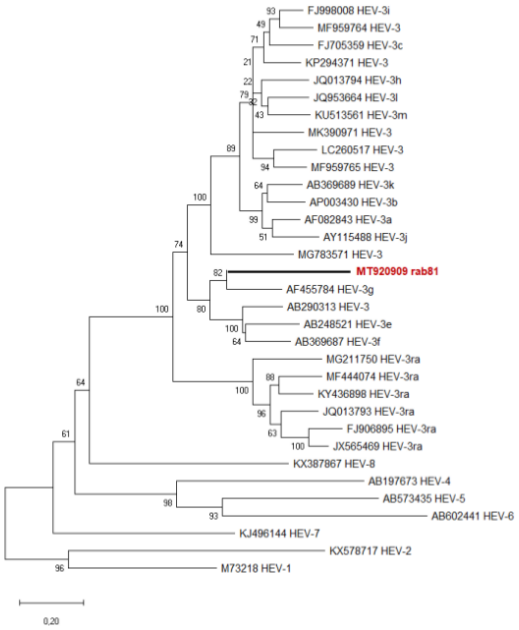


Figure S1: Molecular phylogenetic analysis of seven partial HEV sequences (a-g) as determined by SimPlot analysis



S1

e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FJ705359 HEV-3c
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KU513561 HEV-3m
MK390971 HEV-3
LC260517 HEV-3
MF959765 HEV-3
AB369689 HEV-3k
AP003430 HEV-3b
AF082843 HEV-3a
AY115488 HEV-3j
MG783571 HEV-3
MT920909 rab81
AF455784 HEV-3g
AB290313 HEV-3
AB248521 HEV-3e
AB369687 HEV-3f
MG211750 HEV-3ra
MF444074 HEV-3ra
KY436898 HEV-3ra
JQ013793 HEV-3ra
FJ906895 HEV-3ra
JX565469 HEV-3ra
KX387867 HEV-8
AB197673 HEV-4
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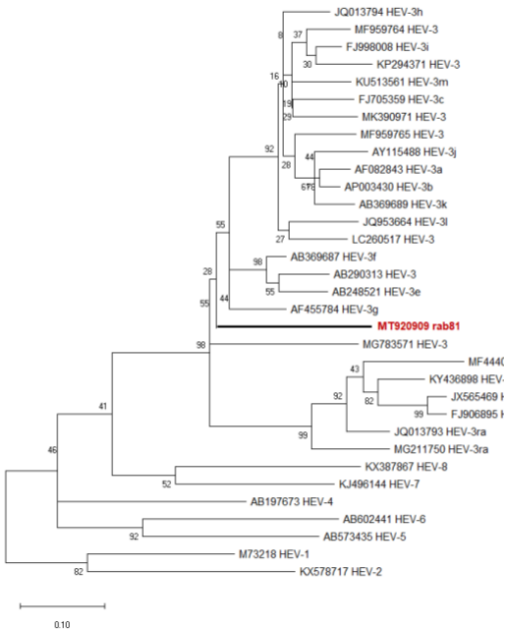
f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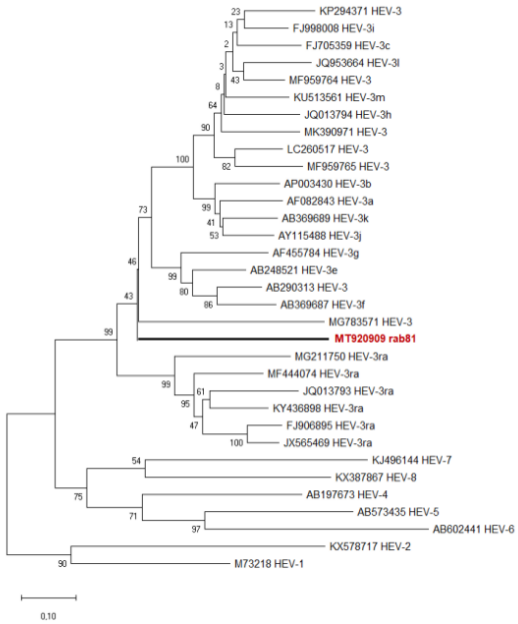
S1

g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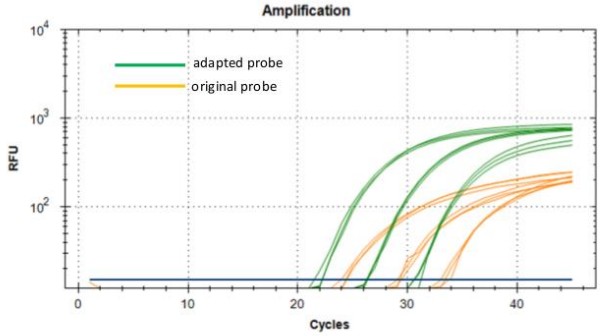
S1

a

b



c

	ct value (average of three)	
dilution	adapted probe	original probe
10^{-3}	30.9	33.4
10^{-4}	26.3	29.2
10^{-5}	22.0	24.3

Figure S2: Nucleotide sequence alignment of quantitative real-time RT-PCR (qRT-PCR) probe target region (a), qRT-PCR (triplicate) at three dilution steps (b) and bean ct-values yielded by original probe or adapted probe (c)

(III) A Modular Hepatitis E Virus Replicon System for Studies on the Role of ORF1-Encoded Polyprotein Domains

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Article

A Modular Hepatitis E Virus Replicon System for Studies on the Role of ORF1-Encoded Polyprotein Domains

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Abstract: Zoonotic hepatitis E virus (HEV) infection is an emerging cause of acute viral hepatitis in developed countries. Known reservoirs of zoonotic genotype 3 (HEV-3) are mainly pigs and wild boar, and to a lesser extent rabbits and deer. Rabbit hepatitis E virus (HEV-3ra) is prevalent in rabbits worldwide and represents a particular risk for zoonotic infection. Current understanding of the molecular mechanisms of HEV pathogenesis is incomplete, particularly due to the limited availability of efficient and reliable cell culture systems. In order to identify genomic regions responsible for HEV propagation in cell culture, we developed a modular chimeric reporter replicon system based on cell culture-adapted (Kernow-C1/p6 and 47832mc) and rabbit-derived HEV strains. Replication in HepG2 cells was monitored on the basis of a *Gaussia* luciferase reporter gene that was inserted in place of the open reading frame (ORF) 2 of the HEV genome. Luciferase activity of rabbit HEV-derived replicons was significantly lower than that of Kernow-C1/p6 and 47832mc replicons. Serial exchanges of defined ORF1 segments within the Kernow-C1/p6 replicon backbone indicated that HEV replication in HepG2 cells is not determined by a single domain but rather by an interplay of longer segments of the ORF1-derived nonstructural polyprotein. This implies that a specific combination of viral factors is required for efficient HEV propagation in cell culture.

Keywords: hepatitis E virus; replicon; luciferase readout; ORF1; nonstructural polyprotein domains

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1. Introduction

Hepatitis E virus (HEV) is a common cause of acute viral hepatitis worldwide [1]. The disease hepatitis E (HE) is usually subclinical and self-limiting; however, in some cases, fulminant hepatic failure is observed. Moreover, complications can arise in immunocompromised patients leading to chronic hepatitis mainly in solid-organ transplant recipients [2], or fetomaternal outcomes in pregnant women with mortality rates of up to 20% [3]. The virus contains a linear, single-stranded positive-sense RNA genome, which is capped at the 5′ end and polyadenylated at the 3′ end. The viral genome contains untranslated regions (UTR) at its 5′ and 3′ ends and three open reading frames (ORF). The ORF1 encodes a non-structural polyprotein that harbors the enzymatic activities of a methyltransferase, a protease, a macrodomain, a helicase, and an RNA-dependent RNA polymerase (RdRp), as well as a Y domain and a hypervariable region (HVR) of unknown function. ORF2 encodes the capsid protein and ORF3 encodes a small, multifunctional accessory protein [4].

Human infections were predominantly caused by strains of the *Orthohepevirus A* species of the *Hepeviridae* family: The genotypes HEV-1 and HEV-2 are transmitted solely between humans mainly by the fecal–oral route in endemic regions of Africa and Asia, where access to clean drinking water is often limited. In contrast, autochthonous HE cases in Europe and North America are mainly caused by zoonotic genotype HEV-3 with the

main reservoir in pigs or wild boar and to lesser extent in rabbits and deer. Transmission is provoked by consumption of undercooked pork and meat products [5].

To monitor and elucidate transmission and clinical course of HEV infection, several animal models have been established. Although wild boar and pigs are highly susceptible to HEV [6] and can exhibit chronic infections [7], these animals do not exhibit any clinical symptoms beyond viremia and fecal virus shedding [8,9]. Similarly, chronic infections or fetomaternal complications could not be reproduced in HEV-infected non-human primates [10]. As alternative, European rabbit (*Oryctolagus cuniculus*) was developed as an alternative model for human HEV infection [11]. Rabbits can be experimentally infected with HEV-3 strains [12] and are associated with a specific subgenotype of HEV (HEV-3ra), which, in turn, can infect humans [13,14]. Furthermore, rabbits exhibit complications upon infection with HEV similar to humans, especially during pregnancy. Both chronic HEV infection [15] and poor fetomaternal outcomes [16] have been reported.

Many molecular aspects of HEV replication still remain unknown because the virus is challenging to efficiently propagate in cell culture. While some progress has been made in recent years, particularly with the development of cell culture models based on HEV-3 strains Kernow-C1 [17] and 47832c [18], the efficient cultivation of HEV in vitro is still limited to specific strains and cell lines. Both HEV strains were isolated from persistently infected patients and exhibit specific insertions within the HVR of the nonstructural protein encoding ORF1, which are critical for maintenance in cell culture [19,20]. In addition, optimized and well-adapted protocols allowed replication of HEV strains without particular insertions and production of high titers in cell culture [21]. However, the knowledge on conditions for HEV replication level in cell culture is still lacking.

Reverse genetics and subgenomic replicon systems in particular are powerful tools to elucidate the genetic characteristics responsible for efficient virus replication in vitro. In the context of HEV, notable examples include Nguyen et al. [22] and Cordoba et al. [23], demonstrating that HEV host specificity is governed not only by the ORF2-encoded capsid protein but also by the nonstructural protein of ORF1, or Tian and colleagues [24], who proved that intergenotypic recombination of fragments from the HVR or X region of ORF1 does not abolish HEV replication in vitro. However, few unique HEV strains have been used as an initial point for development of reverse genetics and replicon systems [25–27]. These strains are typically selected based on efficient growth in cell culture. In particular, the Kernow-C1/p6 system [19] has become a gold standard among HEV cell culture systems.

To evaluate the role of different segments of the ORF1-encoded polyprotein in a cell culture adapted replicon system, we built upon the p6/Luc replicon and assembled *Gaussia* luciferase expressing replicons based on HEV strains 47832mc [26], rabbit HEV strains rab52 [28], and rab81 [28,29]. As the reporter in these constructs substitutes 377 nucleotides of the ORF2/ORF3 overlap region, they cannot express ORF2- and ORF3-derived proteins. As a consequence, infectious particles are not formed [19]. This eliminates some of the complexity of the viral life cycle on one hand, but allows on the other for a more precise assessment of the replicase. Finally, we assembled chimeric replicons based on the p6 replicon backbone by partitioning ORF1 into three fragments and exchanging each of the fragments of p6 separately with the corresponding fragments from 47832mc, rab52, and rab81 strains.

