

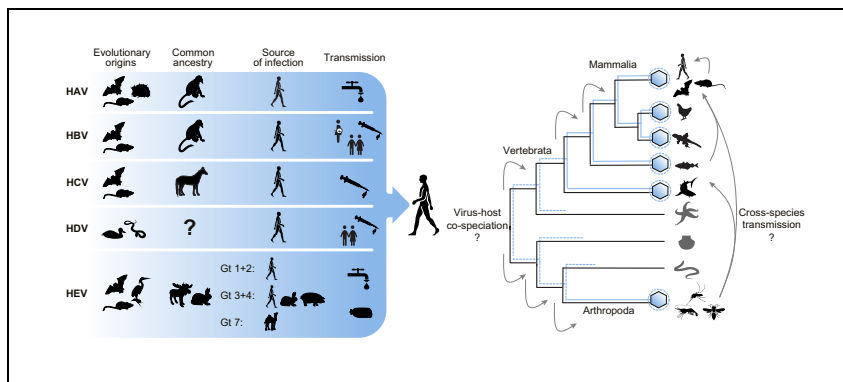


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Evolutionary biology of human hepatitis viruses

Graphical abstract



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Summary

Hepatitis viruses are major threats to human health. During the last decade, highly diverse viruses related to human hepatitis viruses were found in animals other than primates. Herein, we describe both surprising conservation and striking differences of the unique biological properties and infection patterns of human hepatitis viruses and their animal homologues, including transmission routes, liver tropism, oncogenesis, chronicity, pathogenesis and envelopment. We discuss the potential for translation of newly discovered hepatitis viruses into preclinical animal models for drug testing, studies on pathogenesis and vaccine development. Finally, we re-evaluate the evolutionary origins of human hepatitis viruses and discuss the past and present zoonotic potential of their animal homologues.



Evolutionary biology of human hepatitis viruses

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Summary

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Introduction

Approximately 1.3 million people die annually from viral hepatitis worldwide.¹ These deaths are predominantly associated with cirrhosis and hepatocellular carcinoma (HCC) resulting from chronic infections with hepatitis B virus (HBV; 887,000 deaths) and hepatitis C virus (HCV; 399,000 deaths),² as well as hepatitis and liver failure resulting from acute infections with hepatitis A virus (HAV; 11,000 deaths) and hepatitis E virus (HEV; 44,000 deaths).¹ Worldwide, approximately 5% of people infected with HBV are simultaneously infected with hepatitis delta virus (HDV).^{1,3}

The recent discoveries of novel hepatitis viruses from animals allow revisiting the enigmatic evolutionary origins of human hepatitis viruses. In chapter 1 of this review, we discuss the diverse animal homologues of all human hepatitis viruses that were discovered over the last decades. In chapter 2, we analyse hepatitis virus evolution based on the unique genomic and morphologic properties of human and non-human hepatitis viruses. In chapter 3, we discuss the level of evolutionary conservation of the characteristic infection patterns of human hepatitis viruses and review the ability to translate the recent virus discoveries into tractable animal models. Finally, in chapter 4, we discuss the evolutionary origins of human hepatitis viruses in the context of a plethora of newly discovered animal homologues. In this chapter, we also evaluate the potential of animal homologues for past and present cross-species transmission and compare macro-evolutionary patterns of the different hepatitis virus families.

Chapter 1: Milestones towards the understanding of the evolutionary origins of hepatitis viruses

In the following section, we outline the path towards the discovery of human hepatitis viruses

and provide detail on the huge expansion in the number of animal homologues discovered during the last decade.

The discovery of human hepatitis viruses

The path towards the discovery of human hepatitis viruses started with the differentiation between 2 forms of transmissible jaundice. Infectious hepatitis linked to epidemic outbreaks of faecal-orally transmitted jaundice (termed hepatitis A) was differentiated from a parenterally transmitted jaundice with a relatively longer incubation period (termed hepatitis B).^{4,5} Following the discoveries of the causative HAV and HBV in 1973 and 1970,^{6,7} the HBV-associated HDV was discovered in 1980.⁸ In parallel, it was noted that most cases of post-transfusion hepatitis were neither linked to infection with HAV, nor HBV.⁹ However, it was not until 1989 that the causative HCV was finally described.¹⁰ HEV was discovered in 1983 as the cause of a predominantly waterborne acute non-A, non-B hepatitis.¹¹ The discoveries of human hepatitis viruses were followed by milestones of major clinical relevance, including tools for reliable diagnosis (e.g., described in^{12,13}), the development of direct-acting antiviral treatments against HCV and preventive vaccinations against HAV and HBV¹⁴ (Fig. 1). Notably, scientific progress was not evenly distributed among human hepatitis viruses. The clinical relevance of HBV and HCV likely contributed to the enormous achievements attained continuously for these 2 viruses (Fig. 1).

A new era of virus discovery

The first discoveries of non-human hepatitis viruses were ground breaking, yet sporadic. In 1978, a genetically distant relative of HBV, the woodchuck hepatitis virus (WHV), was identified in American woodchucks, a marmot-like rodent species.¹⁵ The discovery of WHV was followed by the discovery of duck hepatitis B virus (DHBV),

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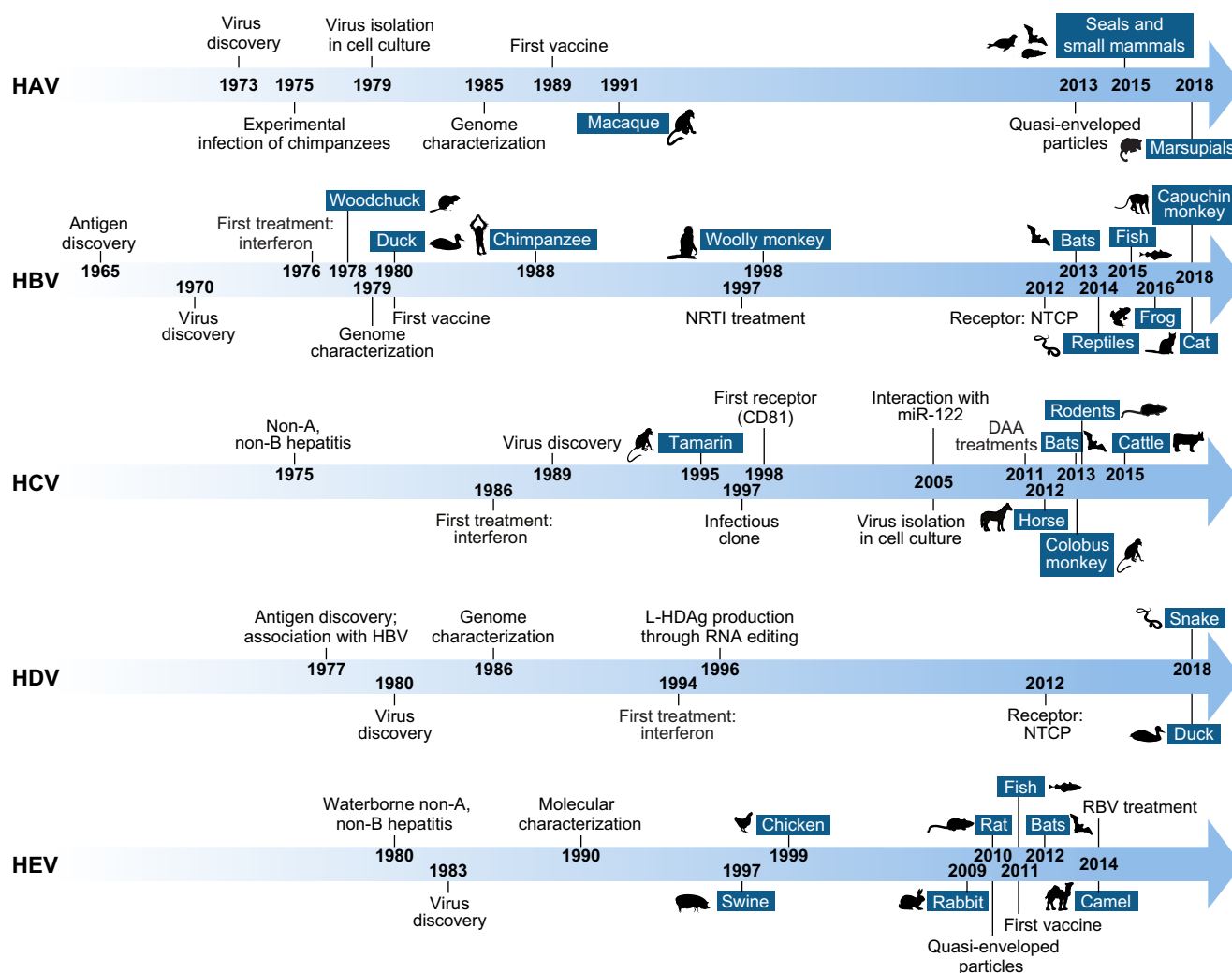


