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Surveying the global virome: Identification and characterization of HCV-related animal hepaciviruses

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Abstract

Recent advances in sequencing technologies have greatly enhanced our abilities to identify novel microbial sequences. Thus, our understanding of the global virome and the virome of specific host species in particular is rapidly expanding. Identification of animal viruses is important for understanding animal disease, the origin and evolution of human viruses, as well as zoonotic reservoirs for emerging infections. Although the human hepacivirus, hepatitis C virus (HCV), was identified 25 years ago, its origin has remained elusive. In 2011, the first HCV homolog was reported in dogs but subsequent studies showed the virus to be widely distributed in horses. This indicated a wider hepacivirus host range and paved the way for identification of rodent, bat and non-human primate hepaciviruses. The equine non-primate hepacivirus (NPHV) remains the closest relative of HCV and is so far the best characterized. Identification and characterization of novel hepaciviruses may in addition lead to development of tractable animal models to study HCV persistence, immune responses and pathogenesis. This could be particular important, given the current shortage of immunocompetent models for robust HCV infection. Much remains to be learned on the novel hepaciviruses, including their association with disease, and thereby how relevant they will become as HCV model systems and for studies of animal disease. This review discusses how virome analysis led to identification of novel hepaci- and pegiviruses, their genetic relationship and characterization and the potential use of animal hepaciviruses as models to study hepaciviral infection, immunity and pathogenesis. This article forms part of a symposium in Antiviral Research on “Hepatitis C: Next steps toward global eradication.”

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1. Introduction

Viral zoonoses account for most of the emerging human viral diseases (Wolfe et al., 2007). Humans are constantly exposed to a vast pool of animal viruses but the biological and epidemiological barriers to interspecies virus transmission are high and therefore the majority of viruses that infect wildlife and domestic animals do not readily infect humans (Parrish et al., 2008). Sustained contact between humans and animals increases the likelihood of emergence of a virus adapted to infect and replicate in humans either directly or through intermediate hosts (Morse et al., 2012). Similar modes of transmission and tissue tropism is common for viruses transmitted between animals and humans, as seen e.g. for influenza virus and human/simian immunodeficiency virus (HIV/SIV). Pathogenesis can be absent or similar, but also dramatically different when transmitted to a novel host.

Viruses that have long circulated in a particular host are typically well adapted and may cause low pathology, whereas pathogenesis may immediately become apparent in the novel host, exemplified by transmission of Ebola virus from bats to humans. Study of certain animal viruses can therefore provide new insights into the natural history of human viruses, and, in some instances, surrogate models for investigating the prevention and treatment of human disease (Bukh, 2012; Evans and Silvestri, 2013; Wobus et al., 2006). Moreover, identification of evolutionary conserved or divergent genomic regions of related viruses can help identify nucleotide or protein sequences important for entry, replication, host and tissue specificity, immune responses and pathogenesis. Thus, in addition to understanding animal disease, identification of animal viruses is of importance to map zoonotic reservoirs, elucidate the origin and evolution, and develop model systems for important human viruses. Survey of animal viromes is an efficient way to identify animal homologs of human viruses.

Around 150 million people worldwide are chronically infected with hepatitis C virus (HCV), and therefore at increased risk for developing liver cirrhosis and hepatocellular carcinoma. HCV infection is typically persistent with only 20–30% of cases resolving after acute infection (Hoofnagle, 2002). Interferon-based antiviral therapy only leads to ~50% sustained viral response and is often poorly tolerated. Recent introduction of interferon-free regimens of direct-acting antivirals have tremendously improved both measures (Scheel and Rice, 2013). HCV, except for the enigmatic GB virus B (GBV-B), is the only member of the genus *Hepacivirus*, one of the four genera in the positive strand RNA virus family, *Flaviviridae* (Smith et al., 2014). Its origin therefore remains elusive. The HCV genome of ~9600 nts is composed of one long ORF flanked by 3′ and 5′ untranslated regions (UTRs). The ORF derived polyprotein is cleaved by host and viral proteases into structural (Core, E1, E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins. In addition, an alternative reading frame protein (ARFP) has been described (Gottwein and Bukh, 2008). Robust HCV replication has only been observed in hepatocytes, and appears at least in part to be determined by requirement for the entry factors CD-81, SR-BI, claudin-1 and occludin (Ploss and Evans, 2012) as well as the liver-specific micro-RNA-122 (miR-122) (Wilson and Sagan, 2014).

The recent identification of equine, rodent, bat and Old World primate hepaciviruses – kick-started by deep sequencing virome analysis – is intriguing, since it vastly broadens the

Hepacivirus genus and may provide clues to the origin of HCV. The animal homologs also have the potential to offer tractable models for HCV. Humans are the only species naturally infected by HCV, whereas chimpanzees can be experimentally infected. However, since the chimpanzee is no longer available for most NIH-sponsored research, there currently is a lack of immunocompetent animal models for the study of HCV persistence, immunity and pathogenesis. It remains unclear, however, to what degree pathogenesis of animal hepaciviruses mirror that seen for HCV in humans, and therefore to what extent these viruses will become useful as model systems or for understanding animal disease. This review discusses the importance of global virome analysis, our current knowledge of the animal hepaciviruses and their potential as models to study hepacivirus infection.

2. Characterizing the global virome

The global virome comprises all viruses that infect life forms, or simply all viruses that exist in nature. Whereas some viruses are restricted to a specific host, others can infect several species. Thus, the “virome of a specific species” should include only those viruses that *reside in* and *most frequently* infects that specific host. The virome of a host may also include endogenous viruses or virus-like elements and viruses that infect microbes inhabiting that host (Virgin, 2014). Developments of high-throughput sequencing platforms resulted in dramatic reductions in costs and dramatic increases in unbiased discovery of novel sequences, including those of animal viruses (Lipkin, 2013). Some of these new viruses are so different from known viruses that they can only be classified as the first recognized members of new virus families (Kapoor et al., 2010). Although these advances theoretically promise complete genetic characterization of the global virome, better tools to identify viruses highly divergent from any known pathogen are needed (Anthony et al., 2013; Delwart, 2013).