2. Results

2.1. Construction of Luciferase Reporter Replicons Based on Different HEV-3 Strains

We constructed a set of novel luciferase reporter replicons based on HEV strains rab52, rab81, and 47832mc (Figure 1A). These strains represent different subclades of HEV-3, with pairwise ORF1 amino acid sequence identities between 84.9% and 92.2% (Table 1). The overall replicon architecture is based on the p6Luc replicon [19], which contains an ORF-encoding *Gaussia* luciferase in place of the first 377 nucleotides of ORF2 (positions 5359 to 5735). This deletion disables expression of functional capsid protein and removes

all but the first eleven nucleotides of ORF3. The luciferase reporter gene was inserted in equivalent positions of rab52-, rab81-, and 47832mc-derived replicons.

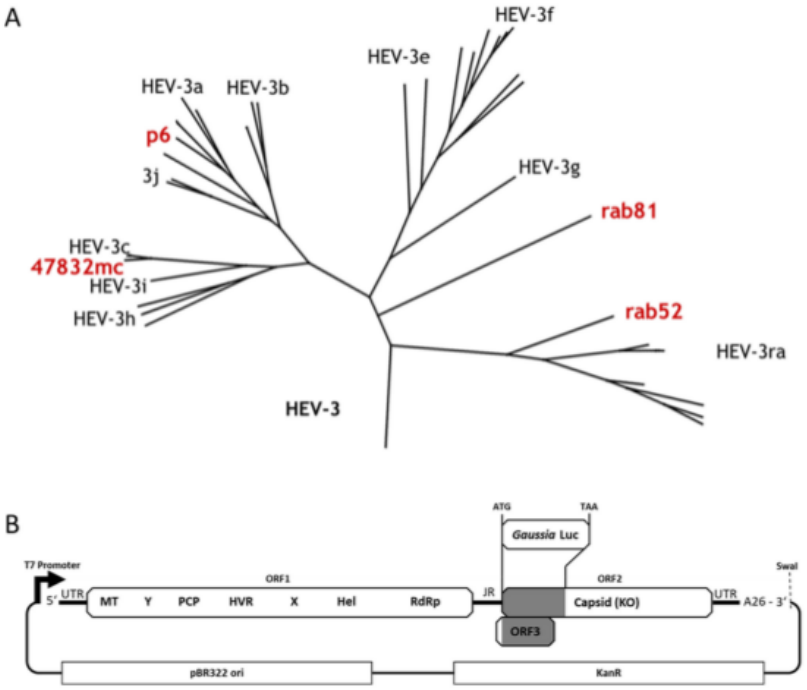

Figure 1. (**A**) Phylogenetic overview of the model strains used in this work. Depicted in red are HEV-3 strains p6 [17], 47832mc [26], rab52 [28], and rab81 [28,29]. The tree was constructed in Geneious using the Maximum Likelihood method. Subgenotype and strain labels were then added manually. (**B**) Schematic overview of parental replicon plasmid constructs. The sequence of each replicon was inserted downstream of a T7 promoter for in vitro transcription. A *Gaussia* luciferase (Luc) reporter gene was inserted at the start codon of ORF2, deleting 377 nucleotides of ORF2, or 380 nucleotides in the case of the rab81-based replicon, and almost the entirety of ORF3. Each construct contains a polyA tail of exactly 26 nucleotides and a SwaI restriction site directly downstream of the polyA tail for linearization prior to transcription. All constructs were cloned with the same minimal pMK2 plasmid backbone, which contains a pBR322-derived origin (ori) of replication, and a kanamycin resistence gene (KanR). MT = methyltransferase; Y = Y-domain; PCP = papain-like cysteine protease; HVR = hypervariable region; X = X-domain (macrodomain); Hel = helicase; RdRp = RNA-dependent RNA polymerase.

Table 1. Pairwise nucleotide and amino acid sequence identities of the four reporter replicon sequences and the corresponding ORF1-encoded proteins.

Replicon Nucleic Acid Sequence Identity				ORF1 Nucleic Acid Sequence Identity				ORF1 Amino Acid Sequence Identity			
	rab81	**rab52**	**47832mc**		**rab81**	**rab52**	**47832mc**		**rab81**	**rab52**	**47832mc**
rab52	77.7%			rab52	74.4%			rab52	86.7%		
47832mc	78.4%	77.5%		47832mc	75.1%	73.5%		47832mc	86.5%	85.3%	
p6	78.5%	77.1%	83.5%	p6	75.5%	73.4%	81.3%	p6	87%	84.9%	92.2%

All constructs were generated with the same plasmid backbone (pMK2) and contain a T7 promoter upstream for in vitro transcription, a 26 nucleotide polyA tail followed by a SwaI recognition site for DNA linearization prior to transcription (Figure 1B). For consistency, the p6/Luc replicon was reassembled in the same vector. Additionally, a negative control replicon was constructed by introducing three point mutations into the sequence encoding the conserved GDD motif of the RdRp, resulting in an inactivated GAA mutant. Finally, the 5′ ends of rab81 and rab52 were adapted to enable in vitro transcription with T7 RNA polymerase. The resulting replicons are referred to as p6LucA26, p6GAALucA26, 47832mcLucA26, rab52LucA26, and rab81LucA26 (Table S1).

2.2. Rabbit HEV-Based Replicons Generate Low Luciferase Activity

After electroporation of HepG2 cells with in vitro transcribed (ivt) RNAs, reporter gene expression was detectable for all four constructs (Figure 2). The luciferase activity for the RdRp GAA mutant replicon remained at mock control background level. Luciferase activity was detected for ivt RNAs of each replicon construct from the first day after transfection and typically reached peak expression on the second or third day. Hereafter, the signal decreased but remained detectable for approximately five days for the rabbit HEV replicons and at least a week for p6 and 47832mc replicons (Figure 2). For parental p6LucA26 in particular, approximately 3×10^4 relative light units (RLU) were measured on the first day. On day three after transfection, peak luciferase activity reached 1.5×10^6 RLU and decreased to approximately 10^4 RLU one week after transfection. In comparison to p6, 47832mc yielded higher luciferase activity (maximum: 3.6×10^6 RLU at day three) and retained slightly higher expression levels in the long term (5.9×10^4 RLU vs. 2.2×10^4 RLU at day seven). In contrast, both the rab81- and rab52-based replicons displayed only low luciferase levels, with peak activities of 227 RLU at day three and 203 RLU at day two, respectively. Both replicon activities dropped to base line over seven days and were no longer distinguishable from the mock background. It is notable that the peak maxima were all reached by day three regardless of whether the respective replicon was based on cell culture adapted or non-adapted strains.

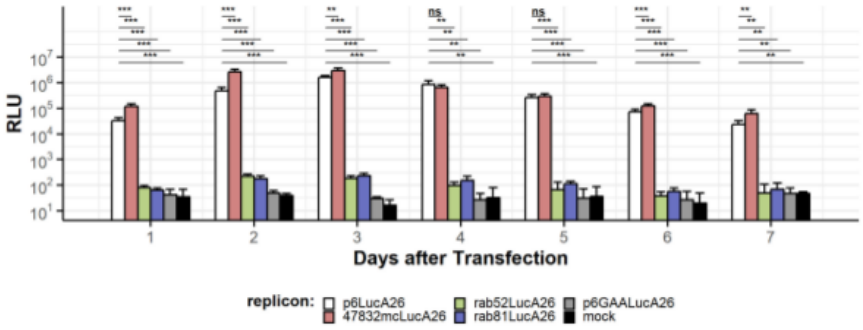

Figure 2. Luciferase activity of four parental HEV replicons in comparison to mock-transfected control (H_2O) and inactivated RdRp GAA mutant replicon based on p6LucA26. HepG2 cells were transfected with replicon ivtRNA by electroporation and seeded across four wells of a 96-well plate as technical quadruplicate. Replication was estimated by measuring total luciferase activity (relative light units; RLU) of each replicon. Error bars indicate standard deviation and asterisks indicate significance level (*** $p \leq 0.001$; ** $0.001 < p \leq 0.01$; ns $0.05 < p \leq 1$). The data depict a representative experiment of three separate transfections.

2.3. Construction and Evaluation of Chimeric ORF1 HEV Replicons

In order to investigate the different activities of the HEV replicons, the influence of corresponding ORF1-derived nonstructural polyproteins was analyzed by generation of

chimeric ORF1 replicons based on the p6-Luc backbone. For this purpose, the ORF1 was divided into three fragments spanning the 5′-UTR, and coding region for methyltransferase, Y-domain, putative protease (MYP, corresponds to nucleotide positions 1–2139 in p6), coding region for hypervariable region, X-domain, helicase (VXH, corresponds to nucleotide positions 2144–4059 in p6) as well as RdRp encoding region and junction region (RJ, corresponds to nucleotide positions 4064–5347 in p6) (Figure 3A). The fragments are connected to each other by tetranucleotides, which are highly conserved across HEV-3 genomes (positions in p6: 2140–2143 "GGUC"; 4060–4063 "UGCC"; 5348–5351 "AUGG") and consequently serve as convenient overhangs for a type IIS restriction-ligation-based recombination strategy. The fragments were inserted into the respective p6LucA26 constructs, resulting in nine chimeric constructs (Figure 3A).

After transfection of HepG2 cells, luciferase signals were monitored over a period of seven days (Figure 3B). In general, all tested replicons with exchanged subgenomic fragments produced measurable luciferase signals, higher than those observed for mock transfected and RdRp knockout (p6GAALucA26) controls. First, the positive control p6LucA26 showed a luciferase readout of 10^4 RLU already on day one, and reached the maximum value of 1.5×10^6 at day three, followed by a decrease to just below the initial value (9.8×10^3 RLU) over the course of the observation period. Substitution of the p6 parental domains by those of 47832mc resulted in similar kinetics of luciferase activity compared to the p6 control but with slightly higher values: Exchange of MYP domain led to increased activity with a value of 2.1×10^6 RLU already on day two followed by maintenance of increased values from day five until the end of the observation period. Insertion of VXH.47832mc domain led to higher values at day four (1.1×10^6 RLU vs. 8.1×10^5 RLU) and chimeric RJ.47832mc replicon peaked at day three (1.8×10^6 RLU vs. 1.5×10^6 RLU). In contrast, while the RJ fragments from rab52 or rab81 demonstrated high activity within the respective p6 chimera, chimeric replicons with subgenomic fragments MYP and VXH from both strains induced a dramatic reduction on the replicon activity at about two orders of magnitude compared to parental p6. Insertion of MYP.rab52 or MYP.rab81 showed peak values at day four of about 7.5×10^3 and 1.3×10^4 RLU compared to 8×10^5 RLU for the p6 replicon. Similarly, VXH.rab52 and VXH.rab81 domains caused a reduction to about 7×10^3 RLU compared to 8×10^5 RLU for the p6 replicon. Furthermore, apart from a delayed increase, the local maximum of the luciferase expression curve is not as pronounced with the chimeric replicons. Rather than the distinct expression peak exhibited by p6 on day three post transfection, the curve plateaus from day three until day five, followed by a slight decrease between day five and day seven of the experiment. Interestingly, although VXH domains of rab52 and rab81 have low sequence identity (Table 2), the luciferase activity curves of both chimeric replicons are almost identical.