Fig. 1. Selected milestones in hepatitis virus research. References include for HAV^{6,19,25,26,28,73,107,219–221} for HBV^{7,15–18,30,32–35,67,222–225} for HCV^{9,10,21,37,38,42–45,71,214,226–230} for HDV^{8,47,67,91,146,231–233} and for HEV^{11,48,50,51,54,56,59,74,133,234–238}. Due to space constraints, only selected milestones are shown. Animal pictograms represent host orders in which distinct hepatitis viruses were detected. DAA, direct-acting antiviral; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HEV, hepatitis E virus; miR-122, micro RNA-122; NRTI, nucleoside reverse transcriptase inhibitor; NTCP, sodium taurocholate co-transporting polypeptide; RBV, ribavirin.

Key point

Diverse animals host hepatitis viruses, but ongoing zoonotic transmission is documented only for hepatitis E virus.

HBV variants in apes, and a divergent HBV species termed woolly monkey hepatitis B virus (WMHBV).^{16–18} For hepatitis viruses other than HBV, knowledge on potential non-human hosts remained scarce. In the 1990s, a distinct HAV type was detected in macaques¹⁹ and HEV was recovered from swine.²⁰ In 1995, a non-human primate (NHP) virus distantly related to HCV, termed GB virus-B (GBV-B) was isolated from a laboratory tamarin.²¹ The tamarin had been inoculated with the serum of a patient with hepatitis, but since GBV-B was never detected in humans, it was most likely a tamarin virus.²²

Over the last decades there has been an explosive expansion of the recognized viral diversity in animals, driven by novel sequencing techniques and an unprecedented focus on zoonotic pathogens which followed the identification of highly pathogenic viruses such as Ebola virus and SARS-coronavirus in bats.^{23,24} A plethora of HAV-

related viruses were recovered from various mammalian species during 2015–2018, including predominantly bats and rodents, but also tree shrews, seals and marsupials.^{25–29} HBV-related viruses were detected in bats during 2013–2015^{30,31} and in a domestic cat in 2018.³² A distinct HBV species termed capuchin monkey hepatitis B virus (CMHBV) was described in 2018.³³ Endogenous and exogenous viruses distantly related to HBV were detected in reptiles, fish and amphibians.^{34–37} HCV-related viruses were detected in horses in 2012, and evidence for sporadic spill-over infections of the horse-associated viruses into dogs and donkeys was found.^{38–41} Soon afterwards, highly diverse HCV-related viruses were detected in bats and rodents,^{42,43} in cattle,^{44,45} and in black-and-white colobus monkeys.⁴⁶ Hepatitis D-like agents were recently detected in ducks and snakes.^{47,48} Zoonotic HEV genotypes were discovered in wild boars,

camelids, rabbits and rats.^{49–52,253} In addition, divergent HEV-related viruses were described in bats, ferrets, rodents, birds, and fish.^{53–59} In sum, homologues of all human hepatitis viruses exist in diverse animals. Apart from the detection of zoonotic HEV strains, none of the recent studies into viral diversity in animals revealed any direct ancestor of human hepatitis viruses, as outlined below.

Chapter 2: Conservation and *de novo* emergence of unique properties of hepatitis viruses

Human hepatitis viruses are assigned to diverse virus families and genera (Table 1). Namely, they belong to the families *Picornaviridae*, genus *Hepatitisvirus* (HAV), *Hepadnaviridae*, genus *Orthohepadnavirus* (HBV), *Flaviviridae*, genus *Hepacivirus* (HCV), and *Hepeviridae*, genus *Orthohepevirus* (HEV). The genus *Deltavirus* (HDV) is unassigned to any virus family. In general, human and non-human hepatitis virus homologues resemble each other in major genomic properties such as structure of the genomic nucleic acid, open reading frame (ORF) composition, genome length and presence and type of noncoding regions (Table 1, Fig. 2). Nonetheless, there are striking differences among viruses from the same family or genus. Hypothetically, *de novo* emergence of genomic features and recombination events during evolution may have contributed to the rise of viruses eventually pathogenic to humans. A prototypic example for micro-evolutionary events within animal reservoirs enabling efficient human infection is the recombination event leading to the functional tetherin antagonist in chimpanzee-associated ancestors of the HIV-1 group M.⁶⁰

Envelopment might not be conserved among animal hepatitis viruses

Recent studies revealed that HAV and HEV, thought previously to be non-enveloped viruses, exist in an enveloped form in blood, referred to as quasi-envelopment (Fig. 2).^{73,74} In contrast to enveloped viruses, there is no hint of virus-encoded proteins in the quasi-envelope of HAV and HEV, which raises questions on how quasi-enveloped viruses enter susceptible cells (summarised in⁷⁵). Interestingly, some non-primate HAV- and HEV-related viruses (henceforth, hepatoviruses and hepeviruses) lack critical genome structures involved in quasi-envelopment. This includes the apparent absence of a C-terminal VP1 capsid protein extension termed pX in some HAV-related bat viruses²⁵ and the lack of short amino acid motifs termed late domain motifs in several divergent hepeviruses from diverse mammals and birds.²⁹ In contrast, HBV-related viruses (henceforth, hepadnaviruses) are enveloped viruses, yet fish were recently found to host a non-enveloped *Hepadnaviridae* sister family,

termed *Nackednaviridae*.³⁶ The discovery of nakednaviruses suggested that envelopment emerged *de novo* in hepadnaviruses capable of infecting mammals and ultimately humans.³⁶ A hallmark of HCV infection is the formation of lipoviral particles, which incorporate both host-derived lipoproteins and virus-derived glycoproteins.⁷⁶ Interestingly, equine hepacivirus capsid proteins associate with intracellular lipid components, suggesting similarities between the replication of HCV and non-human HCV-related viruses (henceforth, hepaciviruses).⁷⁷ However, whether the formation of lipoviral particles is evolutionarily conserved among non-human hepaciviruses remains unknown.

On the one hand, it is thus possible that envelopment and quasi-envelopment are not conserved among animal hepatitis viruses. On the other hand, ancestral animal hepatitis viruses may exploit unknown strategies for envelopment that differ from those found in human viruses.²⁹

Recombination events occurred during hepatitis virus evolution

Recombination events among viruses can occur when 1 cell is co-infected with 2 different viruses that interact during replication. For all human hepatitis viruses, recombination has been exhaustively described.^{78–83} The plethora of recently discovered viruses has enabled revisiting the occurrence of recombination events during the genealogy of human hepatitis viruses and their animal homologues.

For hepatoviruses there is evidence for recombination in the coding sequence of very distantly related viruses associated with different host orders, which hints at a broad host range.²⁹ In addition, variations in the 5'-genome ends of animal hepatoviruses harbouring the internal ribosome entry site (IRES) hint at ancient recombination events involving different viral families.²⁵ For hepadnaviruses, the mosaic genome structure of extinct and extant HBV genotypes suggests that recombination has been a major micro-evolutionary feature of HBV evolution.^{80,190} Recombination among viruses associated with different host species was also described among primate and bird hepadnaviruses.⁸⁴ Whether inter-host recombination events also occurred during the genealogy of the newly identified bat and cat hepadnaviruses demands further investigation. For non-human hepaciviruses, recombination events among diverse hosts have not been unambiguously proven yet.⁸⁵ However, as with hepatoviruses, hints for ancient recombination events are found in the 5'-genome ends of non-primate hepaciviruses, likely involving viruses belonging to different genera.⁴² For hepeviruses, there is evidence for recombination involving different host orders, similar to hepatoviruses.²⁹ Importantly, the camelid-associated HEV genotypes 7 and 8 show

Key point

The *de novo* emergence of virus properties such as envelopment and recombination events may have contributed to the rise of viruses eventually pathogenic for humans

Table 1. Properties of human hepatitis viruses.