The focus of most viral discovery studies has been the identification and characterization of human viruses because of their clinical and public health importance (Wylie et al., 2012). A comprehensive survey of the global virome will likely reveal a plethora of animal homologs of human viruses and thereby lead to a better understanding of the origin of human viruses and potentially of human and animal disease (Anthony et al., 2013). Further, this knowledge will enable rapid identification of natural reservoirs for emerging viral pathogens and development of means to prevent future cross-species virus transmissions. Unbiased deep sequencing approaches have paved the way for identification of a number of previously unknown hepaci- and pegiviruses, specifically by identifying canine/equine and Old World primate hepaciviruses and equine and bat pegiviruses (Chandriani et al., 2013; Epstein et al., 2010; Kapoor et al., 2011; Lauck et al., 2013; Quan et al., 2013).

3. HCV molecular epidemiology

HCV is a highly diverse virus grouped into 7 major genotypes and numerous subtypes (a, b, c, etc.), with ~30% and ~20% differences, respectively, at the nucleotide and amino acid level, (Smith et al., 2014). Distinct differences are observed in geographic distribution, with genotype 1 dominating in the Americas, Europe and Japan; genotypes 2 and 3 are also prevalent in these regions. Genotypes 3 and 6 are widespread in South and Southeast Asia,

and genotypes 4 and 5 are most common in Africa but spreading to Europe. The chronic nature of HCV infection and large quasispecies population within infected individuals contribute significantly towards the rapid pace of viral evolution (Gray et al., 2011). The primary driver of HCV diversity is the error-prone viral RNA-dependent RNA polymerase (Gottwein and Bukh, 2008). In addition, viral RNA recombination occurs at a low and potentially underestimated level (Galli and Bukh, 2014).

Most of our current understanding of HCV origin is based on the comparative genetic and evolutionary analysis of HCV sequences recovered from samples collected worldwide since the discovery of the virus in 1989 (Choo et al., 1989). HCV variants in Sub-Saharan Africa and South East Asia have the greatest genetic diversity, consistent with the hypothesis that the HCV variants now predominant in developed countries have been circulating for hundreds of years and were disseminated from these places (Simmonds, 2013). Studies of the natural history and epidemiology of HCV further indicate that the HCV epidemic in Western countries expanded through the last 100 years (Gray et al., 2013; Magiorkinis et al., 2009; Pybus and Gray, 2013). The subsequent global dissemination, in particular of HCV subtypes 1a and 1b, was probably accelerated after World War II through medical treatments, immunization, blood transfusion and injection drug use. The HCV major genotypes and subtypes have been predicted to derive from a common ancestor 100 to 300 years ago (Gray et al., 2013; Magiorkinis et al., 2009; Smith et al., 1997), although these numbers may be underestimations (Simmonds, 2013).

The endemic focus of HCV in Central Africa and more recent pandemic spread is highly reminiscent of the emergence of HIV-1, for which a chimpanzee origin has been demonstrated (Peeters and Delaporte, 2012). This motivated investigations of the possible existence of HCV-like viruses in non-human primates. Until 2011, the only other classified member of the *Hepacivirus* genus was the enigmatic GBV-B, isolated from laboratory tamarins (Simmonds, 2013; Simons et al., 1995; Stapleton et al., 2011). Since then, our knowledge on hepacivirus diversity has been transformed by the identification of HCV homologs in a range of mammalian species, including horses, Old World monkeys, rodents and bats (Burbelo et al., 2012; Drexler et al., 2013; Kapoor et al., 2011; Kapoor et al., 2013b; Lauck et al., 2013; Quan et al., 2013). Widely divergent hepaciviruses infecting the same rodent or bat species suggests that hepaciviruses have been transmitted between species, although much remains to be learned on the evolutionary history of hepaciviruses. Thus, a zoonotic origin of HCV in humans is a possibility, but with considerable uncertainty about the identity of the source species (Pybus and Gray, 2013).

4. Animal homologs of HCV

Until 2011, GBV-B was the only known homolog of HCV. Initially, a serum sample of a surgeon (initials GB) suffering from acute hepatitis was used to infect tamarins and infected animals developed acute hepatitis. The transmissible agent was termed GB agent (Deinhardt et al., 1967). In 1995, genomic sequencing of tamarin passaged GB agent revealed the existence of two distinct virus species, termed GBV-A and -B (Simons et al., 1995). Since then, several studies have failed to find GBV-B virus in humans, including the surgeon GB, other South American primate species, Old World monkeys and apes further adding to its

obscure origin (Stapleton et al., 2011). However, genetic analyses of the only genome sequence available for GBV-B confirm it as a member of genus *Hepacivirus* (Stapleton et al., 2011). Meanwhile, the more distantly related GBV-A has been assigned to the recently suggested genus *Pegivirus* (persistent GB viruses) and a new name, simian pegivirus (SPgV), has been proposed (Stapleton et al., 2011). To this genus also belongs human pegivirus (HPgV), originally known as GBV-C/HGV although it has no historical connection to the GB sample and does not cause hepatitis. In contrast to HCV, HPgV is a widespread human infection (1–4% of healthy blood donors) that can persist for years with no clinical symptoms (Stapleton et al., 2011), although its immunomodulatory impact may improve prognosis for patients co-infected with HIV (Bhattarai and Stapleton, 2012) and possible other infectious agents such as Ebola virus (Lauck et al., 2014).