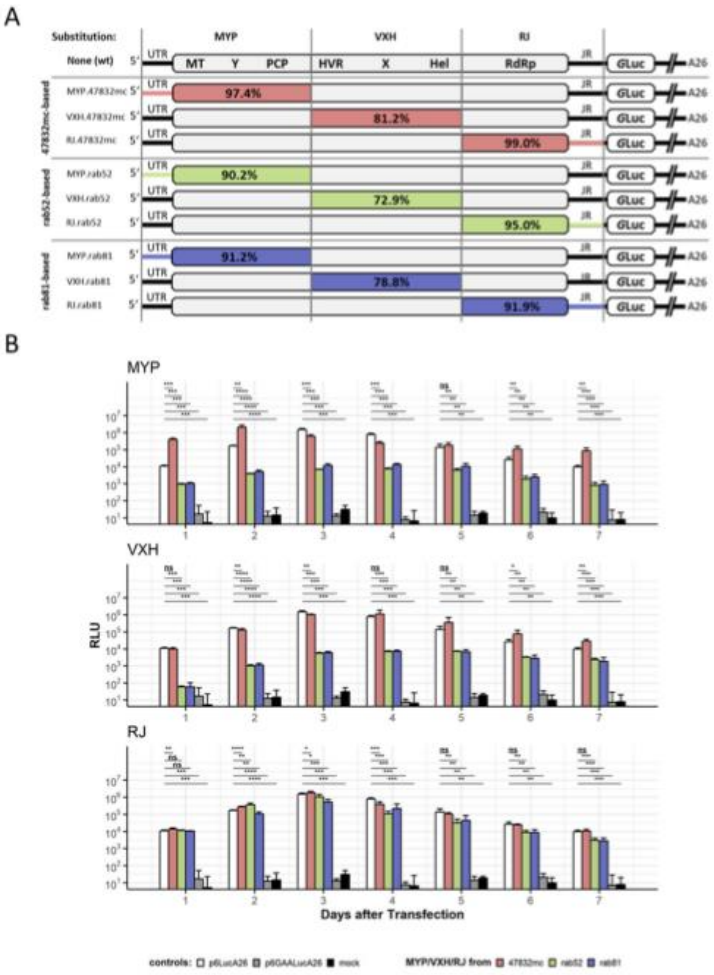

Figure 3. (**A**) Structure of nine chimeric replicons that were constructed using p6LucA26 as backbone. Subgenomic fragments containing partial sequences of ORF1 (MYP, nucleotide positions 1–2139 in p6; VXH, nucleotide positions 2144–4059 in p6; RJ, nucleotide positions 4064–5347 in p6) were replaced with the corresponding sequences from HEV strains 47832mc, rab52, and rab81. Percentages annotated in the highlighted fragments represent pairwise amino acid sequence identity of the encoded proteins with the corresponding ones encoded by parental p6 replicon. Multiple amino acid sequence alignments of each ORF1-encoded protein segment are included in the Supplementary Materials (Figures S1–S3). (**B**) HepG2 cells were transfected with replicon ivtRNA by electroporation and seeded across four wells of a 96-well plate as technical quadruplicates. Replication was estimated by measuring total luciferase activity (RLU) of each replicon. Error bars indicate standard deviation; asterisks indicate significance level in reference to p6LucA26 (**** $p \leq 0.0001$; *** $0.0001 < p \leq 0.001$; ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$; ns $0.05 < p \leq 1$). The data depict a representative experiment of three separate transfections. Note that the controls (p6LucA26, p6GAALucA26, and mock) are shown in each plot for illustrative purposes. The data depicted are derived from a single experiment with all chimeric replicons and one group of controls.

Table 2. Pairwise nucleotide/amino acid sequence identities of the subgenomic fragments/protein domains in the chimeric constructs/proteins.

Nucleotide Sequence Identity											
MYP				**VXH**				**RJ**			
	rab81	rab52	47832mc		rab81	rab52	47832mc		rab81	rab52	47832mc
rab52	76.9%			rab52	68.6%			rab52	79%		
47832mc	79.1%	78.4%		47832mc	67.7%	63.7%		47832mc	79.9%	81.4%	
p6	80.4%	78.5%	84.9%	p6	67.7%	63.7%	75.3%	p6	79.4%	80.6%	84.7%
Amino Acid Sequence Identity											
MYP				**VXH**				**RJ**			
	rab81	rab52	47832mc		rab81	rab52	47832mc		rab81	rab52	47832mc
rab52	88.5%			rab52	81%			rab52	91.9%		
47832mc	90.9%	91.1%		47832mc	77.7%	72.8%		47832mc	92.4%	95.5%	
p6	91.2%	90.2%	97.4%	p6	79.2%	72.9%	82%	p6	91.9%	95%	99%

3. Discussion

Here, we describe the establishment of a modular luciferase replicon system based on subgenomic fragments of different HEV strains. Starting with the re-assembly of the parental p6 reporter replicon and the generation of the p6 GAA RdRp knockout mutant, we further assembled three novel replicons based on HEV-3c strain 47832mc, HEV-3ra strain rab52, and HEV-3 (putative novel subgenotype) strain rab81. Luciferase expression curves of all constructs are characterized by a distinct peak, followed by a significant decrease of luciferase activity. This behavior is consistent with the original description of the p6/Luc replicon [19] and is attributed to the absence of capsid protein synthesis due to insertion of the *Gaussia* luciferase coding sequence. As previously observed by Nguyen et al. [22], the peak of luciferase activity occurred earlier in our experiments, likely due to the difference in transfection protocols or cell lines. The cell culture isolate 47832mc-derived replicon demonstrated similar and even higher luciferase activity compared to the positive control p6LucA26. In contrast, the replicons based on rab52 and rab81 demonstrated markedly lower activity. The similarity of the luciferase kinetics for these two replicons is surprising, as the sequence identity between both replicons is not particularly high (77.8% RNA sequence identity for the complete replicon sequences). Therefore, this similar activity cannot be explained by the close similarity of the two strains and needs further investigation. Although the luciferase expression kinetics of each full-length replicon may show the level of adaptation to growth in cell culture, this does not explain the replication efficiency of a given strain. In order to elucidate the influence of different ORF1 related subgenomic fragments on total replicon activity, we next decided to generate chimeric replicons of the different strains.

Substitution of N-terminal region, termed 'MYP' for the domains it contains, methyltransferase, Y-domain, and protease, resulted in a decrease of the luciferase expression peak by two orders of magnitude when MYP fragments of rab52 or rab81 were used. On the other hand, the activity of the construct containing the MYP fragment of 47832mc substantially exceeded the unmodified replicon. While the expression peak was only slightly higher, it was reached a day earlier. Furthermore, on the first day of the observation period, the MYP.47832mc chimeric replicon had already reached a luciferase output 36-fold higher than the p6LucA26 replicon. This property is not fully reflected by the 47832mcLucA26 replicon, which exceeds p6LucA26 but not to this extent. The difference of almost three orders of magnitude between the "best" and the "worst" MYP-chimeric construct (MYP.47832mc and MYP.rab52, respectively) represents the largest single difference between any two chimeric replicons and stresses the importance of this fragment for HEV replication in vitro. This is interesting, especially in light of recent reports of otherwise unremarkable HEV strains,

which still grow efficiently in cell culture [21]. It poses the question which functions of this region cause this impressive difference in luciferase expression between the different MYP fragments. Firstly, the methyltransferase is involved in RNA capping [30], which is directly related to and crucial for translation of newly synthesized HEV RNA [31] in the host cell. The methyltransferase and protease domains are also relevant for suppression of retinoic acid inducible gene I (RIG-I) -mediated detection of the viral RNA and subsequent activation of the interferon pathway [32]. The protease domain has chymotrypsin-like cleavage and inhibition patterns, has been implicated in processing of the viral proteins [33], and inhibits the host interferon pathway [32,34]. The function of the Y-domain remains unknown, although a mutagenesis screen has revealed critical amino acid residues and secondary structure motifs within the sequence [35]. The 5′-terminal end of the HEV genome interacts with the viral RNA polymerase to facilitate genome replication [36]. In-depth investigation of this sequence and detailed examination of each subdomain could prove valuable.

The middle region, termed VXH for its HVR, X-domain, and helicase part, was of particular interest. The p6 replicon contains an insertion within the HVR and three amino acid exchanges within the X-domain, which are required for efficient growth in cell culture. Shukla et al. described modified constructs without insertion, which caused up to 50-fold decreased luciferase expression, and without the three X-domain mutations, which reduced the output by a factor of 2.3 to 5.1 [19]. This is in accordance with the decrease in the observed luciferase signal when the VXH fragments of rab52 or rab81 were used here. Interestingly, the expression kinetics of the VXH.rab52 and VXH.rab81 chimeric replicons were almost identical. This is surprising because the overall sequence identity between the two VXH fragments is rather low. In fact, VXH.rab52 contains the characteristic HEV-3ra insertion, while VXH.rab81 does not. Nevertheless, both fragments appear to have an almost identical effect on the activity of the replicon. On the other hand, VXH.47832mc only slightly affects the luciferase expression curve. This is consistent with the similarity of the expression kinetics of p6LucA26 and 47832mcLucA26. The VXH.47832mc fragment is derived from a cell culture adapted strain and also contains an insertion in the HVR, which is critical for growth in cell culture [20]. The influence of these insertions on virus replication is still not fully understood, though some evidence exists that they contain nuclear localization signals [20,37]. However, nuclear localization of the polyprotein by itself does not appear to improve replication in cell culture [37]. Notably, Scholz et al. demonstrated that exchanging the insertion in 47832mc for the insertion of p6 attenuates the virus beyond recovery [20]. The sequence length of VXH fragment exchanged in this work is larger, which may account for the comparably lower difference between the replicons. On the other hand, it is possible that the effect of the insertion is dependent on the sequence context of the HVR it is embedded in. When comparing the two strains, it is evident that the insertion of p6 is shorter by 15 nucleotides (171 nucleotides vs. 186 nucleotides), but the overall length of the HVR + insertion is identical between both strains (Figure S4).

The C-terminal region, RJ, contains the RdRp and the junction region. The subgenomic promoter, which controls the transcription of the subgenomic RNA with the reporter gene, is located at the 3′-end of the RdRp coding region. Of all fragments examined, RJ had the weakest effect on replication level, measured by luciferase signal release. RJ.47832mc increased the expression slightly, which is intriguing, given the 99% sequence identity on the amino acid level to RJ.p6 (Table 2). In contrast, RJ.rab81 decreased it by a small amount. Interestingly, RJ.rab52 yielded the highest values on day two, before dropping back to the level observed with the unmodified replicon, and subsequently reach the luciferase expression levels similar to the RJ.rab81 chimera. Both RJ.rab52 and RJ.rab81 contain the K1634 variant of RdRp (ORF1-polyprotein, nomenclature based on HEV-1; the actual amino acid positions are 1706 in Kernow-C1/p6, 1663 in rab52, and 1641 in rab81), an amino acid exchange that is known to increase replication compared to the G1634 residue originally found in p6 [38]. Despite this, the RJ regions of rab52 and rab81 as a whole do not increase luciferase signal compared to the RJ.p6 fragment. The subgenomic promoter

within the fragment may further influence the luciferase expression curve [36,39]. For detailed work on the function of the RdRp, it is advisable to keep the subgenomic promoter sequence unchanged.