	HAV	HBV	HCV	HDV	HEV
Virus family, genus	<i>Picornaviridae</i> , <i>Hepatovirus</i>	<i>Hepadnaviridae</i> , <i>Orthohepadnavirus</i>	<i>Flaviviridae</i> , <i>Hepacivirus</i>	Unassigned, <i>Deltavirus</i>	<i>Hepeviridae</i> , <i>Orthohepevirus</i>
Genome type	Positive-sense linear ssRNA	Circular, partially dsDNA (full length negative-sense, partial positive-sense), replication via reverse transcription	Positive-sense linear ssRNA	Viroid-like, negative-sense circular ssRNA	Positive-sense linear ssRNA
Approx. genome length (nt)	7,500	3,200	9,600	1,700	7,200
Virion diameter (nm)	27–32	42	55–65	36–43	30–34
Envelope	No/quasi- enveloped	Yes	Yes	Yes	No/quasi-enveloped
Course of infection	Acute ⁶¹	Acute/chronic (children 30–90%; adults <5%) ⁶²	Acute/chronic (80–85%) ⁶³	Acute/chronic (>80% if superinfection) ⁶⁴	Acute/chronic (<1%) ⁶⁵
Predominant transmission routes	Mainly faecal-oral, parenteral	Vertical, parenteral, sexual	Parenteral	Parenteral, sexual	Faecal-oral, food- borne, parenteral
Cellular receptor	Unknown ⁶⁶	NTCP, heparan sulfate proteoglycans ^{67,68}	CD81, SR-B1, LDL receptor, claudin-1, occludin ^{69–71}	NTCP, heparan sulfate proteoglycans ^{67,72}	Unknown

dsDNA, double-stranded DNA; nt, nucleotide; NTCP, sodium taurocholate co-transporting polypeptide; ssRNA, single-stranded RNA.

evidence of recombination at the boundary of ORFs encoding non-structural and structural proteins.²⁹ Whether recombination events in these HEV strains contribute to their zoonotic potential is an intriguing question. Notably, recombination events likely enabled the rise of the family *Hepeviridae per se*, as the different hepevirus ORFs are derived from diverse alphavirus- and astrovirus-like ancestors.⁸⁶

To summarise, recombination was likely a frequent event during the genealogy of hepatitis viruses. Whether transmission to humans is a consequence of recombination events in animal reservoirs requires further elucidation.

Distinct features of HDV

HDV is not a complete virus but a subviral agent with a very short, circular RNA genome which can replicate autonomously within a cell but requires the surface proteins of HBV for cell release and uptake (Fig. 2).⁸⁷ Of all human hepatitis viruses, the least is known about the origin of HDV. HDV has been hypothesised to have originated from plant viroids, via recombination with cellular mRNA, or from RNA intermediates of HBV.^{88–90} However, these theories are conflicting and not supported by experimental evidence. Interestingly, HDV-like agents were recently detected in a pool of oropharyngeal and cloacal samples of ducks and in various tissues of snakes.^{47,48} The apparent lack of detectable hepadnaviruses in these preliminary investigations seems consistent with the lack of a predicted large delta antigen containing the C-terminal farnesylation signal required for interaction with the HBV envelope (Fig. 2).^{91,92} On the one hand, this may indicate that the unique association of HDV with HBV is not evolutionarily conserved. On the other hand, divergent helper viruses and divergent

mechanisms of envelopment cannot be excluded based on present knowledge. Although snake and duck HDV-like agents are genetically clearly distinct from human HDV, they show typical HDV genome properties, including the ORF encoding the small delta antigen, genomic and antigenomic ribozymes mediating autocatalytic cleavage and an extremely high degree of genomic self-complementarity of the circular genome. The detection of these divergent viruses in ancient vertebrates strongly suggests that other HDV-like viruses exist, which might include ancestors of human HDV.

Chapter 3: Evolutionary conservation of infection patterns

The comparison of hepatitis virus infection patterns among human and non-human hosts might reveal unique viral properties that eventually enabled human infection. For example, chronic courses of viral infections in animal reservoirs were found to be a strong predictor of human-to-human transmissibility after zoonotic introduction into humans.⁹³ Here, we compare transmission routes, organ tropism, disease outcomes, receptor usage and immune evasion strategies among human and non-human hepatitis viruses.

Conservation of transmission routes among hepatitis viruses

Human hepatitis viruses differ in their transmission routes. HAV and HEV are mainly transmitted through the enteric route. Similarly, faecal shedding has been observed invariably in non-human hosts of hepatoviruses and hepeviruses (Table 2). In contrast, HBV and HDV are transmitted via blood and other body fluids, including semen and vaginal secretions.^{87,94} In addition, there is a

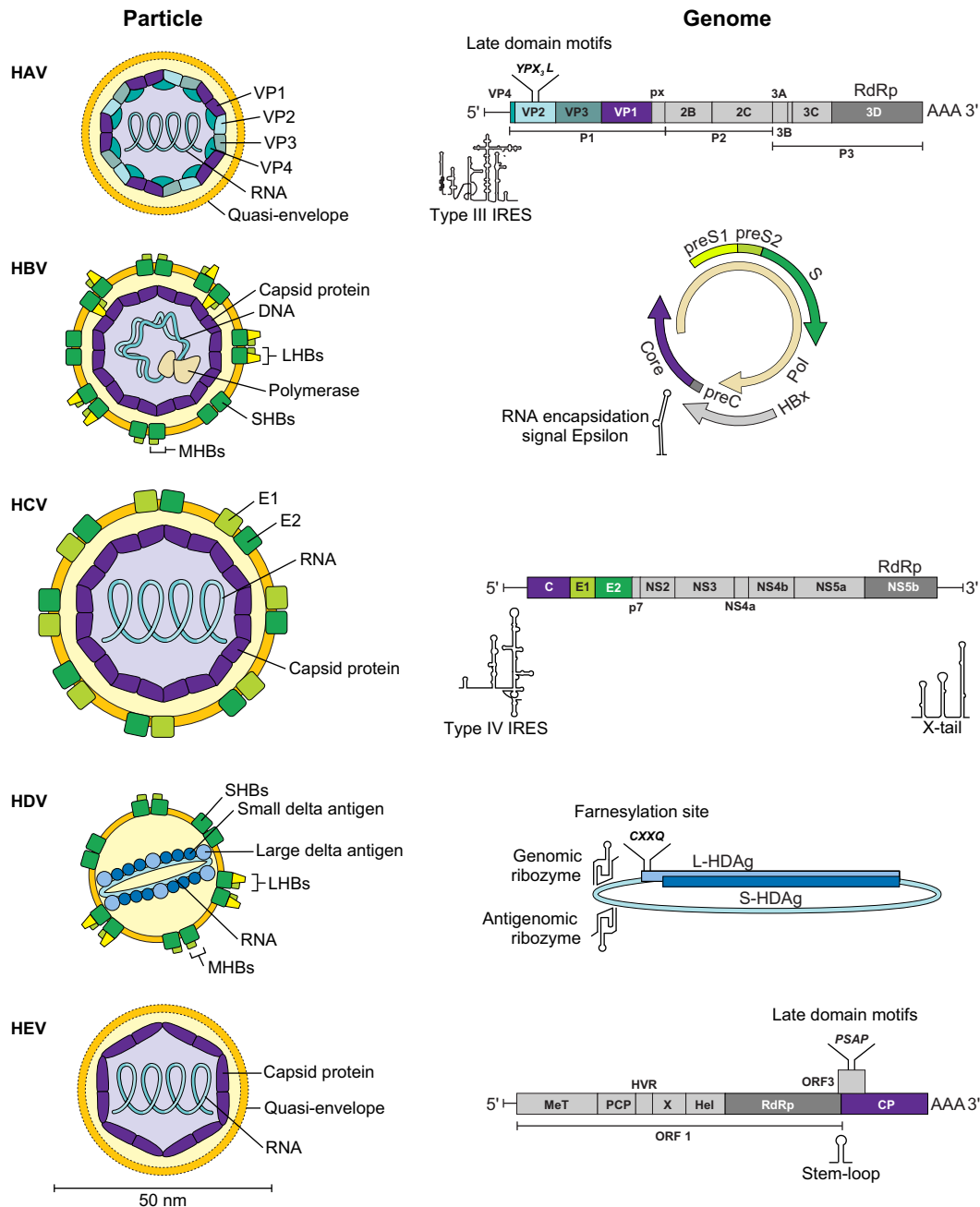


Fig. 2. Virus particles and genome characteristics of human hepatitis viruses. C, core protein; CP, capsid protein; E, envelope protein; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HEV, hepatitis E virus; Hel, Helicase; HVR, hypervariable region; IRES, Internal ribosomal entry site; LHBs, large surface protein; L-HDAg, large hepatitis delta antigen; MHBs, medium surface protein; MeT, methyltransferase; NS, non-structural protein; ORF, open reading frame; PCP, papain-like cysteine protease; Pol, polymerase; RdRp, RNA-dependent RNA polymerase; S, surface protein; SHBs, small surface protein; S-HDAg, small hepatitis delta antigen; VP, viral protein; X, X-domain/ADP-ribose-binding module. Important secondary structures are depicted below genomes.