Identification of non-primate hepacivirus (NPHV) initially in dogs and subsequently in horses was the first indication of a wider distribution of hepaciviruses in mammals (Burbelo et al., 2012; Kapoor et al., 2011). More recently, identification of a diverse range of viruses found in bats, rodents and Old World primates, further expanded the host range of hepaciviruses (Drexler et al., 2013; Kapoor et al., 2013b; Lauck et al., 2013; Quan et al., 2013)(Figure 1, Table 1). Similarly, several novel pegiviruses have recently been identified, with one report in bats in 2010 (Epstein et al., 2010), followed by several recent reports of novel equine (EPgV, including Theiler's disease associated virus, TDAV), rodent (RPgV) and bat (BPgV) pegiviruses (Chandriani et al., 2013; Drexler et al., 2013; Firth et al., 2014; Kapoor et al., 2013a; Kapoor et al., 2013b; Quan et al., 2013). TDAV is of particular interest, as strong epidemiological evidence linked this virus to liver disease in horses, making it the first pegivirus associated with disease. These new discoveries have fundamentally revised our knowledge of viral diversity and host ranges of hepaci- and pegiviruses.

4.1. Canine and equine hepaciviruses

In 2011, high-throughput sequencing of canine respiratory samples revealed the presence of a virus related to HCV, tentatively named canine hepacivirus (CHV) (Kapoor et al., 2011). Hepacivirus infection in dogs, however, came with a couple of curious observations. Firstly, the limited genetic diversity observed among CHV variants, even among dogs from unrelated shelters, was atypical for RNA viruses, including HCV. Secondly, subsequent studies undertaken to understand the genetic diversity and epidemiology of CHV were unsuccessful in identifying additional dogs positive for RNA or antibodies (Burbelo et al., 2012; Lyons et al., 2012), except for a single sero-positive farm dog (Lyons et al., 2014) (Table 2). This suggested that CHV might be a sub-clinical, and unorthodox or recently introduced infection in dogs, replicating at low levels if at all. Further, the identification of virus in respiratory samples was a surprise, given the strict hepatotropism of HCV and GBV-B, the lymphotropism of pegiviruses, and respiratory transmission generally being unusual for *Flaviviridae* (although shedding of pestiviruses such as bovine viral diarrhea virus (BVDV) occurs through most body fluids).

A serology based discovery approach was subsequently used to identify the natural host and species tropism of CHV (Burbelo et al., 2012). Recombinant CHV-NS3 helicase protein was

used as antigen in a luciferase based assay to determine the antibody prevalence in a range of animal species including dogs, deer, pigs, horses and cows. CHV-NS3 antibodies were present in around 35% of horses and in a single cow. Using PCR, several genetically diverse hepacivirus genomes were identified from viremic horses (~14% nucleotide divergence), and the broader term non-primate hepacivirus (NPHV) was suggested to refer to this group of viruses. Subsequent studies confirmed that NPHV naturally infects horses worldwide, typically with 2–7% being RNA positive and 30–40% being sero-positive (Table 2). An increased prevalence has been reported among Thoroughbreds and competition horses (Gemaque et al., 2014; Pfaender et al., 2014), and it will be interesting to understand whether genetic factors or the increased use of injections and close contact with other racehorses is the primary risk factor.

A recent study over a period of only 4 months reported an NPHV chronicity rate of less than 40%, and likely as low as 20% (Pfaender et al., 2014), which is lower than that observed for HCV (~70%). Infections can persist, with viremia documented in a horse serially sampled over 6 months (Lyons et al., 2014; Lyons et al., 2012) and in other horses followed for longer periods (unpublished data and (Pfaender et al., 2014)) (Table 1). Clearance of the virus in two closely followed animals coincided with mild elevations in liver enzymes. When various organs of an infected horse were analyzed, high viral RNA amounts were observed in the liver, and lower amounts in the spleen and other organs. Negative strand RNA, a hallmark of viral replication, could only be observed in the liver. Some lymphocyte infiltration was also observed in histological examination of liver tissue (Pfaender et al., 2014).

NPHV remains the genetically closest relative of HCV (Figure 1). Substantial similarity between NPHV and HCV exists even in the envelope proteins, which is among the most variable portions of the HCV genome. Moreover, the number and position of cysteine residues in the envelope protein E2 indicate that also the tertiary structure of NPHV E2 may be more similar to HCV than to other genetically related viruses (Kapoor et al., 2011) (Table 1). The NPHV genome resembles that of HCV, with a long ORF encoding a polyprotein predicted to yield similar structural and non-structural proteins. The 5′UTR resembles that of HCV but with a longer stem-loop I, and only a single miR-122 seed site (Burbelo et al., 2012). The type IV internal ribosome entry site (IRES) is similar in sequence and predicted structure (except for the absent prediction of a stem-loop IV) to that of HCV (Burbelo et al., 2012). The NPHV IRES and is capable of driving translation of a downstream ORF, whereas replication elements in the NPHV 5′ UTR appear functionally distinct from those of HCV (Stewart et al., 2013).

Similar to HCV, the NPHV core protein appears to be processed by cellular signal peptide protease and to have a lipid droplet localized phase, at least when individually expressed (Tanaka et al., 2014). Also similar to HCV, the NS3-4A protease is capable of cleaving human MAVS to inactivate the RIG-I pathway and thereby the innate immune response (Parera et al., 2012; Patel et al., 2012), although it remains to be shown whether the somewhat different equine MAVS is cleaved. Comparative sequence analysis revealed that the single CHV strain identified in dogs is highly similar (99.7% at the nucleotide level) to one NPHV strain (NZP1) among the more diverse strains from horses. Occasional

transmission, natural or by medical procedures such as vaccination, could have transmitted NPHV to dogs, where low-level presence and/or replication could occur, restricted to a narrow sequence space. Further studies should explore the infection prevalence, tissue tropism, persistence and pathogenesis of NPHV in horses.

4.2. Rodent hepaciviruses

To increase our understanding of hepacivirus host species and to potentially identify small animal models for HCV, we and others initiated methodical searches for hepaciviruses in several species of wild rodents (Table 2). Results of our survey of North American rodents revealed presence of several genetically diverse rodent hepaciviruses (RHV) in serum samples from deer mice (*Peromyscus sps.*) (Kapoor et al., 2013b), a widely distributed small rodent that is used experimentally in studies of hantaviruses. In addition, RHV was found in two other species, the hispid pocket mouse (*Chaetodipus hispidus*) and the desert wood rat (*Neotoma lepida*). Shortly thereafter, another study reported RHV in European bank voles (*Myodes glareolus*) and South African four-striped mice (*Rhabdomys pumilio*) after performing a large survey of rodents in Europe, Africa, Thailand and Mexico (Drexler et al., 2013). Overall, RHV was found with a prevalence of 2–3% in positive species from the two studies (Drexler et al., 2013; Kapoor et al., 2013b), although higher prevalence was found in a more recent study in rats (*Rattus norvegicus*) from New York City (Firth et al., 2014), and by Drexler et al. when using a more sensitive assay on serum samples (Table 1 and 2).