Future work will expand the scope of both host cell lines and model replicons. Additional replicons based on different HEV strains, either cell culture adapted or non-adapted wildlife- or human-derived, as well as the corresponding chimeras, should reveal in more detail viral factors that are required for growth in vitro. On the other hand, different host cell lines will show how host factors interact with different HEV genotypes. Beyond HepG2, other cell lines, such as HuH-7, PLC/PRF/5, and A549, have shown promise within some cell culture protocols. Indeed, in light of our results with the 47832mc replicon, the accompanying A549 subclone D3 [40] appears to be a promising system for future experiments.

4. Materials and Methods

4.1. HEV Strains, Plasmids, and Cell Culture

Plasmids containing the full-length sequences of Kernow-C1/p6 (GenBank acc. JQ679013) and p6-Luc were generously provided by Patricia Farci (National Institutes of Health, Bethesda, MD, USA). Clone 47832mc (GenBank acc. MN756606) was a kind gift from Reimar Johne and Johannes Scholz (Federal Institute for Risk Assessment, Berlin, Germany). Synthetic DNA fragments encompassing the rab52 (GenBank acc. KY436898) genome were produced by Eurofins Genomics (Ebersberg, Germany). Synthetic DNA fragments encompassing the rab81 (GenBank acc. MT920909) genome were produced by Twist Bioscience (San Francisco, CA, USA). The 5′ ends of rab81 and rab52 were adapted as follows: The first nucleotide of rab81 was mutated to G to enable in vitro transcription with T7 RNA polymerase. The 5′ end of rab52 (KY436898) is truncated and was therefore extended and adapted to the consensus sequence of HEV-3ra 5′ UTRs. Plasmids containing the *Gaussia* luciferase reporter gene for insertion in rab52, rab81, and 47832mc replicons were ordered from Twist Bioscience (San Francisco, CA, USA).

HepG2 cells were purchased from CLS (Eppelheim, Germany) and grown in DMEM (Gibco 52100, ThermoFisher, Waltham, MA, USA), supplemented with $NaHCO_3$ and Na-pyruvate, and 10% fetal bovine serum, including 10 mg/L gentamicin (PAN-Biotech, Aidenbach, Germany), and 250 mg/L amphotericin B (PAN-Biotech, Aidenbach, Germany) in cell culture flasks with vented caps (Corning, Corning, NY, USA) in a humidified incubator with 5% CO_2.

4.2. PCR and Cloning

Subgenomic fragments were selected as follows: First, the genomes were scanned for conserved tetranucleotides to serve as recombination sites. Subsequently, the candidate sites were narrowed down to represent the functional domains [41] of the HEV-encoded proteins. Finally, optimal overhangs were selected using the data on overhang ligation fidelity by Potapov et al. [42] in order to maximize assembly efficiency.

The fragments were amplified from plasmid templates using Q5 High Fidelity DNA Polymerase (NEB, Ipswich, MA, USA) or Phusion Hot Start Flex DNA Polymerase (NEB, Ipswich, MA, USA). PCR products were gel purified using the Gel extraction Kit (Qiagen, Hilden, Germany) or the Wizard SV Gel and PCR purification Kit (Promega, Madison, WI, USA). All gel extractions were done by cutting out bands from a Sybr Safe (ThermoFisher, Waltham, MA, USA) stained agarose gel using blue light transillumination. Purified fragments were either used directly for assembly or first cloned with PCR cloning plasmid pMiniT 2.0 using the PCR cloning Kit (NEB, Ipswich, MA, USA). The resulting plasmids were amplified in *Escherichia coli* [43] and verified by restriction digest and sequencing analysis. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) or the PureYield Plasmid Miniprep System (Promega, Madison, WI, USA) and digested using Esp3I (NEB, Ipswich, MA, USA) or Anza BpiI (ThermoFisher, Waltham, MA, USA), according to the manufacturer's instructions and column-purified or gel extracted as described above. Fragments were ligated using T4 DNA ligase (NEB, Ipswich, MA,

USA) and used to transform *E. coli* DH5α cells. All inserts were sequenced using the dideoxy chain termination method (Sanger sequencing) at Eurofins (Ebersberg, Germany). Backbone plasmid pMK2 was constructed by PCR amplification of the backbone of pET-19b (Novagen) and inserting the kanamycin resistance gene from pcDNA3-EGFP (a gift from Doug Golenbock; Addgene Plasmid #13031). Cloning and sequencing primer sequences are provided in the Supplementary Materials (Table S2). A listing with descriptions of all replicon plasmids (Table S1) and cloning intermediates (Table S3) is also provided in the Supplementary Materials.

4.3. In Vitro Transcription and Transfection

Template DNA was generated by transforming *E. coli* with the appropriate, replicon-containing plasmids and growing an overnight culture in LB medium with 50 µg/mL kanamycin. Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The plasmids were linearized using SwaI (NEB, Ipswich, MA, USA). Linearized plasmids were purified using the Wizard SV Gel and PCR purification Kit (Promega, Madison, WI, USA) and quantified using a Nanodrop 2000c spectrophotometer (ThermoFisher, Waltham, MA, USA). Replicons were transcribed from 1 µg linearized DNA template using the HiScribe T7 ARCA mRNA Kit (NEB, Ipswich, MA, USA) according to the manufacturer's instructions. If needed, the reactions were scaled as appropriate. ivtRNA was subsequently purified using the provided LiCl solution, and resuspended in nuclease-free water. Success of transcription reactions was confirmed by Nanodrop measurement and by running the RNA preparations on a 1% agarose gel. For transfection, the purified RNA was diluted to 125 ng/µL in a volume of 40 µL in nuclease-free water. A total of 1 µL was used for agarose gel analysis of the transfection mix. HepG2 cells were trypsinized and washed with and resuspended in OptiMEM (ThermoFisher, Waltham, MA, USA). After counting using a Neubauer chamber without trypan blue exclusion, the suspension was adjusted to 3.3×10^6–3.5×10^6 cells/mL. A total of 360 µL of this suspension was mixed with the diluted ivtRNA and transferred into a 4 mm electroporation cuvette (VWR, Radnor, PA, USA). The cells were then immediately electroporated using a Square Wave pulse for 20 ms at 300 V in a GenePulser XCell electroporation device (Bio-Rad, Hercules, CA, USA). After at least ten minutes of regeneration inside the cuvette, the cells were transferred into 1.2 mL of DMEM. This suspension was then seeded in four wells of a 96-well plate at 100 µL per well and incubated in a humidified incubator at 37 °C with 5% CO_2. Medium was exchanged daily and the supernatants were collected and stored frozen at −80 °C. Each transfection was done at least three times.

4.4. Luciferase Reporter Assay

Initially, luciferase activity was measured using the Pierce Gaussia Flash Assay Kit (ThermoFisher, Waltham, MA, USA) and an infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). A total of 20 µL supernatant of cultures transfected with *Gaussia*-luciferase expressing HEV replicons were transferred to black 96-well plates (ThermoFisher, Waltham, MA, USA). Substrate working solution was prepared by diluting coelenterazine according to the instructions of the kit. A total of 50 µL working solution was injected per well, followed by 1s of shaking and light measurement with 0.5 s integration time. Alternatively, we diluted coelenterazine (Carl Roth, Karlsruhe, Germany) in PBS with 5 mM NaCl according to [44]. For statistical analysis, each group was compared to the positive control, p6LucA26, in an unpaired, two-tailed *t*-test. *p* values were adjusted for multiple testing using the FDR method [45]. Data analysis and visualization was done using R [46] with packages readxl [47], tidyr [48], tibble [49], rstatix [50], dplyr [51], ggplot2 [52], ggpubr [53], and ggh4x [54] in RStudio [55].

5. Conclusions

We established a modular HEV replicon system and demonstrated that the different luciferase outputs of chimeric replicons reflect the activity of their donor replicons. Our

work provides an easy and efficient procedure to identify viral factors required for replication in vitro as well as potential bottlenecks. Beyond replicons, this basic approach is transferable to infectious cDNA clones and to study and compare the effect of different viral sequences in vivo.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11030355/s1, Table S1: List of replicon plasmids. Table S2: Sequences of cloning and sequencing primers.Table S3: List of cloning intermediates. Figure S1: Multiple amino acid sequence alignment of the ORF1-MYP-encoded protein segment from the model strains p6, 47832mc, rab52, rab81. Figure S2: Multiple amino acid sequence alignment of the ORF1-VXH-encoded protein segment from the model strains p6, 47832mc, rab52, rab81. Figure S3: Multiple amino acid sequence alignment of the ORF1-RJ-encoded protein segment from the model strains p6, 47832mc, rab52, rab81. Figure S4: Comparison of the HVR insertions of p6 and 47832mc.

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Table S1: List of replicon plasmids

Name	Length (nt)	description
Unmodified replicons		
pMK2-p6LucA26	9704	Full-length replicon p6LucA26 in plasmid backbone pMK2; derived from JQ679013 and p6-Luc [19]
pMK2-p6GAALucA26	9704	RdRp GDD->GAA mutant of full-length replicon p6LucA26 in plasmid backbone pMK2; derived from JQ679013
pMK2-47832mcLucA26	9697	Full-length replicon 47832mcLucA26 in plasmid backbone pMK2; derived from MN756606
pMK2-rab52LucA26	9573	Full-length replicon rab52LucA26 in plasmid backbone pMK2; derived from KY436898
pMK2-rab81LucA26	9500	Full-length replicon rab81LucA26 in plasmid backbone pMK2; derived from MT920909
MYP chimeras		
pMK2-p6(MYP.47832mc)LucA26	9703	Chimeric replicon p6(MYP.47832mc)LucA26 in plasmid backbone pMK2; p6LucA26 backbone with nt 1-2139 replaced by the homologous fragment (nt 1-2138) of 47832mcLucA26
pMK2-p6(MYP.rab52)LucA26	9705	Chimeric replicon p6(MYP.rab52)LucA26 in plasmid backbone pMK2; p6LucA26 backbone with nt 1-2139 replaced by the homologous fragment (nt 1-2140) of rab52LucA26
pMK2-p6(MYP.rab81)LucA26	9705	Chimeric replicon p6(MYP.rab81)LucA26 in plasmid backbone pMK2; p6LucA26 backbone with nt 1-2139 replaced by the homologous fragment (nt 1-2140) of rab81LucA26
VXH chimeras		
pMK2-p6(VXH.47832mc)LucA26	9704	Chimeric replicon p6(VXH.47832mc)LucA26 in plasmid backbone pMK2; p6LucA26 backbone with nt 2144-4059 replaced by the homologous fragment (nt 2143-4058) of 47832mcLucA26
pMK2-p6(VXH.rab52)LucA26	9575	Chimeric replicon p6(VXH.rab52)LucA26 in plasmid backbone pMK2; p6LucA26 backbone with nt 2144-4059 replaced by the homologous fragment (nt 2145-3931) of rab52LucA26
pMK2-p6(VXH.rab81)LucA26	9509	Chimeric replicon p6(VXH.rab81)LucA26 in plasmid backbone pMK2; p6LucA26 backbone with nt 2144-4059 replaced by the homologous fragment (nt 2145-3865) of rab81LucA26
RJ chimeras		
pMK2-p6(RJ.47832mc)LucA26	9704	Chimeric replicon p6(RJ.47832mc)LucA26 in plasmid backbone pMK2; p6LucA26 backbone with nt 4064-5347 replaced by the homologous fragment (nt 4063-5346) of 47832mcLucA26
pMK2-p6(RJ.rab52)LucA26	9704	Chimeric replicon p6(RJ.rab52)LucA26 in plasmid backbone pMK2; p6LucA26 backbone with nt 4064-5347 replaced by the homologous fragment (nt 3936-5219) of rab52LucA26
pMK2-p6(RJ.rab81)LucA26	9704	Chimeric replicon p6(RJ.rab81)LucA26 in plasmid backbone pMK2; p6LucA26 backbone with nt 4064-5347 replaced by the homologous fragment (nt 3870-5153) of rab81LucA26

Table S2: Sequences of cloning and sequencing primers.