high risk of perinatal transmission of HBV.⁹⁵ Like in humans, both perinatal and horizontal HBV transmission has been described for gibbons.⁹⁶ For non-primate hosts, vertical hepadnavirus transmission is known to be effective in rodents and birds.^{97,98} Based on a higher prevalence in female tent-making bats compared to males, predominantly sexual virus transmission has been suggested for the Tent-making bat HBV (TBHBV)²⁵⁴. Transmission routes for other bat or

cat hepadnaviruses remain unknown.^{30,32} HCV is a blood-borne virus. However, perinatal transmission rates are much lower for HCV than HBV and sexual transmission is rare.^{63,99} HCV is mainly transmitted via blood transfusions, sharing of equipment in injecting drug use and reuse of injection needles in healthcare,^{1,63} which raises questions on the transmission mode of HCV in scattered prehistoric human populations. Here, fights, use of weapons or tools as well as cultural

Table 2. Evolutionary conservation of infection patterns.

	Hallmarks of human infection	Hallmarks of infection in non-human animals	
		Naturally or experimentally infected with autochthonous virus	Experimentally infected with human virus
HAV	Long faecal shedding; Quasi-envelopment; Never chronic but may cause protracted infections of up to one year; Faecal-oral transmission	Bat, rodent, hedgehog, shrew: Acute infection; Faecal shedding; Hepatotropism ²⁵ Woodchuck: Mild fever; Faecal shedding; Hepatotropism ¹⁰⁵	Chimpanzee: Faecal shedding; Quasi-envelopment; Acute hepatitis ^{73,106,107} New and Old World monkeys: Faecal shedding; Hepatotropism ¹⁰⁸⁻¹¹⁰ Guinea pig: Faecal shedding; Hepatotropism; Subclinical disease ¹¹¹
HBV	Oncogenic (HCC); Age-dependent chronicity rate; Vertical, parenteral, sexual transmission; Acute, fulminant courses in adults	New World primates: Acute and chronic; Hepatitis; Vertical transmission ^{17,33} Old World primates: Acute and chronic; Horizontal and vertical transmission; Hepatitis ^{96,112,113} Bats: Apparently acute and chronic; Sexual transmission ^{30,254} Woodchuck: Acute and chronic; Hepatitis; Oncogenic; Hepatotropism ¹¹⁴ (summarised in ¹¹⁵) Squirrels: Hepatitis ¹¹⁶ , Oncogenic ¹¹⁷ Cat: Viremia ³² Duck: Acute and chronic ¹¹⁸ ; Hepatitis; Vertical transmission; Not oncogenic; No exclusive hepatotropism (summarised in ^{115,119})	Chimpanzee: Acute and chronic; Hepatitis; Oncogenic (summarised in ^{120,121}) Tupaia: Acute and chronic; Hepatitis ¹²² ; Low viremia (summarised in ¹¹⁵)
HCV	Oncogenic (HCC); High chronicity rate; Parenteral transmission	Cattle: Apparently acute and chronic; Hepatotropism ^{44,45} Horse: Apparently acute and chronic; Hepatotropism ¹⁰² Rat: Hepatotropism ¹²³ Bank vole: Hepatotropism ⁴² Marmoset: Acute and chronic; Hepatitis ¹²⁶ Tamarin: Acute hepatitis; High viral titres ¹²⁷	Chimpanzee: Acute and chronic; Hepatitis; Oncogenic ^{124,125} (summarised in ¹²¹) Tupaia: Mild hepatitis; Acute and chronic; Oncogenic ¹²⁸
HDV	HBV-dependent infection; Co-infection with HBV: Mild to severe acute disease; Superinfection with HBV: Mostly chronic, worsens disease outcome compared to HBV mono-infection	Snake: No hepatotropism; Apparently independent of HBV ⁴⁸	Chimpanzee: Hepatotropism; HBV co-infection and superinfection; Co-infection: Mild disease; Superinfection: Acute severe disease, >50% subclinical chronic HDV infection ^{129,130} (summarised in ¹³¹); Woodchuck: Uses WHV envelope; Hepatotropism; Acute and chronic (summarised in ¹³¹)
HEV	Low chronicity rate; Quasi-envelopment; Mainly faecal-oral transmission; Zoonotic	Rabbit: Faecal shedding; Acute and chronic hepatitis; Viremia ¹³² Rat: Faecal shedding; Apparent hepatotropism; Mild hepatitis ^{133,134} Ferret: Faecal shedding; Viremia; Acute and chronic hepatitis ¹³⁵ Bat: Faecal shedding; Viremia ⁵⁴ Swine: Faecal shedding; Viremia; Acute and chronic ^{136,137} Moose: Faecal shedding; Mild hepatitis ¹³⁸ Chicken: Hepatitis-splenomegaly syndrome ⁵⁹ Camel: Faecal shedding; Viremia ^{51,52}	Chimpanzee: Severe acute hepatitis ¹³⁹ Macaque: Faecal shedding; Viremia; Hepatitis ^{140,141} Swine: Faecal shedding; Mild hepatitis ¹⁴²

HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HEV, hepatitis E virus; HCC, hepatocellular carcinoma; WHV, woodchuck hepatitis virus.

or religious practices such as tattooing, circumcision, acupuncture or scarification might have enabled virus transmission.¹⁰⁰ Transmission routes among non-human hepaciviruses are unclear. In small mammals, transmission of hepaciviruses through biting and scratching associated with mating and territorial behaviour is conceivable.¹⁰¹ In horses, parenteral transmission of equine hepacivirus has been suggested,^{102,103} possibly including sexual transmission and veterinary practices.^{41,102} Transmission via human-

aided routes would suggest a relatively recent spread of this virus among equines worldwide and is consistent with the low virus diversity found in equines.^{38,41,102,104} Similar transmission routes are conceivable for cattle hepaciviruses.^{44,45}

In sum, virus transmission seems to be evolutionary conserved among enteric hepatitis viruses and their homologues, while transmission routes of non-human hepadnaviruses and hepaciviruses are poorly understood.

Determinants of hepatotropism are poorly understood

For HAV and HEV, little is known about the viral and host factors that promote liver tropism in humans. In bat hepatoviruses, but in none of the other non-primate hepatoviruses, almost equal amounts of virus were detectable in the liver and spleen.²⁵ However, whether this is due to sequestered macrophages containing hepatovirus genetic material, as observed during experimental infections of mice with HAV,¹⁴³ or due to extrahepatic viral replication remains to be determined.

For HBV and HDV, after low-specific binding to heparin-sulfate proteoglycans (summarised in¹⁴⁴), hepatocyte entry is mediated via the sodium taurocholate co-transporting polypeptide (NTCP for the human receptor, Ntcp for the homologous receptor in animals).⁶⁷ These liver-specific receptors likely govern the hepatotropism of HBV and HDV.⁶⁷ Interestingly, the avian hepadnavirus DHBV does not show a strict hepatotropism (summarised in¹¹⁹). This is potentially explained by the usage of a receptor that differs from that used by HBV. Notably, even among mammalian hepadnaviruses, Ntcp usage might not be conserved, as illustrated by the apparent lack of hNTCP usage of some bat hepadnaviruses and WHV.^{30,145} In the snake delta-like agent, the apparent lack of hepatotropism may be associated with the absence of a detectable hepadnavirus (Table 2).⁴⁸

For HCV, an important, yet not exclusive factor determining hepatotropism in humans is its interaction with the liver-specific micro RNA-122 (miR-122).¹⁴⁶ All known non-human hepaciviruses contain at least one miR-122 binding site in their 5'-genome ends, which may contribute to their apparently conserved hepatotropism.^{147,148}

Liver tropism is thus a widely, yet not perfectly conserved attribute of the animal homologues of human hepatitis viruses. Because the factors determining hepatotropism of human hepatitis viruses are not entirely understood, systems allowing experimental infections with the newly discovered animal viruses will hopefully provide urgently needed insights into the determinants of viral hepatotropism.