Histopathological signs of liver inflammation and high viral RNA concentrations and negative strand RNA in the liver suggested RHV hepatotropism in bank voles and rats (Drexler et al., 2013; Firth et al., 2014). Recombinant serology for two distinct hepacivirus lineages in bank voles demonstrated seroprevalence rates of around 8% and 12%, respectively. Co-occurrence of RNA and antibodies was found in around 5% of PCR-positive bank vole sera. Overall, RHV genome organization was similar to that of other hepaciviruses. In contrast to all other hepaciviruses analyzed, two lineages of rodent hepaciviruses from European bank voles contained a pegivirus-like IRES (Drexler et al., 2013). This could possibly originate by recombination between rodent hepaci- and pegiviruses in co-infected animals (Galli and Bukh, 2014). RHV strains contain an isolate-dependent number of miR-122 binding sites in the 5′ UTR. In the 3′ UTR, no poly(U) tract was found, however, the terminal stem-loop resembled that of HCV for certain RHV isolates (Drexler et al., 2013; Kapoor et al., 2013b). Thus, there appear to be little similarity in sequence, length or inferred RNA secondary structure in the 3′ UTR sequences across the hepacivirus genus. Putative alternative reading-frame proteins were predicted in some but not all RHV strains, however, these did not resemble the corresponding HCV protein (Drexler et al., 2013). The genetic diversity of RHV exceeded that observed for hepaciviruses infecting either humans or horses, with strains within single animal species, deer mice or bank voles, as divergent from each other as HCV is to GBV-B (Figure 1).

4.3. Bat hepaciviruses

Hepaciviruses infecting bats were reported in two insectivorous species from Kenya (Quan et al., 2013), and antibody positive serum was found in bats from West and Southern Africa (Drexler et al., 2013) (Table 1 and 2). Many rodent and bat pegiviruses were also discovered

(Drexler et al., 2013; Firth et al., 2014; Kapoor et al., 2013b; Quan et al., 2013), greatly expanding the diversity of previously reported bat pegiviruses (Epstein et al., 2010). Phylogenetic trees based on helicase and polymerase coding sequences of bat hepaci- and pegiviruses (BHV and BPgV) supported clustering into 3 hepacivirus and 3 pegivirus clades (Figure 1 and (Quan et al., 2013)). Within the *Hepacivirus* genus, the viruses were derived from two species of African bats (*Hipposideros vittatus*, *Otomops martiensseni*).

One clade of BHV was distinct from, but most closely related to GBV-B, whereas two other clades occupied a basal position relative to the NPHV and HCV clades. Twenty near full-length genome sequences were determined, representing all clades in which the BHV and BPgV were identified. Diversity between lineages of BHV was similarly large as that between RHV strains (Figure 1), and the BHV genome organization was similar to that of other hepaciviruses (Table 1). Alternative reading-frame proteins were predicted in some but not all BHV strains (Quan et al., 2013), whereas the UTRs were not yet characterized. In positive bat species, the RNA prevalence was around 5%. Whereas serologic responses to BHV were not tested, sera with cross-reactivity to HCV antigen were around 10%, indicating a somewhat higher seropositivity for BHV antibodies (Drexler et al., 2013; Quan et al., 2013). Together, these studies demonstrated that several highly divergent species of hepaci- and pegiviruses infect rodents and bats.

4.4. Primate hepaciviruses

A high-throughput sequencing based study of serum samples from black-and-white colobus monkeys (*Colobus guereza*) further revealed the presence of guereza hepacivirus (GHV) (Lauck et al., 2013). The detection of GHV in colobus monkeys represents the first documented natural infection of a non-human primate with a hepacivirus and expands the known host range of hepaciviruses to Old World monkeys. While possessing a genome organization typical of hepaciviruses, including a type IV IRES, the GHV genome was predicted to encode an exceptionally long NS5A protein, the C-terminal region of which would contain long intrinsically disordered stretches of amino acids. Among three isolates recovered from different individuals, ~15% nucleotide diversity was observed. GHV is phylogenetically placed at a similar distance to GBV-B and certain RHV and BHV isolates, and is therefore not grouping specifically with other primate hepaciviruses (Figure 1), arguing against a deeper co-evolutionary pattern of hepacivirus diversification.

5. Phylogeny of hepaciviruses indicates a lack of co-speciation

Comparative analysis of genomic organization and phylogenetic relatedness can be used to delineate members of hepaci- and pegivirus genera into respective clusters (Kapoor et al., 2013b; Stapleton et al., 2011). The basal radiation into at least three different lineages and an exceptionally high genetic diversity observed among rodent and bat hepaciviruses suggests that these animals could be ancestral hosts of hepaciviruses, which then subsequently infected other mammalian species (Pybus and Gray, 2013). It is also possible, although less likely, that rodents and bats became infected with genetically highly distinct hepaciviruses from other mammalian species.

Phylogenetic analysis of all known hepaciviruses generally reveals a lack of co-speciation among different viruses, including distinct origins of the primate hepaciviruses, HCV, GHV and GBV-B (if this is considered a primate virus; Figure 1). Notably, unlike the genetically diverse subtypes of HCV, the hepaciviruses found in rodents do not form compact genetic clusters and the only confirmed non-human primate hepacivirus, GHV, is genetically more similar to rodent and bat viruses than to HCV. Even the different hepacivirus species identified in single rodent species, such as deer mice or bank voles, are not genetically more similar to each other than to hepaciviruses found in primates, horses or bats. Since viruses found in individual species cannot be delineated into separate clusters long-term co-evolution is unlikely. These surprising observations and the expanded host range of hepaciviruses suggest that hepaciviruses can cross species barriers and that cross-species transmission might have aided their genetic diversification. Experimental animal infection studies addressing the cross-species transmission potential and mode of transmission of animal hepacivirus are required to obtain better understanding of their origin and evolution.