Name	Sequence (5‘ -> 3‘)	Source	Used in
32c_3af	AGTCGAAGACATGGTCAACATCTGGTTTTTCTAG	this work	Cloning of VXH.47832mc
32c_3ar	CAGTGAAGACTTGACCATGTCCGGGTGTAC	this work	Cloning of MYP.47832mc
32c_4f	AGTCGAAGACTATGCCTCAGGAGCTTACG	this work	Cloning of RJ.47832mc
32c_4r	AGTCGAAGACGAGGCATATATAACAAGCCCTG	this work	Cloning of VXH.47832mc
32c_5r	AGTCGAAGACTCCCATGGGCAATGCACAA	this work	Cloning of RJ.47832mc
32c_6f	AGTCGAAGACCTGATGTTAGGATTCTAGTTCAAC	this work	Cloning of terA26.47832mc
52_3f	AGTCGAAGACTTGGTCCACGTCTGG	this work	Cloning of VXH.rab52
52_3r	AGTCCGTCTCGGACCAAGTTTTAGTATAAAGAGTC	this work	Cloning of MYP.rab52
52_4f	AGTCGAAGACTATGCCGCAGGAGCTTAC	this work	Cloning of RJ.rab52
52_4r	AGTCGAAGACTCGGCATATACAGGAGACCC	this work	Cloning of VXH.rab52
52_5r	AGTCGAAGACTCCCATGGGCAATGCAG	this work	Cloning of RJ.rab52
52_6f	AGTCGAAGACTCGATGTCAGGATCCTTGTTC	this work	Cloning of ter.rab52
52fw_bHel	CCAGATTAGTGCCTACCACC	this work	sequencing
52fw_Hel	TGCTGGCTGTACCGTTAACC	this work	sequencing
52fw_HVR	GTCCAGCTGCCACCTTCTG	this work	sequencing
52fw_MT	ACCTACCACACTACCTCTTAC	this work	sequencing
52fw_ORF2	ATACCTCATGACATCGATCTTG	this work	sequencing
52fw_PCP	GTGAAGAGTGTGACCAAGAAG	this work	sequencing
52fw_Rdrp	GTTATATCATCTGGTCCGGTC	this work	sequencing
52rv_ORF2	GTCTGATCGTACTCAGCAGC	this work	sequencing
52s_3f	ACTTGGTCCACGTCTGG	this work	sequencing
52s_4f	TATGCCGCAGGAGCTTAC	this work	sequencing
52s_4r	CGGCATATACAGGAGACCC	this work	sequencing
52s_5r	TCCCATGGGCAATGCAG	this work	sequencing
81.3'250	ACTGTTGACCACCCTGCACG	this work	sequencing
81_3f2	AGTCGAAGACTTGGTCAACATCTGGTT	this work	Cloning of VXH.rab81
81_3r	AGTCGAAGACTTGACCAAGTCCGAGTATACAAG	this work	Cloning of MYP.rab81
81_4f	AGTCCGTCTCATGCCGCAGGAGCTTAC	this work	Cloning of RJ.rab81
81_4r	AGTCGAAGACGCGGCATGTAAAGCAACC	this work	Cloning of VXH.rab81
81_5r	AGTCCGTCTCCCCATGGGCAATGCACAG	this work	Sequencing; Cloning of RJ.rab81

Table S2 (continued)

Name	Sequence (5‘ -> 3‘)	Source	Used in
81_6f	AGTCGAAGACCTGATGTCAGGATTCTGGTC	this work	Cloning of ter.rab81
AmpRp_rv	CTGAGAAGACCATACTCTTCCTTTTTCAATATTATTGAAGC	this work	Sequencing Cloning of pMK2
BbsI_T(26)CC	GAAGACGCTTTTTTTTTTTTTTTTTTTTTTTTTCC	this work	Cloning of terA26.rab52 terA26.rab81 terA26.47832mc
BbsI_T7p	GAAGACTTCTGCTAATACGACTCACTATA	this work	Cloning of MYP.rab81 MYP.47832mc
Cloning of Analysis Forward Primer	ACCTGCCAACCAAAGCGAGAAC	NEB	sequencing
Cloning of Analysis Reverse Primer	TCAGGGTTATTGTCTCATGAGCG	NEB	sequencing
Esp3I_T(26)CC	CGTCTCGTTTTTTTTTTTTTTTTTTTTTTTTTCC	this work	Cloning of GLuc_terA26.p6
Esp3I_T7p	CGTCTCGCTGCTAATACGACTCACTATA	this work	Cloning of MYP.p6 MYP.rab52
F1610	CTTGAGGCCCTTTATAGTGC	[29]	sequencing
F2991c	CTATTCAACAGGGTGACGTTG	this work	sequencing
F5086	GGACCTTACAAATTCTATTATACAG	[29]	sequencing
F6485	CGACAATCAACATGAACAGGAC	[29]	sequencing
F705	GTGCAGGGTATAACCATGATG	[29]	sequencing
GLuc_fw_Kp6_O2	CTGACGTCTCCATGGGATCACCATGGGAGTCAAAGTTCTGTTTG	this work	Cloning of GLuc-terA26.p6
K2_rv	CCTTGATTACGGTAGTGGAG	this work	sequencing
KanR_fw	CTGAGAAGACAGAGTATGATTGAACAAGATGGATTGC	this work	Cloning of pMK2
KanR_rv	CTGAGAAGACAGAGTCAGAAGAACTCGTCAAGAAG	this work	Cloning of pMK2
Kp6/GAA_fw	CTGACGTCTCGCAGCTTCGGTGGTCCTCTGTAGCG	this work	Cloning of RJ_GAA.p6
Kp6/GAA_rv	CTGACGTCTCAGCTGCACCCTTAAAGGCAGCAACAC	this work	Cloning of RJ_GAA.p6
Kp6_3af	CAGTCGTCTCTGGTCAACATCTGGCTTTTC	this work	Cloning of VXH.p6
Kp6_3ar	CAGTCGTCTCTGACCAGGTCCGGGTATAC	this work	Cloning of MYP.p6
Kp6_4f	CAGTCGTCTCATGCCACAAGAGCTTACCGTG	this work	Cloning of RJ.p6
Kp6_4r	CAGTCGTCTCTGGCATGTAAAGCAGGCCCTG	this work	Cloning of MYP.p6
Kp6_5r	CAGTCGTCTCCCCATGGGCGATGCAACAAAC	this work	Cloning of RJ.p6

Table S2 (continued)

Name	Sequence (5‘ -> 3‘)	Source	Used in
Ori_fw	CTGAGAAGACGAGACTGTCAGACCAAGTTTACTC	this work	Cloning of pMK2
p15A_seq_rv	GAGTCAGTGAGCGAGGAAG	this work	sequencing
p6_F1750	GGACGACGGTGGTTGA	this work	sequencing
p6_F2700	AGACTGTGATTGGCTGG	this work	sequencing
p6_F3250	GATGTTGATGTGGTGGTTG	this work	sequencing
p6_F4000	TCCTGCCAGATTAGTGC	this work	sequencing
p6_F4900	GGTCCTCTGTAGCGAC	this work	sequencing
p6_F600	TTGTATGCYGCACTACATCT	this work	sequencing
p6_F6750	CCAATGGCGAGCCGAC	this work	sequencing
p6_R1900	ATGTAGCAGTGCAATCCAG	this work	sequencing
p6_R2900	TGGTATATGCCCGAGCC	this work	sequencing
p6_R4300	CTCCACCATGGCCTCAA	this work	sequencing
p6_R5300	CCAATAAGGTTATGTACCAG	this work	sequencing
p6_R6100	ATGRAGGCGCTCACTAG	this work	sequencing
p6_R6800	TCGTACTCAGCGGCAGT	this work	sequencing
pACYC_Seq	AATAGGCGTATCACGAGGC	this work	sequencing
pMK2_fw	CGGGATCCGAAGACTGAAAATTTAAATACGAAAGGGCCTCGTGATAC	this work	Cloning of pMK2
pMK2_rv	CGGGATCCGAAGACAGGCAGTCCTAGCGGAGTGTATACTGG	this work	Cloning of pMK2
Primer_2F	GACTACTATTATTGCTACGGC	[29]	sequencing
Primer_3F	GAGATGATTCAATGGTCTTATG	[29]	sequencing
Primer_4R	CACATAAAATGTTTTAGAATGC	[29]	sequencing
R2429	CACAATCCGACTCGAATAAGG	[29]	sequencing
R3157	CAGCAAATGCGGTGGTAATGAC	[29]	sequencing
R5625	CATACAAGACAAGATTAGTGCC	[29]	sequencing

Underlined nucleotides represent the recognition sites of the restriction endonucleases used for cloning.