Chronic courses of infection occur in diverse hepatitis virus hosts

Prehistoric human populations were small and scattered, raising the question of how hepatitis viruses survived in these populations. This is illustrated by the disappearance of HAV in isolated populations due to the lifelong immunity HAV infections engender (summarised in²⁵). In contrast to prehistoric humans, other hepatovirus hosts such as small mammals were abundant and widespread, which presumably aided viral survival in these populations.²⁵

In contrast to HAV, both HBV and HCV establish chronic infections in humans, which presum-

ably favoured virus maintenance in prehistoric human populations and among NHPs. Chronic hepatitis virus infections are commonly defined as infections that persist for more than 6 months for HBV and HCV, and 3 months for HEV.^{149–151}

The chronicity rate varies drastically among human hepatitis viruses. Chronic HEV infections are very rare (<1% of infections) and almost exclusively occur in immunocompromised patients.¹⁵² In contrast, chronic HCV infections are very frequent at up to 80–85% (Table 1).⁶³ For HBV, the chronicity rate varies depending on the age at which the infection occurs, with rates of up to 90% for infected neonates, 30% for children aged 1–5 years and less than 5% for older children and adults (summarised in⁶²).

Interestingly, chronic courses of infection are not unique for human HBV and HCV. Similar to human HBV infection, the chronicity rate of the rodent-associated WHV is age-dependent. While animals infected as newborns generally develop chronic infections, WHV usually causes acute self-limiting infections in animals infected at older ages (summarised in¹¹⁵). Chronic courses of hepadnavirus infections also occur in ducks, squirrels, and NHPs (Table 2).^{30,96,112,113,116–118,153} Whether chronic hepadnavirus infections occur in bats is unclear.³⁰

Among non-human hepaciviruses, studies reporting chronic infections are scarce. Chronic hepacivirus infections with prolonged viremia for more than 6 months were sporadically observed in experimentally infected horses and naturally infected cattle.^{44,102,154} The chronicity rates in horses and cattle thus seem to be much lower than in human HCV infections.^{44,102,103} For other non-human hepaciviruses, whether chronic infections occur remains unknown. Similarly, chronic courses of hepevirus infections in non-human hosts are largely unknown. Nonetheless, an apparent chronic HEV infection in wild boars and prolonged viral shedding in experimentally infected immunocompromised domestic swine, rabbits, and ferrets suggest that chronicity is not limited to human HEV infections.^{132,135,137,155}

To conclude, many questions regarding chronic infections in animal hepatitis viruses are still unanswered. Translational animal models to assess therapeutics for chronic hepatitis B and E, and investigating viral pathogenesis will require some correlate of chronic infection, which may be conceivable given the evolutionary conservation of chronicity among several animal hepadnaviruses and hepaciviruses.

Mechanisms leading to HCC differ among hepatitis viruses

Chronic hepatitis virus infections can result in severe disease outcomes, including cirrhosis and HCC.¹⁵⁶ In humans, chronic HBV infections result in HCC in 15–40% of cases¹⁵⁷ and chronic HCV infections in approximately 2.5% of cases.¹⁵⁸

Key point

Chronicity may have aided hepatitis B and hepatitis C virus survival in scattered prehistoric human populations.

Interestingly, woodchucks infected with WHV and ground squirrels infected with the genetically related ground squirrel hepatitis virus are at a high risk of developing HCC when infected at birth (summarised in¹⁵⁹) (Table 2). While oncogenesis in woodchucks is mainly caused by *N-myc* gene activation via targeted insertion of hepadnaviral DNA into host DNA, oncogenesis in ground squirrels is associated with an activation of *c-myc* genes.¹⁶⁰ For human HBV, the mechanisms of oncogenesis remain poorly understood. HBV DNA fragments can integrate into the hepatocyte genome during viral replication.¹⁶¹ However, in contrast to rodent hepadnaviruses, no specific integration site associated with oncogenesis has been identified. Accumulation of integration events into genes that may enhance oncogenesis, such as those encoding the telomerase reverse transcriptase (*TERT*), a histone H3 lysine 4 methyltransferase (*MLL4*), and cyclin E1 (*CCNE1*) have been described,^{162,163} but their contribution to oncogenesis is unclear. Furthermore, integration sites in tumour cells seem to be enhanced in sites critical for chromosome stability, such as in proximity to telomeres.¹⁶³ In addition, the viral HBx protein promotes oncogenesis through complex interactions with the host cell, such as altering the expression of host oncogenes and tumour suppressors, stimulating cell-cycle entry by activating cyclins and cyclin-dependent kinase pathways and blocking apoptosis (summarised in¹⁶⁴). In humans, HBx also interacts with the structural maintenance of chromosomes (Smc) complex Smc5/6 and stabilises the extrachromosomal cccDNA (covalently closed circular DNA) produced during HBV replication.¹⁶⁵ Interestingly, HBx presumably emerged *de novo* in mammals during hepadnavirus evolution.³⁷ Although the role of the HBx protein in non-human hepadnavirus infections is unknown, conservation of the HBx gene in mammalian orthohepadnaviruses suggests that HBx-mediated oncogenesis may be conceivable in diverse mammals. In concordance, ducks infected with avian hepadnaviruses lacking an HBx do not develop HCC (summarised in¹¹⁵).

In contrast to HBV, integration of viral genetic material into the host genome does not occur during HCV infections. Here, the core protein and non-structural proteins NS3 and NS5A promote HCC development by altering the expression of host genes involved in diverse oncogenic pathways, including proteins involved in cell-cycle control (such as p53, p21, cyclins) and apoptosis (summarised in¹⁶⁴). Strikingly, HCC development has not been observed in any non-human hepacivirus host yet. Hypothetically, oncogenesis might either be unique to human HCV infections, or oncogenesis in animals has not been observed due to the relatively short life spans of the investigated animals, e.g. mice and rats infected with rat hepaciviruses.¹⁶⁶ In contrast to these rodents, some bat species have a comparably long life span

of up to 30 years.¹⁶⁷ However, whether bats can get chronically infected with hepaciviruses and if this infection leads to oncogenesis is unknown. In addition, it has been hypothesised that bats may have a lower risk of developing cancer than other mammals, potentially because of their unique physiological and immunological properties.¹⁶⁸ In horses and cattle, chronic courses of hepacivirus infections have been described and it would be intriguing to study whether these large and relatively long-lived animals develop HCC. However, costly long-term infection studies and longitudinal epidemiological investigations will be needed to address this question.

If and how animals infected with hepatitis viruses develop HCC is not well understood. HCC can be induced in mice, but this requires genetic engineering of the mouse genome, the use of chemotoxic agents, injection of tumour cells or xenograft approaches (summarised in¹⁶⁹). Both HBV and HCV can cause chronic infections and HCC in chimpanzees, but using chimpanzees as animal models is strongly restricted for ethical reasons. An essential step towards an animal model for oncogenic hepatitis is the identification of a tractable, long-living host with oncogenic mechanisms similar to human HBV and HCV infections.

Receptor usage differs across hepatitis virus homologues

A crucial step during viral infection and a major factor limiting cross-species transmission is the interaction of viral proteins with host cell membrane structures enabling viral attachment and entry into cells. Strikingly, the cellular receptor molecules of HAV and HEV are still unknown. Recent data revealed that contrary to textbook knowledge, TIM1 is not essential for HAV entry into hepatocytes or epithelial cells.^{66,170}

HBV host specificity seems to be determined by a few essential amino acids of the cellular receptor NTCP/Ntcp. Interestingly, human HBV can use the Ntcp of great apes and New World monkeys, but 1 specific amino acid exchange in the Ntcp is needed for a cross-species transmission of human HBV to Old World monkeys.^{33,171} Similarly, 1 amino acid exchange in the woodchuck Ntcp enables an efficient infection with HBV.¹⁷² However, additional unidentified host-specific co-factors might be essential for viral entry and infection. For example, while HDV infection of mouse hepatocytes can be enabled by alteration of just 3 amino acid residues in the murine Ntcp, HBV infection is not possible in these animals.¹⁷³ In addition, HBV infection can be enabled in pig and macaque hepatocytes expressing the human NTCP, but not in similarly engineered hepatocytes of mouse, rat, and dog origin.¹⁷⁴ Strikingly, HBV can readily infect primary hepatocytes of the Asian tree shrew,¹⁷⁵ which implies that both the HBV cellular receptor and potential co-factors are conserved in this animal.