6. Animal hepaciviruses as potential HCV ancestors

The high genetic diversity observed among BHV and in particular RHV strains raises the possibility that other hepaciviruses, including HCV, may have originated from rodents or bats (Drexler et al., 2013; Kapoor et al., 2013b; Quan et al., 2013). NPHV remains the closest relative of HCV (Figure 1); however, a relatively small number of non-human species have been screened for hepaciviruses (Table 2) and it is possible that additional hepaciviruses will be identified in other hosts. A comprehensive evaluation of animal viromes will be ideal to estimate the extent of zoonosis and cross-species transmission of hepaciviruses. Serology-enabled approaches, such as the one used to study the host tropism of NPHV (Burbelo et al., 2012), will in addition be useful for such studies.

Given our current knowledge of hepacivirus phylogeny (Figure 1), it is conceivable that HCV originated from a single zoonotic source (cross-species transmission event) and then subsequently evolved, diversified and disseminated in humans giving rise to the highly diverse HCV genotypes and subtypes. Were the major HCV genotypes derived by individual transmission events, it would be expected that HCV phylogeny would overlap that of hepaciviruses in the source animal (Pybus and Gray, 2013). To obtain a better understanding of the origin of HCV and the zoonotic potential of hepaciviruses, it is important to further study their mode of transmission. HCV is primarily transmitted parenterally and a similar route may therefore be anticipated for other hepaciviruses. It remains unclear, however, whether parenteral transmission alone can account for the apparently efficient viral spread in horse, and probably rodent and bat, populations.

It remains to be explored whether airborne, foodborne or vectorborne transmission routes are exploited by hepaciviruses, as is known for flaviviruses (vectorborne) and pestiviruses (viral shedding and direct contact). Such routes of transmission could broaden the panel of culprits to be considered for the origin of HCV in humans. The historical use of horse serum derived anti-toxins for human therapeutics could lead to speculation that HCV arose from equine NPHV, given that NPHV is genetically most similar to HCV, yet equidistant to the individual genotypes. Challenges to this speculation in particular includes whether the most

recent common HCV ancestor has existed in times of equine serum for human use (medical or otherwise), although it may have in time of domesticated horses dating back to around 3500 B. C. (Outram et al., 2009). Another challenge is the comparatively little sequence diversity among horse viruses sequenced from different countries compared to HCV (Burbelo et al., 2012; Lyons et al., 2014; Lyons et al., 2012; Pfaender et al., 2014; Tanaka et al., 2014), although a wider geographical characterization could possibly change that. It is also possible that HCV has circulated in the human population as long as it has existed, and that common hepacivirus ancestors are pre-historic to the human race. Further epidemiologic and transmission studies are necessary to expand our knowledge on hepacivirus species tropism, cross-species transmission and evolution.

7. Animal hepaciviruses as models for HCV

Despite recent breakthroughs in HCV therapy (Scheel and Rice, 2013), vaccine efforts lag far behind and would be aided by a better understanding of hepacivirus immune responses and a pre-clinical model for testing vaccine candidates (Liang, 2013). The only true animal model for HCV, the chimpanzee, is no longer available for most NIH-sponsored research. Several models of immunodeficient mice engrafted with human liver cells have been developed (Bukh, 2012) (Vercauteren et al., 2014). Robust HCV infection is observed in this model, which can be used for studies of inhibition of viral entry and replication, but with the obvious shortcoming in studies of adaptive immune responses. Efforts are ongoing to improve this model to concurrently reconstitute mice with human liver and immune cells (Vercauteren et al., 2014). More recently, mice engineered to express human versions of the HCV entry factors were described. These permitted the entire HCV lifecycle when crossed to innate immune deficient strains, such as STAT1, IRF1 and IRF7 knock-outs (Dorner et al., 2013; von Schaewen and Ploss, 2014). Drawbacks of this model include low levels of replication and an increasing need to blunt innate immune pathways to achieve more efficient replication. Another entry factor engineered model in the ICR mouse background apparently allowed HCV replication without immune suppression, but with very low viremia (Chen et al., 2014). Absent immune-competent animal models with robust HCV infection, homolog hepaciviruses in their natural hosts could potentially provide useful surrogate models for the study of HCV.

An ideal surrogate model should resemble HCV, including its hepatotropism, ability to establish persistent infection, associated immune responses and pathogenesis (Bukh, 2012). GBV-B infection of New World monkeys has been used as a surrogate model, and an infectious clone has been established (Bukh, 2012; Bukh et al., 1999). However, persistent infection appears to be rare (Takikawa et al., 2010). Although the recently identified animal hepaciviruses could hold promise as HCV models, characterization has focused on genetic attributes and little is known on interactions with the host and immune responses (Table 1). Information of liver tropism is becoming evident, at least for NPHV and RHV (Drexler et al., 2013; Pfaender et al., 2014). The presence of 1–2 miR-122 sites in the 5′ UTR of most hepaciviruses (Table 1) may further indicate that they typically are hepatotropic, albeit the importance of the miR-122 interaction was so far only established for HCV (Wilson and Sagan, 2014) and may even be dispensable for GBV-B, despite its hepatotropism (Sagan et al., 2013).