Table S3: List of Cloning of intermediates

Name	Length (nt)	description
MYP Fragments		
pMiniT2.0-T7pMYP.47832mc	4771	subgenomic fragment MYP.47832mc with T7 promoter in pMiniT2.0
pMiniT2.0-T7pMYP.rab52	4771	subgenomic fragment MYP.rab52 with T7 promoter in pMiniT2.0
pMiniT2.0-T7pMYP.rab81	4773	subgenomic fragment MYP.rab81 with T7 promoter in pMiniT2.0
pMiniT2.0-T7pMYP.p6	4770	subgenomic fragment MYP.p6 with T7 promoter in pMiniT2.0
VXH Fragments		
pMiniT2.0-VXH.47832mc	4536	subgenomic fragment VXH.47832mc in pMiniT2.0
pMiniT2.0-VXH.rab52	4407	subgenomic fragment VXH.rab52 in pMiniT2.0
pMiniT2.0-VXH.rab81	4341	subgenomic fragment VXH.rab81 in pMiniT2.0
pMiniT2.0-VXH.p6	4534	subgenomic fragment VXH.p6 in pMiniT2.0
RJ Fragments		
pMiniT2.0-RJ.47832mc	3904	subgenomic fragment RJ.47832mc in pMiniT2.0
pMiniT2.0-RJ.rab52	3904	subgenomic fragment RJ.rab52 in pMiniT2.0
pMiniT2.0-RJ.rab81	3902	subgenomic fragment RJ.rab81 in pMiniT2.0
pMiniT2.0-RJ.p6	3902	subgenomic fragment RJ.p6 in pMiniT2.0
pMiniT2.0-RJ_GAA.p6	3902	subgenomic fragment RJ_GAA.p6 in pMiniT2.0; contains RdRp GDD->GAA codon exchanges
Gaussia Luciferase reporter and terminal fragment of p6LucA26		
pMiniT2.0-insGLuc-terA26.p6	4881	subgenomic fragment insGLuc-terA26.p6 in pMiniT2.0
3' Terminal fragments of 47832mc, rab52, rab81, for combination with *Gaussia* luciferase reporter fragments		
pMiniT2.0-terA26.47832mc	3995	subgenomic fragment terA26.47832mc in pMiniT2.0
pMiniT2.0-terA26.rab52	3998	subgenomic fragment terA26.rab52mc in pMiniT2.0
pMiniT2.0-terA26.rab81	3991	subgenomic fragment terA26.rab81 in pMiniT2.0
Gaussia Luciferase reporter fragments for combination with corresponding _terA26 of 47832mc, rab52, rab81		
32cLuc_GL1 in pTwist Amp High Copy	3121	contains 11nt upstream of the ORF2 start codon, a *Gaussia* luciferase reporter inserted at the ORF2 start codon (deleting 377nt of the HEV genome), and 317nt of the 47832mc genome
52Luc_GL1 in pTwist Amp High Copy	3121	contains 11nt upstream of the ORF2 start codon, a *Gaussia* luciferase reporter inserted at the ORF2 start codon (deleting 377nt of the HEV genome), and 317nt of the rab52 genome
81Luc_GL1 in pTwist Amp High Copy	3121	contains 11nt upstream of the ORF2 start codon, a *Gaussia* luciferase reporter inserted at the ORF2 start codon (deleting 380nt of the HEV genome), and 317nt of the rab81 genome
Cloning of backbone plasmid for replicon constructs		
pMK2	2101	Cloning of vector pMK2; Kanamycin resistance gene, pBR322-derived origin of replication

Figure S1: Multiple amino acid sequence alignment of the ORF1-MYP-encoded protein segment from the model strains p6, 47832mc, rab52, rab81. The sequences were alignmed with MUSCLE in Geneious. Dots indicate residues which are identical to the corresponding position in p6. Numbers indicate the amino acid position in p6.

Figure S2: Multiple amino acid sequence alignment of the ORF1-VXH-encoded protein segment from the model strains p6, 47832mc, rab52, rab81. The sequences were alignmed with MUSCLE in Geneious. Dots indicate residues which are identical to the corresponding position in p6. Numbers indicate the amino acid position in p6.

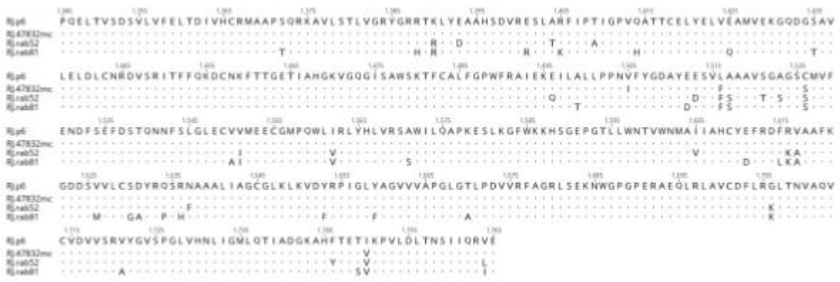

Figure S3: Multiple amino acid sequence alignment of the ORF1-RJ-encoded protein segment from the model strains p6, 47832mc, rab52, rab81. The sequences were alignmed with MUSCLE in Geneious. Dots indicate residues which are identical to the corresponding position in p6. Numbers indicate the amino acid position in p6 [19,29].

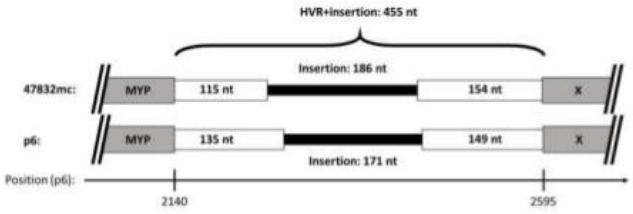

Figure S4: Comparison of the HVR insertions of p6 and 47832mc. While the insertion of p6 is shorter than the insertion within the HVR of 47832mc by 15 nucleotides, remainder of the p6 HVR is longer by the same amount. As a consequence, the total lengths of both HVRs are identical [19,29].

4 Own Contributions to Publications

Publication I:

Priemer, G.; Cierniak, F.; Wolf, C.; Ulrich, R. G.; Groschup, M.H.; Eiden, M. “Co-Circulation of Different Hepatitis E Virus Genotype 3 Subtypes in Pigs and Wild Boar in North-East Germany, 2019.”
Pathogens 2022, 11, 773, doi:10.3390/pathogens11070773.

Grit Priemer: methodology, formal analysis, investigation, resources, data curation

Filip Cierniak: methodology, software, writing - original draft preparation, writing - review and editing, visualisation

Carola Wolf: Conceptualisation, methodology, resources

Rainer G. Ulrich: validation, writing - review and editing

Martin H. Groschup: Conceptualisation, validation

Martin Eiden: Conceptualisation, methodology, validation, formal analysis, investigation, data curation, writing - original draft preparation, writing - review and editing, visualisation

Publication II:

Cierniak, F.; von Arnim, F.; Heckel, G.; Ulrich, R.G.; Groschup, M.H.; Eiden, M. “A Putative Novel Hepatitis E Virus Genotype 3 Subtype Identified in Rabbit, Germany 2016.“ **Viruses** 2021, 13, 1065, doi:10.3390/v13061065.

Filip Cierniak: methodology, software, investigation, data curation, writing - original draft preparation, writing - review and editing, visualization

Felicitas von Arnim: methodology, investigation, resources

Gerald Heckel: validation, writing - review and editing, supervision

Rainer G. Ulrich: validation, writing - original draft preparation, writing - review and editing, supervision

Martin H. Groschup: Conceptualization, supervision, funding acquisition, writing - review and editing

Martin Eiden: Conceptualization, methodology, validation, data curation, writing - original draft preparation, supervision, project administration, writing - review and editing

Publication III:

Cierniak, F.; Ulrich, R.G.; Groschup, M.H.; Eiden, M. “A Modular Hepatitis E Virus Replicon System for Studies on the Role of ORF1-Encoded Polyprotein Domains.” **Pathogens** 2022, 11, 355, doi:10.3390/pathogens11030355.

Filip Cierniak: Conceptualization, methodology, software, investigation, data curation, writing - original draft preparation, writing - review and editing, visualization

Rainer G. Ulrich: validation, writing - original draft preparation, writing - review and editing, supervision

Martin H. Groschup: Conceptualization, writing - review and editing, supervision, funding acquisition

Martin Eiden: Conceptualization, methodology, validation, resources, data curation, writing - original draft preparation, supervision, project administration

The above information is confirmed by signature:

Filip Cierniak	Prof. Dr. Rainer G. Ulrich

5 Discussion

The goal of this study was to provide a perspective on zoonotic HEV in Germany, with a particular focus on the rabbit / rabbit HEV model system, in a One Health context. The overarching goal of the project was to further the understanding of zoonotic HEV, with a focus on pigs and especially rabbits as the animal reservoir hosts in Germany. This was achieved by diagnostic screening and phylogenetic analysis of HEV sequences from pigs in north-eastern Germany (publication I), sequence determination and phylogenetic analysis of a novel rabbit HEV genome (publication II) which stood out in an earlier screening study [93] due to its phylogenetic divergence from the cluster of rabbit-associated HEV sequences, and assessment of this sequence information for replication in cell culture by an RGS. The low replication of rabbit HEV isolates in cell culture made it effectively impossible to recover detectable titers of infectious virus for infection experiments, and only barely allowed detecting the replication of those strains in a luciferase reporter assay. This dilemma led to a more complex, generalized approach to the HEV RGS workflow than originally intended, with a systematic comparison of subgenomic fragments of rabbit HEV with established cell culture isolates of HEV (publication III).

5.1 HEV in swine in north-eastern Germany

In order to identify potential candidate strains, broad screening of putative host populations is the tool of choice. Publication I is an investigation into the current phylogenetic distribution of zoonotic HEV in the region of Mecklenburg-Western Pomerania conducted in cooperation with the LALLF. A sample of almost 400 pigs and wild boars was tested for the presence of HEV RNA. The analysis showed that approximately 10% of the animals were positive for virus RNA. The viruses detected in publication I encompass a wide range within the genotype HEV-3, but no other genotypes were identified (publication I, table 1). This further confirms that, in Germany, HEV-3 is the predominant genotype circulating in pigs. These results are in agreement with many similar studies. A high seroprevalence of HEV was detected in several regions of Germany [242,243] and other European countries, such as Spain [244], France [245], or Italy [246]. Taken together, these findings demonstrate that HEV circulates in many European populations of wild boars and domestic pigs. One striking exception to the rule is the recent study conducted by Westphal and colleagues, who investigated boars and deer in an enclosed game reserve in northern Germany and found no evidence of HEV infection [247]. This may be a proof that the transmission between different boar or pig populations plays a major role in the epidemiology of zoonotic HEV. In line with this, the wide variety of HEV-3 subtypes (3a, 3c, 3e, 3f, 3k, and four 3i-like sequences, thus

representing all major subclades of HEV-3 with the exception of HEV-3ra) detected in publication I indicates that the HEV population is not traceable to a single infection event but rather that HEV has been introduced into the population several times independently. The high prevalence of HEV infection in commercial pigs in Germany and other countries, as well as the trade and movement of pigs between farms has been discussed as a possible reason [248]. This poses a considerable food safety risk, especially for immunocompromised patients. Studies from other regions show that contaminated food products are a major source of HEV infection. While this risk can be mitigated by sufficient heating, studies by Wolff et al. show that other methods of meat processing do not sufficiently inactivate HEV [249,250]. This is reflected by data from France and Switzerland, where raw sausages were identified as a source of human HEV infection [251,252]. A comparable study on HEV infections in humans from the north-eastern Germany is necessary to confirm and quantify the risk.