Key point

Some biological properties, such as hepatotropism, receptor usage or mechanisms leading to HCC differ among human hepatitis viruses and ancestral viruses carried by animals other than primates.

For HCV, a complex interaction of various receptor molecules has been described^{69–71} (Table 1). Like HBV, the host range of HCV seems to be limited by its receptors. Interestingly, rodents can be infected with HCV following modifications of either the host or the virus. On the host side, human liver-chimeric mice susceptible to HCV provide promising opportunities to circumvent host specificity.¹⁷⁶ On the viral side, only 3 amino acid exchanges within the viral envelope proteins of HCV have been shown to allow infection of mouse hepatocytes *in vitro*, but additional adaptations might be necessary for efficient replication *in vivo*.¹⁷⁷ Among non-human hepaciviruses, entry mechanisms have not been well studied, but the inability of the equine hepacivirus to infect human cells *in vitro* suggests a high level of host specificity.¹⁵⁴ Recently detected hepaciviruses might provide exciting new opportunities for animal models, as exemplified by a rat hepacivirus capable of infecting immunocompetent laboratory mice and rats.¹⁶⁶ Notably, any tractable animal model of human hepatitis would greatly benefit from susceptibility to human viruses or recombinants thereof, which has rarely been achieved.

Viral immune evasion strategies differ in host specificity

In addition to viral entry, cross-species transmission is limited by host immune responses and the ability of viruses to evade these responses after host switching. Among other mechanisms, HAV and HCV evade the human innate immune response by cleavage of the mitochondrial antiviral signalling protein (MAVS). MAVS is an essential part of interferon pathways induced by viral double-stranded RNA.¹⁷⁸ MAVS is cleaved by the HAV protease precursor 3ABC¹⁷⁸ and the HCV protease NS3/4A¹⁷⁹ at different sites. Strikingly different levels of host specificity are evident in HAV- and HCV-mediated MAVS cleavage. HAV-mediated MAVS cleavage seems to be rather host-specific based on the inability of human HAV to cleave murine MAVS.¹⁴³ The ability of non-human hepatoviruses to cleave cognate and heterologous MAVS has not been studied yet and requires further investigation. Interestingly, for hepaciviruses, MAVS cleavage via the NS3/4A protease is conserved among equine, bat, rodent, and primate hepaciviruses,^{178,180,181} hence reflecting a conserved immune evasion strategy. Strikingly, human MAVS can be cleaved by all of these diverse non-human hepaciviruses,¹⁸¹ hinting at a less host-specific mechanism compared to HAV.

In conclusion, both virus-receptor interactions and host immune responses can restrict cross-species transmission. Understanding the underlying mechanisms is essential for adapting potential novel animal models to human hepatitis viruses.

Chapter 4: Evolutionary origins of hepatitis viruses

The plethora of newly discovered viruses provides insights into the genealogy of human hepatitis viruses.¹⁸² Here, we discuss hypotheses on the when, the whence, and the where of human hepatitis virus evolution.

The age of human hepatitis viruses is underestimated

For all human hepatitis viruses, projections of the most recent common ancestors (MRCA) based on extant viruses yield surprisingly similar results in the range of several thousand years before present.^{183–185} Hypothetically, environmental factors such as the formation of large human populations, rearing of livestock and changes in land use might have contributed to the rise of hepatitis viruses in humans and explain the similarities of calculated MRCA. However, projections of ancient MRCA need to be treated with caution, because of the technical constraints of bioinformatic programmes, recombination events, and an inevitable sampling bias. These limitations are best illustrated by variations of the projected origins of HBV, which vary by several orders of magnitude.^{186–188} In addition, ancient HBV sequences from mummies and human remains revealed that HBV strains closely related to contemporary strains already occurred in the Neolithic, strongly suggesting that HBV is much older than previously thought.^{189–191} The avian-associated hepadnaviruses were dated to have emerged around 6,000 years ago and thus again in a similar range as those yielded by projections of human-associated hepatitis virus MRCA.¹⁸⁶ Again, the limitations of such projections became evident when endogenous hepadnavirus elements were identified in the genomes of birds and reptiles.^{37,192–195} According to these molecular fossils, hepadnaviruses must have occurred in the Early Mesozoic > 200 million years ago (mya), predating the rise of mammals (Fig. 3A) and again exceeding the MRCA from bioinformatic calculations by several orders of magnitude.¹⁹⁶

Ancient origins of non-human hepatitis viruses

Interestingly, hepatitis viruses are found in diverse vertebrate taxa (Fig. 3A), hinting at a long evolutionary association between vertebrates and hepatitis viruses. The common ancestors of vertebrates date back to the Ordovician, approximately 450 mya, from where they diversified into fish, amphibians, reptiles, birds, and mammals. Notably, all of these vertebrate taxa harbour viruses with some genetic relationship to human hepatitis viruses. Remarkably, viruses and viral elements with genetic or structural relationships to hepatoviruses, hepadnaviruses, hepaciviruses, and hepeviruses also exist in arthropods,^{197–200} which are evolutionarily ancient animals that evolved approximately 500 mya.²⁰¹ These recent

Key point

Human hepatitis viruses have ancient zoonotic origins.