NPHV is the best studied of the novel hepaciviruses. NPHV infection can be persistent, although the chronicity rate appears to be somewhat lower compared to HCV (Pfaender et al., 2014). However, given the many unknowns including transmission and exposure levels, chronicity rates need to be more firmly established through prospective studies or experimental transmission studies. Mildly elevated liver enzymes and some infiltration of lymphocytes into the liver were concurrent with clearance of NPHV infection in two longitudinally followed horses (Pfaender et al., 2014), resembling what is seen for HCV. However, whether fibrosis or other liver pathology results from NPHV infection remains to be determined, as does the kinetics of immune responses following infection. NPHV in horses therefore could be used to study hepaciviral evolution, immune responses, persistence and pathology, and tools for equine immunology are abundant. Further studies are needed to determine how different NPHV natural history is from that of HCV, however. One drawback is the large size and associated animal care costs of horses compared to conventional laboratory animal models.

Given the small size of rodents, RHV e.g. in rats or deer mice that are a commonly used lab model for hantavirus studies, could also be a promising model for HCV. However, less is known about the RHV natural history compared to NPHV, and further studies are warranted at this time to establish rodent models. RHVs are highly diverse (Figure 1) allowing determination of the breadth of immunity to reinfection. Genetically different viruses that infect the same host will also be useful for the identification of genomic regions that are requisite or dispensable for specific biological properties, including virus persistence or hepatotropism. Studying RHV infections in a host amenable to genetic manipulation could provide a powerful approach for unraveling immune mechanisms; the rat may provide such a model. Another possibility would be to adapt one or more RHV strains to infect common lab mice, e.g. using immune-compromised models as an intermediate host, thereby allowing studies of infection in a host amenable to genetic manipulation. However, the use of an unnatural host could alter the course of viral infection and may impose barriers in studies of immune response; whether natural hepaciviral infections occurs in the house mouse (*Mus musculus*) remains to be determined. In addition to being small and easy available, rodent models may also provide the most accessible opportunity to investigate routes of transmission for hepaciviruses. Such studies performed in rodents, including deer mice, have been valuable for understanding hantavirus transmission.

Non-human primate hepaciviruses may be yet another model for HCV. Traditionally, primates have been used in research due to their closer relatedness to humans, and certain niches such as HIV co-infection could specifically be studied in such models. However, the currently known primate hepaciviruses are more distantly related to HCV than for example NPHV (Lauck et al., 2013), and little is known about these viruses and how the infection mirrors HCV so far. It is possible though, that closer related primate viruses will be identified in the future, or that efforts to adapt HCV to infect monkeys will be successful. It is of note though, that at least while chimpanzees have been available for HCV research, interest in GBV-B studies in tamarins and marmosets has been limited (Bukh, 2012).

8. Future perspectives on animal hepacivirus research

Much of our current knowledge of the replication, host interactions, immune responses, and pathogenesis of HCV comes from experimental infection of primates or cell culture systems. *In vitro* models have proven valuable for understanding many functional aspects of virus replication, entry and assembly as well as for inhibitor studies (Steinmann and Pietschmann, 2013). Similarly, establishment of *in vitro* systems for animal hepaciviruses may be warranted to understand whether differences to HCV exist at least in the most important concepts of the viral life cycle (Scheel and Rice, 2013). Establishment of replicons and infectious cell culture systems for HCV has proved to be complex and slow, although the HCV toolbox now established will clearly help such endeavors for novel hepaciviruses. Appropriate hepatocyte cell lines and good antibodies recognizing the novel viruses are however still lacking.

As discussed above, the *in vivo* systems available to study HCV are scarce or compromised (Bukh, 2012). The identification and genetic characterization of animal hepaciviruses therefore provides a unique opportunity to develop tractable animal models. Although initial findings indicate liver tropism for NPHV and RHV, controlled experimental infections are warranted to better study tissue tropism. A better understanding of the tissue tropism, course of infection, pathogenesis and immune responses, e.g. through transmission studies in naive animals, will be important to determine which virus/host provide the better surrogate model for HCV, including whether certain RHV strains are more suitable than others. Given the indications of liver tropism, it will further be of interest to determine to what extent these viruses depend on miR-122. For HCV, the development of chimpanzee infectious consensus clones and productive infection after intra-hepatic RNA inoculation was an important milestone (Kolykhalov et al., 1997; Yanagi et al., 1997). These studies defined all necessary genetic elements for HCV replication and virus production, and fulfilled the modern version of Koch's postulates by linking the viral genome, the course of infection, immune responses and pathology. Similar studies will be important for the novel hepaciviruses. Although much could potentially be learned from the novel hepaciviruses in terms of vaccine strategies and immune responses, such responses would be specific to the virus in study. Chimeric viruses carrying HCV genes of interest could be used to measure HCV specific responses; such chimeras have been developed for GBV-B (Griffin et al., 2008; Haqshenas et al., 2007; Li et al., 2014; Rijnbrand et al., 2005; Takikawa et al., 2006). Generating GBV-B chimeric viruses was not easily achieved; however, this may differ for hepaciviruses more closely related to HCV.

Thus, defining the natural history of RHV, NPHV and GHV infection, the rate of chronicity, the immune determinants of clearance and protection and possible disease association, holds promise for establishing a relevant preclinical model for the development of HCV vaccine strategies and interventions to prevent or reverse virus-associated liver disease. In addition, understanding hepacivirus infection in livestock could prove of veterinarian importance, as was recently shown for TDAV and liver disease in horses (Chandriani et al., 2013). It would be interesting to see additional studies addressing NPHV transmission and the frequent use of parenteral equine products in veterinary medicine.

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Highlights

- Virome analyses enabled discovery of hepaci- and pegiviruses in horses, rodents, bats and non-human primates.
- Equine non-primate hepacivirus (NPHV) is the genetically closest relative of hepatitis C virus (HCV).
- Phylogenetic analysis of hepacivirus species indicates lack of co-speciation with hosts.
- Characterization of animal hepaciviruses may allow development of immunocompetent models to study HCV infection.