Interestingly, one of the animals tested was highly positive for HEV RNA in muscle tissue. The possibility of extrahepatic HEV replication has been known for over 20 years [253]. However, the main known sites of extrahepatic HEV detections are digestive, lymphatic, or neural tissues rather than muscle tissue [254]. HEV RNA has been detected in muscle tissue by other studies [92,255–258]. However, virus RNA titers in muscle tissue were lower than in the liver, which always raises the question whether the detection of HEV RNA was due to virus replication in muscle tissue or due to a contamination during the extraction process. In contrast, in publication I, approximately five-fold higher RNA titers were detected in the muscle tissue compared to the liver samples. Additionally, the tissue examined in publication I was a lateral femoral muscle, which has no direct contact to the liver. This means that contamination during the extraction process is less likely than, for example, in the case of diaphragm tissue, which has been used in some other studies [259–261] and that is in direct contact with the liver. A contamination could still occur due to circulating virus RNA in the bloodstream, although in this case the detectable titers should be lower than in the liver. For a definite proof of HEV replication, negative strand RNA should be attempted in future studies. Nevertheless, the evidence for HEV replication in muscle tissue raises the question which viral or host factors are required. Further characterization of the virus sample is advisable. A first step could be the sequencing of the full-length viral genome, and possibly an attempt at an infection in cell culture. This should then be followed up on by constructing a cDNA clone and reporter replicon for further analysis. Finally, chimeric replicons can be used to further characterize the viral genome and its functionality in comparison to other HEV strains.

5.2 A putative novel rabbit-associated HEV-3 subtype

The strain of rab81 had been first identified in a surveillance study of German wild rabbits and brown hares carried out by Hammerschmidt and colleagues [93]. Based on sequence analysis of a short amplicon from the RdRp domain-encoding sequence of ORF1, the strain was initially assigned to the phylogenetic clade of subtype HEV-3g rather than the rabbit-associated clade of subtype HEV-3ra. However, a conclusive phylogenetic analysis was not possible since the complete genome sequence could not be determined via Next Generation Sequencing (NGS) [93]. Publication II describes the determination of the complete genome sequence of the rab81 strain as well as a comprehensive phylogenetic and recombination analysis of the sequence. Upon generation of the full-length sequence by RT-PCR based recovery and sequencing of overlapping rab81 genome fragments, a comprehensive phylogenetic analysis revealed that this virus strain was not closely related to any other known sequence and did not fit within any of the established subtypes [36]. Recombination analysis revealed inconclusive evidence for a mosaic-like genome composition (publication II, Figure 4b). While bootscan analysis did show differential clustering of distinct genomic regions, the bootstrap support was low, which does not support the hypothesis of a recent recombination event. Interestingly, the genome structure had some similarity to a recently discovered genotype 3/rabbit HEV recombinant [262], including a three nucleotide insertion within the ORF2/ORF3 overlap region, despite not being closely related. The same region is commonly used in diagnostic PCR assays, due to being highly conserved [263,264]. The novel sequence contains a point mutation localized within the probe binding sequence, which drastically reduces the sensitivity of one of the most commonly used RT-qPCR assays [263] (publication II, Figure S2). As a result, this sequence would likely have been missed in many routine screenings. This is a possible reason for the lack of known related sequences. It is likely that similar strains circulate in Germany and possibly other countries but remain undetected. Adapted assays with optimized probes, different target regions can ameliorate this issue. Alternatively, more sophisticated methods may be used, such as NGS, probably combined with target enrichment. These results show that many aspects of HEV epidemiology remain unknown and emphasize the role of the rabbit as HEV host and model organism. In particular, the detection of novel and recombinant HEV strains in wild rabbits is of major interest. Rabbits are not only susceptible for rabbit HEV but can also be infected with isolates from pigs and other species. It is worth considering how this affects the distribution and evolution of the virus and which risk it poses to other host species, especially humans. Further efforts will be needed to reveal more about the genetic variability of the HEV strains circulating in Germany.

5.3 A modular replicon system for HEV

In addition to phylogenetic analysis, the functional characterization of HEV in a cell culture model system was the objective of publication III. For this purpose, two model strains were chosen: Rabbit HEV (HEV-3ra) strain rab52 [93] and newly described non-standard rabbit-derived HEV-3 strain rab81 (publication II). Early efforts were impeded by the low replicative activity exhibited by those two strains in a cell culture system. For comparison, cell culture-adapted clones Kernow-C1/p6 (provided by Dr. Patricia Farci) [114] and 47832mc (provided by Prof. Dr. Reimar Johne) [228] were examined as well. Kernow-C1 was originally isolated from a fecal sample of a patient with chronic hepatitis E and passaged six times in HepG2/C3A cells (hence, “p6”) [208]. The strain grows exceptionally well in cell culture. Remarkably, it is able to infect and replicate in cells from various tissues and several species [208]. The enhanced replicative ability of the Kernow-C1/p6 strain has been traced to a unique insertion of a fragment from the human S17 ribosomal protein-encoding sequence within the HVR of ORF1 and three point mutations within the X domain, which were selected during passaging [114]. Similarly, 47832mc was originally isolated from a serum sample of an organ-transplant recipient with chronic HEV infection (serum sample 47832) [212]. In contrast to several other HEV strains examined by Johne and colleagues (two further serum samples from acutely infected human patients and one liver sample from an infected wild boar), the virus from sample 47832 was successfully passaged in A549 cells, during which it acquired several point mutations (strain 47832c) [212]. Finally, Scholz et al., generated a cDNA clone of the 47832c strain (“molecularly cloned”, thus designated as 47832mc), and were able to recover infectious virus upon transfection of BSR-T7/5 cells [228]. In another striking similarity to the Kernow-C1/p6 strain, strain 47832 contains an insertion within the HVR of ORF1 [212]. Scholz et al. further showed that this insertion is critical for productive infection of cultured cells [115]. Unlike the insertion of the Kernow-C1/p6 strain, the insertion of 47832 is not of human origin. Instead, it consists of a rearranged fragment of its own ORF1 [212]. Interestingly, the insertions are of different lengths but when considered in the context of their own genomic backbones, they extend each of the HVRs to the exact same length of 455 nucleotides (publication III, Figure S4). Neither of the rabbit-derived HEV strains, rab52 or rab81, contain a similar insertion within the HVR or similar adaptations to growth in cell culture. This may explain, at least in part, why their detectable activity is much lower. In direct comparison, the rab52 and rab81 reporter replicons produced around four orders of magnitude less signal than the Kernow-C1/p6 or 47832mc-based replicons did (publication III, Figure 2). Strikingly, except for the amplitude, the luciferase signal curves of the rabbit-derived replicons were almost identical to those of the culture-adapted replicons, characterized by a peak on day two or three after transfection followed by declining signal

levels. However, these results alone do not explain *why* the rabbit-derived strains replicate so much less efficiently.

Until now, it has been difficult to ensure comparability between different HEV cell culture strains and replicons. Because there are only few, specific HEV strains capable of efficient growth in cell culture, the established systems are typically limited to distinct, singular viruses which are not closely related. This makes it difficult to determine the genomic features responsible for cell culture compatibility. In some cases, there may be a particular feature, such as HVR insertions in the genomes of 47832 and Kernow-C1/p6, which can be associated with growth in cell culture [114,115]. In other cases, growth in cell culture can be observed without any specific genome characteristic [33,241]. This suggests an adaptive mechanism distinct from HVR insertions. However, so far, little is known about this mechanism. Mutations, particularly those that result in altered amino acid residues within the NSP, are expected to influence the replicative fitness of the virus. However, the relatively low sequence identity between the respective replicons makes it impossible to predict which specific domain or genomic region is mainly responsible for the difference in reporter gene expression. At the same time, due to the number of different substitutions, it is hardly feasible to examine each one individually. Instead, publication III demonstrates an approach to characterize individual genomic regions of HEV with regard to their contribution to viral replication. The subdivision of the viral genomes into equivalent subgenomic fragments made it possible to assemble chimeric replicons and test the activity of ORF1 domains from different viruses in the same genomic backbone. A homologous subdivision pattern ensured comparability between donor strains. This way, the HEV genome was divided into four fragments: First, the 5'-terminal fragment, which contains the coding sequence of the N-terminal region of the NSP with the MT, Y, and PCP domains ("MYP"). Second, a fragment encoding the HVR, X, and Hel domains ("VXH"). The third fragment ("RJ") encodes the RdRp and carries the intergenic junction region (JR) with the subgenomic promoter. Finally, the 3'-terminal fragment that contains ORF2 and ORF3, or the coding sequence of the reporter gene in the case of a subgenomic replicon (publication III, Figure 3 A).

Publication III further shows that the 5’-terminal subgenomic fragment (“MYP”), plays a vital role in determining HEV replication rates in HepG2 cells (publication III, Figure 3). Out of the subgenomic fragments examined in the study, MYP showed the highest potential for modulation of the reporter gene expression. Interestingly, substituting MYP from Kernow-C1/p6 with the corresponding fragment of 47832mc significantly increased luciferase output on the first day after transfection, and accelerated the growth kinetics, whereas the corresponding MYP fragments of rab52 and rab81, which lowered GLuc activity by two orders of magnitude. This difference is comparable to a deletion of the HVR insertion in

Kernow-C1/p6 [114], or an exchange of the HVR-containing VXH fragment, which also reduced luciferase activity by two orders of magnitude when the rabbit-derived fragments were used (publication III, Figure 3). The MYP fragment is involved in the regulation of cellular immune responses and RNA replication. Considering that neither Kernow-C1/p6 nor 47832mc showed any mutational adaptations within this region by passaging [114,212], it is possible that high activity of one or several of the functional domains within MYP is a prerequisite for high viral replication, before further adaptations can occur. Surprisingly, exchanging the RJ fragment had comparatively little effect on replicon activity. On the other hand, the RdRp has been a target for optimization of the Kernow-C1/p6 system in the past [211], which means that there is room for improvement within this domain.

A key aspect which aided in establishing context between the different strains and subgenomic fragments was the choice of a uniform replicon assembly strategy to minimize the degrees of freedom that arose from the experimental design. In the past, different authors employed various methods to assemble HEV cDNA clones and replicons. For publication III, the replicon architecture according to Shukla et al. [114] was used as a base line, refined, and applied to the model strains. The reporter and the insertion locus were not changed. The *Gaussia* luciferase (GLuc) ORF is inserted in frame with the start codon of ORF2. This leads to more efficient reporter gene expression compared to an insertion at the start codon of ORF3. Furthermore, 377 nucleotides are deleted from the 5'-terminal end of ORF2, which prevents the formation of infectious virions and thus allows for more direct examination of the replicase activity at a substantially reduced biosafety risk. GLuc is an enzyme which emits light as a by-product of the oxidative decarboxylation reaction that converts coelenterazine to coelenteramide zand has several advantages as reporter compared to alternative systems. It produces a stronger signal than firefly or *renilla* luciferases and is relatively small (185 aa / 555 bp). Furthermore, the protein is secreted very efficiently from within the cell, which allows detection of reporter activity in the cell culture supernatant, without disturbing the cells [265]. A key disadvantage of GLuc is the enzyme's intense but short-lived signal emission due to covalent binding of substrate derivatives and consequently irreversible inactivation of the enzyme [266]. In practice this requires measurement of light output immediately after substrate addition. For future systems, a modified GLuc reporter system with more stable signal output should be considered [267]. Alternatively, NanoLuc (NLuc) could be used instead, which has similar properties as GLuc (strong signal, small size, can be secreted) but catalyzes a more stable reaction with Furimazine as substrate [268].