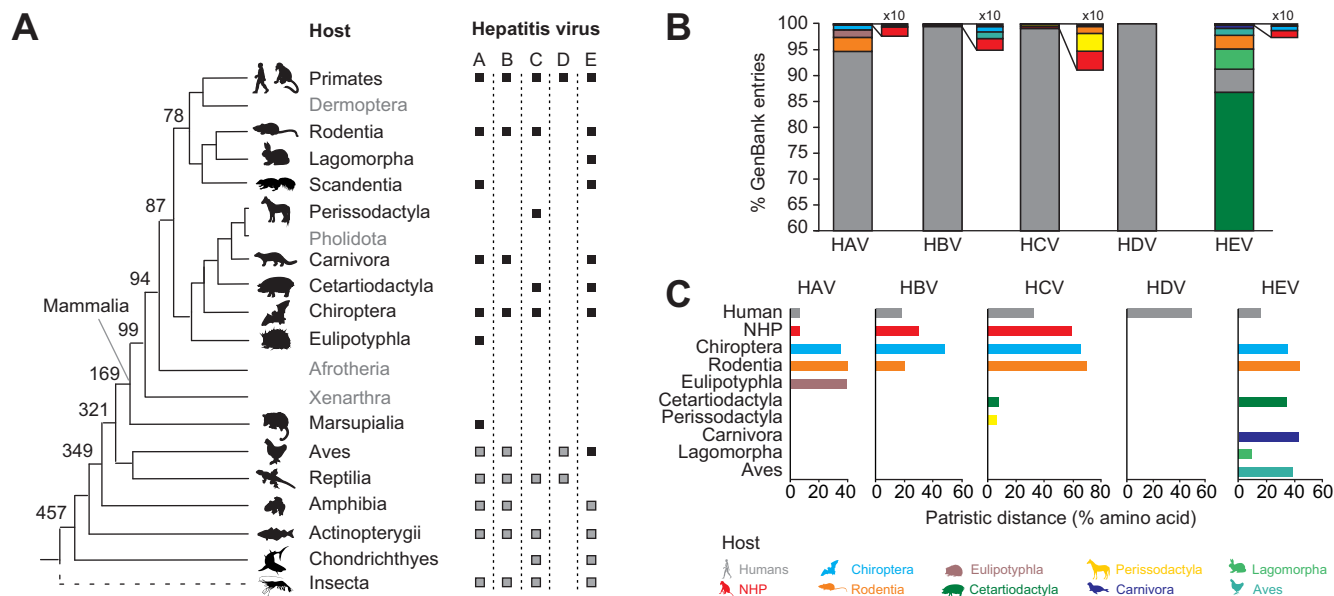


Fig. 3. Hepatitis virus homologues in non-human hosts. (A) Simplified phylogenetic tree of mammalian orders, Aves, Reptilia, Amphibia, Osteichthyes, Chondrichthyes, and Insecta (Phylogeny adapted from^{196,239}). Squares indicate orders for which homologues of human hepatitis viruses were found; black, same virus genus as human representative; grey, viruses distantly related to human hepatitis viruses. Host taxa for which no homologous hepatitis viruses were found are shown without pictograms and are coloured in grey. Numbers next to nodes show approximate time of divergence in million years according to^{196,239} (B) GenBank entries of human hepatitis viruses and their homologues per mammalian host order given in percent of all entries of the search term. Host orders with less than 0.1% of entries are summarised as *others*. Host orders with less than 0.5% of entries are magnified 10-fold to the right. GenBank search terms and final entries per mammalian host taxon were: for HAV (January 17, 2018): “*Hepatovirus [Organism] AND host [All Fields]*”, Human, n = 3,186, Rodentia, n = 90, Eulipotyphla, n = 49, Chiroptera, n = 32, NHP, n = 6, Scandentia, n = 1, and Carnivora, n = 1; for HBV (January 19, 2018): “*Hepadnaviridae [Organism] AND host [All Fields]*”, Human, n = 68,867, NHP, n = 156, Aves, n = 87, Chiroptera, n = 73, Rodents, n = 25 and Cetartiodactyla, n = 10; for HCV (January 18, 2018): “*Hepacivirus [Organism] AND host [All Fields]*”, Human, n = 132,645, NHP, n = 491, Perissodactyla, n = 456, Rodentia, n = 182, Carnivora, n = 25, Cetartiodactyla, n = 18 and Chiroptera, n = 12; for HDV (January 17, 2018): “*Deltavirus [Organism] AND host [All Fields]*”, Human, n = 912; for HEV (January 17, 2018): “*Hepeviridae [Organism] AND host [All Fields]*”, Human, n = 7,601, Cetartiodactyla, n = 3,152, Lagomorpha, n = 456, Rodentia, n = 316, Aves, n = 155, Carnivora, n = 76, Non-human primates, n = 16, Chiroptera, n = 10, Scandentia, n = 2, Eulipotyphla, n = 1 and Perissodactyla, n = 1. (C) Patristic distance (maximum percentage pairwise amino acid distance) per host order calculated in MEGA7²⁴⁰ using translation alignments generated in Geneious 9.1.8.²⁴¹ HAV: A GenBank search with the term “*Hepatovirus*” was performed on January 18, 2018, and all sequences longer than 6,000 nucleotides were selected. Duplicates, cell culture-adapted strains, or viruses isolated from experimentally infected animals were excluded from the dataset. Sorting according to host orders resulted in: Human, n = 101, NHP, n = 5, Chiroptera, n = 4, Rodentia, n = 7 and Eulipotyphla, n = 5 sequences for translation alignments of complete polyproteins. Non-homologous regions (px/3A) were subsequently deleted. For HBV, HCV and HDV, GenBank searches as performed for 3B were used. HBV: All sequences with complete polymerase CDS from non-human hosts were selected. Complete polymerase CDS of reference sequences from²⁴² and the capuchin monkey hepatitis B virus (CMHBV, Acc. No. KY703886) were used. Sorting according to host orders resulted in Human, n = 37, NHP, n = 92, Chiroptera, n = 60 and Rodentia, n = 5 sequences. HCV: All sequences with complete polyprotein CDS from non-human hosts were selected. Complete polyprotein CDS of reference sequences from (<https://hcv.lanl.gov/components/sequence/HCV/search/searchi.html>) and GBV-B (Acc. No. NC001655) were used. Sorting according to host orders resulted in Human, n = 178, NHP, n = 4, Chiroptera, n = 5, Rodentia, n = 15, Perissodactyla, n = 19, Cetartiodactyla, n = 10 sequences. HDV: Sequences with complete HDAG CDS were selected resulting in a total of 158 human-derived sequences. HEV: A GenBank search with the term “*Hepeviridae*” was performed on January 25, 2018, and all sequences longer than 6,000 nucleotides were selected. Duplicates, cell culture-adapted strains, or viruses isolated from experimentally infected animals were excluded from the dataset. Sorting according to host orders resulted in Human, n = 165, Chiroptera, n = 3, Rodentia, n = 18, Cetartiodactyla, n = 93, Aves, n = 11, Carnivora, n = 13 and Lagomorpha, n = 17 sequences. CDS, coding sequences; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HEV, hepatitis E virus; NHP, non-human primates. Bars are coloured according to host taxon.

Key point

Hepatitis viruses are much older than previously thought, with hepatitis B virus ancestors predating the rise of mammals.

observations further corroborate an ancient evolutionary origin of hepatitis viruses.

The genetic diversity of viruses in a given host taxon provides insights into the time of virus evolution within that host. In public databases, the number of HAV, HBV, and HCV genomic sequences originating from humans highly exceeds the number of sequences from their non-human homologues (Fig. 3B). In contrast, the majority of HEV sequences originates from even-toed ungulates, including swine, camels, cattle, deer, sheep, and moose. This is consistent with the high impact of these zoonotic viruses on human health. Interestingly, despite the comparably low number of genomic sequences available for animal homologues of HAV, HBV, and HCV, the genetic diversity

of non-human viruses generally exceeds that of the human-associated viruses (Fig. 3C). This is especially conspicuous for viruses hosted by small mammals, hinting at a longer association of small mammals with hepatitis viruses compared to humans. The comparably low diversity in human hepatitis viruses may additionally be linked to evolutionary pressures favouring certain genotypes,²⁰² the rapid expansion of the human population potentially favouring certain viruses over others, and to extinction events best illustrated by the recent findings of an extinct HBV genotype in human remains.^{190,191}

In conclusion, hepatitis viruses are presumably much older than previously thought. The comparison of hepatitis virus diversity in different host

taxa suggests that evolution in non-human hosts generally preceded the introduction of hepatitis viruses into the human lineage.

Complex macro-evolutionary events during hepatitis virus evolution

Hypothetically, hepatitis viruses may have accompanied speciation of their hosts. Indeed, similarities in the topologies of relatively old vertebrate lineages such as fish, amphibians and birds and their hepato-, hepadna- and hepaciviruses may hint at virus-host co-speciation.^{36,203} In addition, evidence for co-speciation exists for the New World monkey hepadnaviruses CMHBV and WMHBV.³³ However, cross-species transmission must also have occurred during the evolution of hepatitis viruses. Apart from the zoonotic HEV genotypes, examples of cross-species transmission events include hepatoviruses in marsupials that are of likely rodent origin,²⁸ horse-associated hepaciviruses in dogs and donkeys,^{38,41} gorillas infected with chimpanzee hepadnaviruses, orangutans infected with gibbon hepadnaviruses,¹¹⁹ and rat hepeviruses in shrews.²⁰⁴ In addition, the tentative HBV genotype J likely emerged from a recombination event involving human- and gibbon-associated parental strains, hinting at cross-species viral transmission from gibbons to humans (summarised in⁸⁰). Evidence for cross-species transmission events in hepatitis viruses might indicate that humans or their primate precursors acquired hepatitis viruses from another animal host.

It remains unclear when this hypothetical host switching into primates occurred. Nonetheless, a relatively recent introduction of hepatitis viruses into humans or their primate precursors is strongly supported by the lack of genetic diversity among primate viruses compared to non-primate hepatitis virus homologues, and by the complete absence of human viruses clustering in basal positions in phylogenetic reconstructions (Fig. 4A-E).

Interestingly, the frequency of predicted cross-species transmission events varies among the different hepatitis virus families. While cross-species transmission may be relatively frequent in the enterically transmitted hepato- and hepeviruses, it seems to be comparably infrequent in blood-borne hepadna- and hepaciviruses.^{29,36,205} Possible explanations for this observation include potentially easier transmission of highly stable enterically transmitted viruses compared to viruses likely transmitted exclusively through contact with body fluids. Additionally, different levels of host specificity due to receptor interactions or viral immune evasion strategies may play a role, as outlined earlier.

The macro-evolutionary patterns of hepatitis virus are thus complex and likely include both co-speciation and cross-species transmission events. Apart from certain HEV strains, no recent

zoonotic event is evident in the phylogenetic reconstructions of human hepatitis viruses.