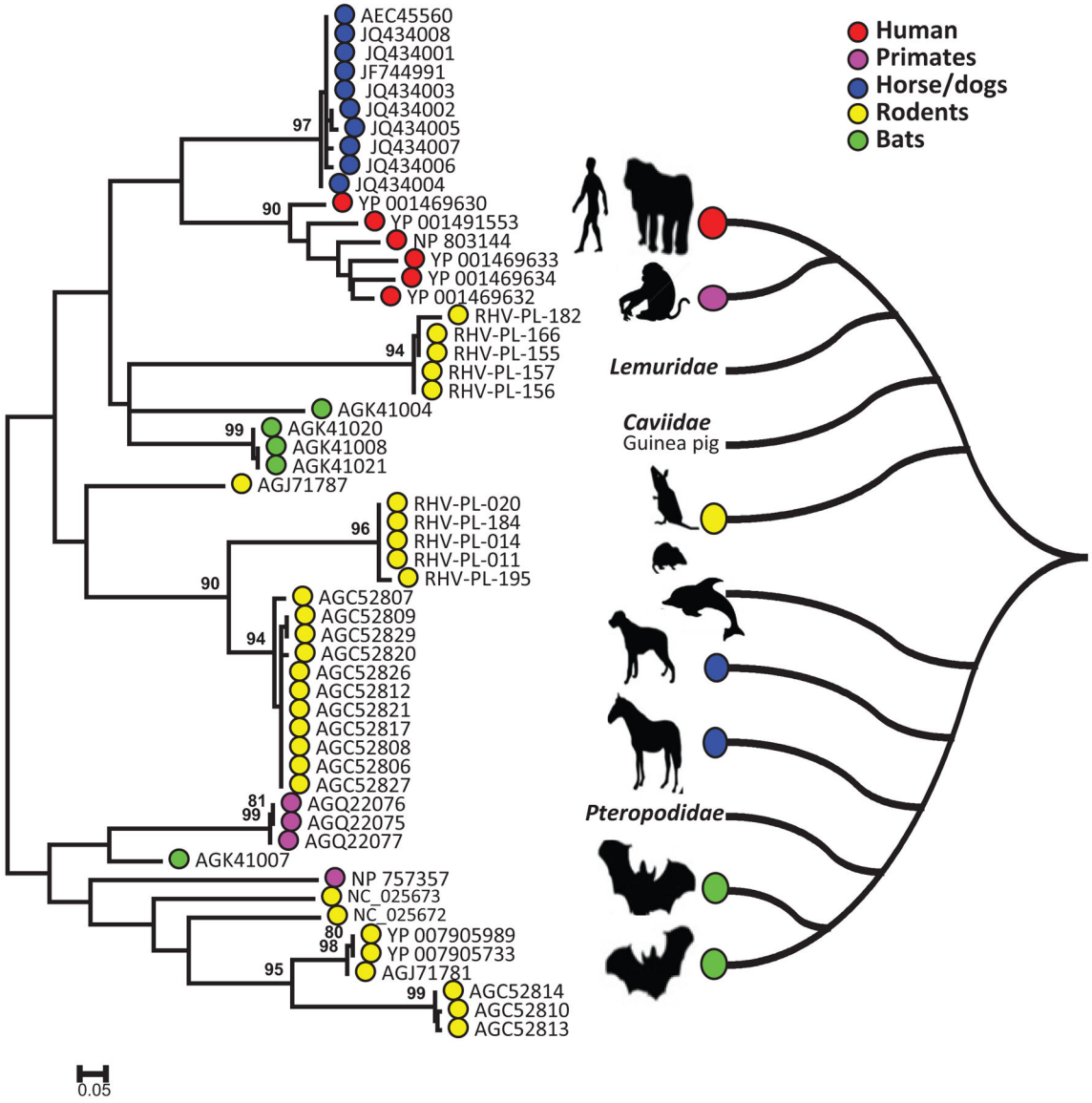

Figure 1.

Phylogenetic analysis of the NS3 (helicase) region of hepaciviruses. Amino acid sequences were aligned with MUSCLE 3.8 (Edgar, 2004), and the trees constructed using maximum likelihood methods as implemented in the MEGA 6 software package (Tamura et al., 2013). Optimum maximum likelihood models for NS3 alignments were determined and used for phylogenetic reconstruction. Phylogenetic analysis used the Le-Gascuel model with gamma (γ) distribution (5 rates) and invariant sites (LG+γ+I). Bootstrap re-sampling was used to determine robustness of grouping. Hepacivirus sequences are annotated using their GenBank accession numbers, except for the unpublished hepacivirus sequences found in deer mice, *Peromyscus leucopus* (labeled as RHV-PL-XXX.). Mammalian phylogeny is merely a representative drawing of published data (Meredith et al., 2011).

Table 1

Molecular and biological properties of hepaciviruses.

	HCV	NPHV	RHV	BHV	GBV-B	GHV
Natural host	Humans	Horses [1]	Rodents [2]	Bats	?	(Non-human primates)
Host for viral identification	Humans	Dogs, Horses	Rodents	Insectivorous African bats	Tamarins	Black-and- white colobus monkey
Experimental infection	Chimpanzees	-	-	-	Tamarins, Marmosets	-
Primary tissue tropism	Liver	Liver	Liver	?	Liver	?
Pathogenesis	Hepatitis, cirrhosis, carcinoma	(Liver inflammation)	Liver inflammation	?	Hepatitis	?
Outcome	Acute or chronic	Acute or chronic	?	?	Acute [3]	?
Chronicity rate	~70%	(~20%)	?	?	-	?
Prevalence	~2%	~3%	~2–3%	~5%	-	?
Seroprevalence	2–4%, higher in risk-groups	~40%	~20%	• 10% [4]	-	?
Transmission	Blood-borne	?	?	?	?	?
Diversity (nt level)	7 genotypes, ~30% diversity	~15% diversity	• 8 clades. Highly diverse.	• 3 clades. Highly diverse.	One isolate	~15% diversity
ORF: No. of predicted proteins	10	10	10	10	10 (or 11)[5]	10
ORF: typical length (amino acids)	3008–3033	2942–2949	2748–3007	2901–3024	2864	3334–3336
Envelope proteins: No. of predicted N- glycosylations (E1/E2)	5–6/11	4/10	0–2/2–6	2/4–5	3/6	4/4
Putative ARFP	Yes	No	Isolate dependent	Isolate dependent	No	Yes
5′UTR: miR-122 interaction	Two seed sites required for replication.	One seed site. Requirement unknown.	Isolate dependent seed number. Requirement unknown.	?	Two seed sites. Possibly dispensable [7].	Two seed sites. Requirement unknown.
3′UTR: long internal poly(U) tract	Yes	?	No	?	No	Not found [6]

NOTES: Entries in parenthesis indicate preliminary findings with confirmation needed.