Additional factors to be considered are the untranslated regions (UTRs) of the HEV genome and the length of the 3' poly(A) tail. The HEV genome contains three UTRs: The 5'-

UTR, the 3'-UTR and the Junction Region (JR) between ORF1 and ORF2/ORF3. The UTRs are regions of the HEV genome with conserved sequence motifs that have a significant influence on HEV replication. The 5' UTR is crucial for the translation of the HEV proteins as it contains the methylguanosine cap structure [269]. Additionally, it may be required as a binding site for the RdRp during genome replication [103]. The JR contains the subgenomic promoter [237] and is therefore an important factor for the expression of ORF2/ORF3, or the reporter gene in accordingly assembled replicons. The 3'-UTR contains a conserved stem loop that is critical for replication and infectivity [270] by binding specifically to the viral RdRp [271]. Notably, none of these UTRs were examined independently in publication III, but in unison with the 'MYP', 'RJ', and 3'-terminal fragments, respectively. Any influence the UTRs may have on the reporter gene expression can therefore not be quantified directly. This should be investigated further in future studies. Furthermore, little is known on the effect of poly(A) length on HEV replication, although the virus is known to possess a polyadenosine tract at the 3'-terminus. A clear correlation between tail length and translation efficiency and RNA stability exists in eukaryotic mRNA [272]. Furthermore, in other viruses with poly(A) tails, such as corona viruses [273] or tick-borne encephalitis virus [274], a clear influence of poly(A) length on the viral life cycle was determined. In view of the work of Panda and colleagues, who describe an infectious HEV cDNA clone with a poly(A) tail of only 5 nucleotides [224], the length of the poly(A) tail is likely not an essential factor for HEV replication. Other authors used different lengths, for example, the 47832mc clone contains 23 nucleotides of poly(A) [228], while for Kernow-C1/p6 the tail is 36 nucleotides long [114]. These lengths are not based on the actual poly(A) tails in infectious virions, due to how difficult it is to determine the precise lengths and was chosen somewhat arbitrarily by the respective authors. Evidently, each length is sufficient to produce functional positive strand virus RNA. However, for direct comparison, it makes sense to normalize the poly(A) tail lengths in order to equalize their influence on virus replication. For publication III, the length of 26 nucleotides was chosen. This length is similar to the size of the repeating unit within the cytoplasmic poly(A)-ribonucleoprotein complex (approximately 24-30 nt) [275]. To clarify which effect the length of the poly(A) tail has on HEV replication, different lengths can be systematically investigated in future studies. An additional element of standardization is added to the 3' terminus by ensuring all constructs are linearized using the restriction endonuclease SwaI. The recognition site of this enzyme (ATTTAAAT) is 8 nucleotides long, which is 2 nucleotides more than in alternative strategies that employ endonucleases such as BglII [225], XhoI [224], or MluI [114], and therefore approximately 16 times less likely to occur by chance in any HEV genome. A disadvantage of Type IIP restriction endonucleases, such as SwaI, is that cleavage with those enzymes results in a "masked" terminus. In the

case of SwaI, that means that rather than releasing the template for an RNA ending in …AAAAAAA, the resulting 3'-terminus of the replicon RNA is …AAAATTT. In principle, an "unmasked" tail would be ideal, however, a type IIS endonuclease, such as SapI, would be required to achieve that [276]. This may be further optimized in the future.

Type IIS endonucleases have a decisive advantage over Type IIP enzymes for replicon assembly. Type IIS enzymes cleave DNA outside their recognition motif. That means that the overhang sequence of the cleavage product is not dependent on the recognition motif and can be chosen non-palindromic. This allows for highly efficient directed assembly with efficiencies near 100%. The most well-known application of Type IIS enzymes is Golden Gate Assembly [277] but they have also been used in specific virus reverse genetics protocols [278]. Crucially, a recent publication [279] quantified ligation efficiencies of 4 nucleotide sticky-ends, a tremendous benefit especially for multi-fragment assemblies. Accordingly, 4 nucleotide recombination sites with high ligation rates but low cross-reactivity were chosen which are conserved across a wide range of HEV genomes and in a position where putative functional domains of ORF1 are not disturbed. This should facilitate replication of this work and similar assays in the future with HEV strains beyond the four model strains investigated in publication III.

6 Outlook

HEV has (re-)emerged as a major zoonotic pathogen both in developing and industrialized countries. This has led to a reevaluation of the epidemiological factors in recent years from a pure human-to-human spread in countries with poor access to sanitation infrastructure to global zoonotic spread as the main infection route. HEV has a uniquely broad host range. Zoonotic transmission to humans has been confirmed from various species, such as swine, deer, camels, and rabbits. As a result, HEV has been described as “one of the most successful zoonotic viral diseases in human history” [13]. Consequently, hepatitis E can only be adequately understood and addressed from a One Health context. Further research is needed to ascertain the spread of HEV in animal hosts around the world, and the presence of related strains in humans. As shown in this work and other publications, HEV infection is highly prevalent in swine (family Suidae), which poses a substantial risk of infection for humans exposed to those animals. Future work should focus on assaying HEV infections in humans, especially in areas of known high prevalence in wild and domestic animals. Phylogenetic analysis can then be used to ascertain how the infection cycles of different hosts are linked.

To understand these infection networks, it is crucial to understand the interactions between the virus and each of its hosts. In addition to humans and swine, the role of rabbits in the infection process deserves further attention. Although there is evidence from several countries that HEV infection in rabbits is common worldwide, the implications for Hepatitis E epidemiology in general and HEV infection in humans in particular are not well understood. Future efforts should therefore focus on determining the epidemiology of HEV in various rabbit populations in comparison to human and swine populations in the same areas. In particular, the role of rabbits as a reservoir for novel HEV strains and as a possible facilitator of recombination between different strains should be considered. It is important that diagnostics are adapted and expanded to detect unknown, divergent strains which evade detection by established methods. The detection range of RT-qPCR-based assays can be increased by modified probes or alternate target regions. Furthermore, the cost-effectiveness of more sophisticated methods, such as NGS combined with target enrichment, should be considered.

However, diagnostic efforts alone are not sufficient to determine the key determinants of the viral life cycle. Only controlled experiments in a laboratory setting can solve this problem. For this purpose, RGS and reporter replicons are invaluable tools. Building on the results and methods presented in this work, it should be possible to further uncover the mechanisms of HEV replication and infection. Furthermore, by specifically studying target regions of the viral genome from different strains in terms of their effect on infectivity and pathogenicity in

different animals, determinants of the host range and interspecies transmission can be addressed. Finally, chimeric replicons could be used to find the right therapeutic agent in a personalized way, even if the specific virus strain in question does not, grow efficiently in cell culture. Using a cell culture isolate as genomic backbone, each domain of the patient isolate can be tested in different inhibitor assays. This represents a potential tool for the scientific understanding of HEV, as well as a tool to combat and cure Hepatitis E from a medical or pharmaceutical perspective.

7 Summary

Hepatitis E virus (HEV) is emerging worldwide as a zoonotic pathogen that has remained largely undetected for decades, if not centuries. Its enormous success can be attributed to the wide range of host species, which can transmit the virus to humans, depending on the viral genotype. As a result, HEV is likely to remain a challenge even when the remaining hepatitis viruses (HAV, HBV, HCV), which are transmitted exclusively between humans, are under control. Although millions of HEV infections occur each year, little is known about this puzzling pathogen. One major issue in HEV research is the lack of reliable model systems. Established animal models are inefficient, expensive, or simply not representative of human HEV. On the other hand, cell culture systems are limited by the slow growth of the virus and inefficient replication and infection. The aim of this work is to with deepen the understanding of zoonotic HEV in animal hosts in Germany. For this purpose, a molecular and phylogenetic characterization of HEV sequences from rabbits and swine was conducted. A novel subtype of the zoonotic genotype HEV-3 was identified in a rabbit sample, further emphasizing the role of rabbits as HEV host species and possible reservoir of zoonotic HEV infections in Germany. On the other hand, a molecular biological screening of pigs and wild boars in Mecklenburg-Western Pomerania indicates a wide range of HEV-3 subtypes circulating in swine in north-east Germany. Furthermore, an optimized replicon system was established in order to enable characterization of various HEV sequences by reverse genetics. As a proof of concept, two rabbit HEV derived replicons were compared with two established, cell culture adapted HEV strains. The influence of different regions of the nonstructural protein on HEV replication was determined and quantified. In particular, a system was established, to reproducibly compare different strains and genotypes. This refined replicon system will enable the characterization of further HEV sequences and thus expand the knowledge on the determinants of the viral life cycle.

8 Zusammenfassung

Hepatitis-E-Virus (HEV) erweist sich zunehmend als zoonotischer Erreger mit weltweiter Verbreitung, der Jahrzehnte, wenn nicht Jahrhunderte, unbemerkt blieb. Der enorme Erfolg von HEV ist auf sein weites Spektrum an Wirtsspezies zurückzuführen, welche das Virus, je nach Genotyp, auch an Menschen weitergeben können. Folglich wird HEV auch dann ein Problem bleiben, wenn die übrigen Hepatitisviren (HAV, HBV, HCV), die ausschließlich zwischen Menschen übertragen werden, unter Kontrolle sind. Trotz Millionen jährlicher HEV-Infektionen ist nur wenig über diesen Erreger bekannt. Eine der größten Hürden in der Erforschung von HEV ist das Fehlen zuverlässiger Modellsysteme. Etablierte Tiermodelle sind aufwendig, und oft nicht repräsentativ für HEV-Infektionen beim Menschen. Zellkultursysteme andererseits sind limitiert durch langsames Viruswachstum und niedrige Titer sowie geringe Infektionseffizienz. Diese Arbeit zielt darauf ab, das Verständnis des zoonotischen HEV bei Tieren in Deutschland zu verbessern. Zu diesem Zweck wurden HEV-Sequenzen aus Kaninchen und Schwein molekular und phylogenetisch charakterisiert. In einer Kaninchenprobe wurde ein neuartiger Subtyp des zoonotsichen Genotyps HEV-3 identifiziert. Dies betont nochmals die Relevanz von Kaninchen als Wirtsspezies und Reservoir zoonotischer HEV-Infektionen in Deutschland. Zudem deutet ein molekularbiologisches Screening von Haus- und Wildschweinen in Mecklenburg-Vorpommern auf die Zirkulation mehrerer HEV-3 Subtypen im Nordosten Deutschlands hin. Des Weiteren wurde ein optimiertes Replikonsystem etabliert, das die Charakterisierung verschiedener HEV-Sequenzen mittels reverser Genetik ermöglicht. Als „proof of concept“ wurden zwei Kaninchen-HEV-abgeleitete Replikons mit zwei etablierten, zellkulturadaptierten Stämmen verglichen. Der Einfluss verschiedener Regionen des Nichtstrukturproteins auf die HEV-Replikation wurde gezeigt und quantifiziert. Insbesondere wurde ein System etabliert, mit dem verschiedene Stämme und Genotypen reproduzierbar miteinander verglichen werden können. Dieses weiterentwickelte Replikonsystem wird in Zukunft die Charakterisierung weiterer HEV-Sequenzen ermöglichen und damit das Wissen über Determinanten des viralen Lebenszyklus vertiefen.

9. Literature

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10 Appendix

10.1 Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde. Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

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