[1] NPHV was originally identified in dogs (Kapoor et al., 2011), but subsequent confirmatory attempts have been unsuccessful (except for a single seropositive dog (Lyons et al., 2014)).

[2] Identified in deer mice, hispid pocket mice, desert wood rats, european bank voles, south african four-striped mice, rats (Drexler et al., 2013; Firth et al., 2014; Kapoor et al., 2013b).

[3] Persistent infection has been reported with recombinant GBV-B (Takikawa et al., 2006).

[4] Based on reactivity with HCV and may therefore be underestimated (Drexler et al., 2013).

[5] The p13 protein of GBV-B may be processed into a p7 protein (equivalent to HCV p7) and a p6 protein (Takikawa et al., 2006).

[6] Complete characterization remains to be done.

[7] A recent study demonstrated miR-122 independent GBV-B replication (Sagan et al., 2013).

Table 2

Surveys of animal hepacivirus RNA and serology (excluding HCV).

	Number of animals positive/total					
Species	**Viral RNA**	**Serology**	**Disease**	**Serum/tissue**	**Geographic origin**	**Reference**
Species with positive samples identified						
Dog	**9/33**	-	Respiratory	Respiratory	U.S.	(Kapoor et al., 2011)
	0/60	-	Healthy	Nasal swap	U.S.	(Kapoor et al., 2011)
	5/19	-	Gastro-intestinal	Liver	U.S.	(Kapoor et al., 2011)
	-	0/80	Unknown	Serum	U.S.	(Burbelo et al., 2012)
	0/197	-	Respiratory/unknown	Respiratory, plasma, lung, liver, spleen	U.K.	(Lyons et al., 2012)
	0/239	-	Unknown	Nasal swap	Germany	(Drexler et al., 2013)
	0/167	-	Unknown	Serum/plasma	Germany	(Drexler et al., 2013)
	0/113	**1/113**	Various	Serum/plasma	U.K.	(Lyons et al., 2014)
	0/100	0/100	Chronic hepatitis	Serum, liver	U.K.	(Bexfield et al., 2014)
	0/255	-	Liver disease/healthy	Liver	The Netherlands	(van der Laan et al., 2014)
Horse	**7/99**	**32/99**	Unknown	Serum	U.S.	(Burbelo et al., 2012)
	3/142	-	Unknown	Serum	U.K.	(Lyons et al., 2012)
	0/40	-	Unknown	Respiratory	U.K.	(Lyons et al., 2012)
	7/210	-	Unknown	Serum/plasma	Germany	(Drexler et al., 2013)
	3/327	**142/327**	Healthy/hepatopathy	Serum/plasma	U.K./France	(Lyons et al., 2014)
	1/1	-	Jaundice	Serum	Hungary	(Reuter et al., 2014)
	11/433	**136/433**	Non-liver related illnesses	Serum	Germany	(Pfaender et al., 2014)
	11/31	**7/31**	Unknown	Serum	Japan	(Tanaka et al., 2014)
Rodents	**13/493 (13/389 in positive species)**	-	Unknown	Plasma	U.S.	(Kapoor et al., 2013b)
	37/4770 (37/1983 in positive species)	-	Unknown	Post-mortem tissues	Thailand, Gabon, South Africa, Germany, the Netherlands, Mexico	(Drexler et al., 2013)
	57/239	**19/97**	Unknown	Serum		
	0/61	-	Unknown	Liver	U.K.	(Lyons et al., 2012)

	Number of animals positive/total					
Species	**Viral RNA**	**Serology**	**Disease**	**Serum/tissue**	**Geographic origin**	**Reference**
Bats	**9/1673 (9/165 in positive species)**	-	Unknown	Serum, kidney, liver, lung	Guatemala, Cameroon, Nigeria, D.R. Congo, Kenya	(Quan et al., 2013)
	0/2939	**13/180**[1]	Unknown	Serum	Gabon, Ghana, Papua-New Guinea, Australia, Thailand, Panama, Germany	(Drexler et al., 2013)
Non-human primates[2]	**3/9**	-	Unknown	Plasma	Uganda	(Lauck et al., 2013)
	0/164	0/164	Unknown	Serum/plasma	Cameroun	(Lyons et al., 2014)
Cow	0/84	**1/84**	Unknown	Serum	U.S.	(Burbelo et al., 2012)
Species with no positive samples identified						
Human	0/362	0/362	Unknown	Serum/plasma	Cameroun	(Lyons et al., 2014)
	0/59	-	Healthy	Serum	Brazil	(Levi et al., 2014)
Donkey	0/16	-	Unknown	Plasma	U.K.	(Lyons et al., 2012)
	0/100	0/100	Various	Serum/plasma	U.K.	(Lyons et al., 2014)
Pig	0/40	-	Unknown	Serum	U.K.	(Lyons et al., 2012)
Deer	-	0/81	Unknown	Serum	U.S.	(Burbelo et al., 2012)
Rabbit	-	0/14	Unknown	Serum	U.S.	(Burbelo et al., 2012)
Cat	0/56	-	Unknown	Plasma	U.K.	(Lyons et al., 2012)
	0/131	0/131	Various	Serum/plasma	U.K.	(Lyons et al., 2014)
	0/452	-	Unknown	Nasal swap	Germany	(Drexler et al., 2013)

[1] Serology for HCV reactive antibodies. Presumably an underestimation of prevalence.

[2] A number of studies unsuccessfully attempted to identify HCV or GBV-B like hepaciviruses in non-human primates prior to the identification of NPHV, and have not been included here.