A recent NHP origin of human hepatitis viruses is unlikely

Since genetic relatedness of hosts facilitates cross-species transmission of viruses²⁰⁶ one could assume human hepatitis virus origins in NHPs, similar to the origins of HIV in monkeys and apes.²⁰⁷ Indeed, relatives of HAV, HBV, and HCV are found in NHPs (Fig. 4A). However, for HAV and HCV these detections are sporadic and the viruses are not genetically diversified in NHPs²² (Fig. 4A/C). In addition, hepaciviruses from NHPs do not share a recent common ancestor with human HCV (Fig. 4C), suggesting a non-recent host switch into NHPs from an unknown source. Among primate HBV, diversified strains from Old World apes are intermixed with human strains and recombination events among these viruses hint at past cross-species transmission.¹¹⁹ However, the relatively large genetic diversity of human viruses compared to viruses from Old World apes may hint at an origin of HBV in the human stem lineage.^{33,208} Interestingly, both the WHMBV and the CMHBV surface proteins can use the hNTCP for cell entry, suggesting zoonotic potential of these divergent HBV species from New World monkeys. Nonetheless, the phylogenetic relationships of primate HBV suggest that neither CMHBV, nor WMHBV are direct ancestors of human HBV.³³ Hence, our current knowledge on primate HAV, HBV and HCV diversity does not support a recent origin of human viruses in NHP. Thus, studies investigating hepatitis virus diversity in diverse NHP species and NHP remains are urgently needed.

The complex where and whence of human hepatitis viruses

Small mammals such as bats and rodents are particularly relevant animal reservoirs of human pathogens.^{209,210} Their important role as hosts for zoonotic pathogens has been linked to their species richness, abundance, worldwide distribution, the synanthropic behaviour of some species, the ability of bats to fly, to gregarious populations of some bat species and to unique immunological properties of bats.^{209,211,212} Interestingly, highly diversified homologues of HAV, HBV, HCV, and HEV were detected in these 2 mammalian orders^{25,28,30,42,43,54,213,214} (Fig. 4), hinting at the importance of small mammals during hepatitis virus evolution.

One can assume that the diversity of viruses is highest in areas of prolonged circulation. Based on this assumption, the origins of human hepatitis viruses can be mainly projected to the Old World (Fig. 5).^{80,100} In addition, diverse viruses assigned to HAV and HBV are found in Old World NHPs which hints at a relatively long association of hepatitis viruses with primates in the Old World

Key point

Both cross-species transmission and co-speciation likely shaped hepatitis virus evolution.

Key point

Small mammals were important hosts during the evolution of human hepatitis viruses.

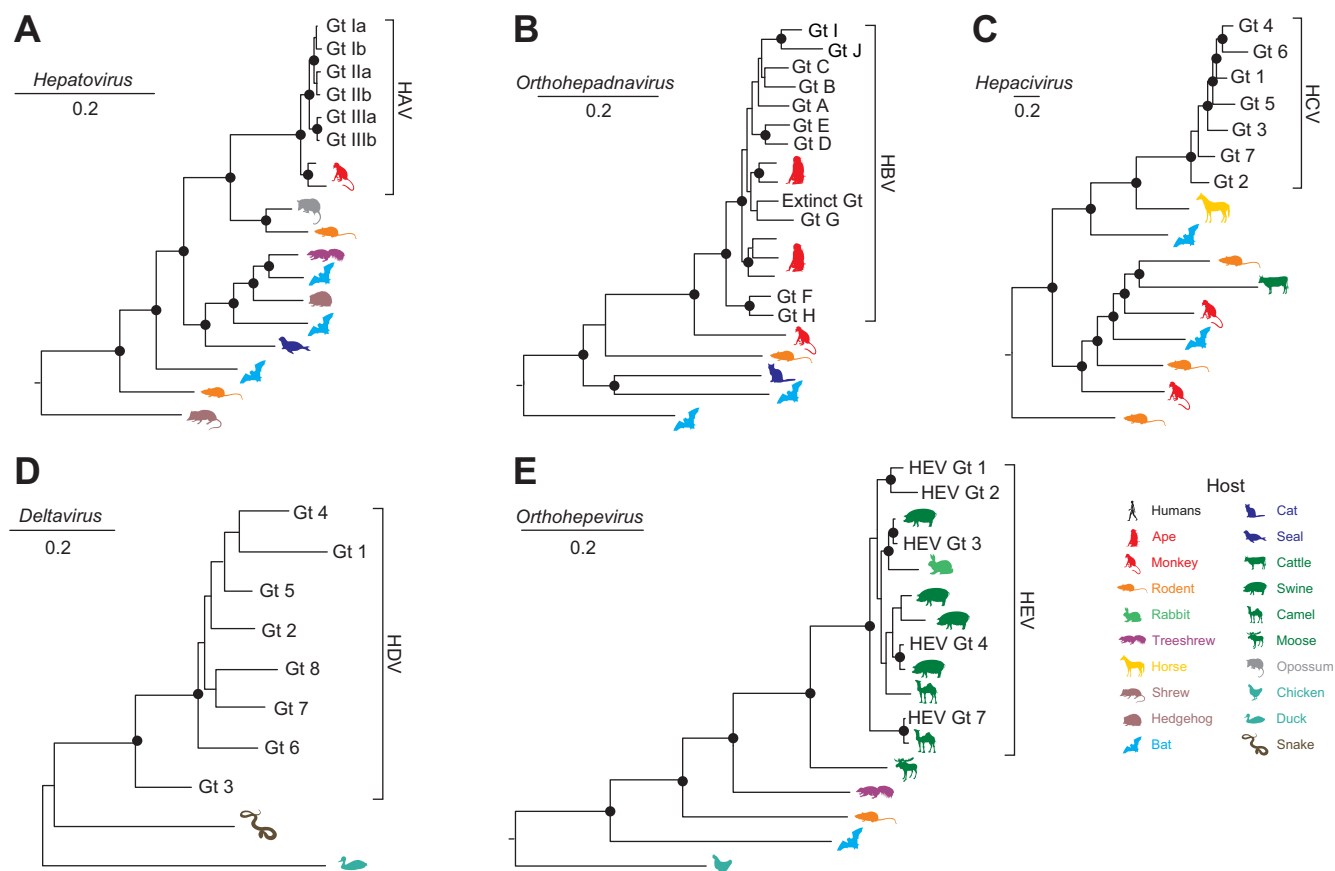


Fig. 4. Phylogenetic relationships of human hepatitis viruses and their vertebrate homologues. Phylogenies are based on amino acid alignments for hepatoviruses, hepaciviruses, deltaviruses, and hepeviruses and a nucleotide alignment for hepadnaviruses generated using MAFFT²⁴³ in Geneious 9.1.8.²⁴¹ Phylogenies were generated using a WAG substitution model (for amino acid sequences) or an HKY substitution model (for nucleotide sequences) excluding all ambiguous data or gaps. Hepadnavirus and deltavirus phylogenies were generated using a Maximum Likelihood algorithm and 1,000 bootstrap replicates in MEGA7.²⁴⁰ For all other virus genera, Bayesian phylogenies were generated using MrBayes V3.1.²⁴⁴ Outgroups were: Hepatoviruses, Avian encephalomyelitis virus, accession number (Acc. No.) NC003990; Hepadnaviruses, Bluegill hepadnavirus, Acc. No. NC030445; Hepaciviruses, Wenling shark virus, Acc. No. KR902729; Hepeviruses, *Pischihepevirus*, Acc. No. HQ731075. The deltavirus phylogeny was calculated without an outgroup. Bayesian phylogenies were run for 2 million generations, sampled every 100 steps. After an exclusion of 5,000 of the total 20,000 trees as burn-in, final trees were annotated with TreeAnnotator from the BEAST package and visualised with FigTree.²⁴⁵ Bayesian posterior probability support of grouping above 0.9 or bootstrap support above 90% is highlighted by filled circles at nodes. The scale bar indicates genetic distance. Animal pictograms are coloured according to their host taxa: Black and red, Primates; Orange, Rodentia; Light green, Lagomorpha; Pink, Scandentia; Yellow, Perissodactyla; Light brown, Eulipotyphla; Light blue, Chiroptera; Dark blue, Carnivora; Dark green, Cetartiodactyla; Grey, Marsupialia; Turquoise, Aves; Dark brown, Reptilia. (A) For hepatoviruses, the complete P1 domain was used. Acc. No. of representative sequences were: M59286, KT452691, KT877158, KT452714, KT452730, KR703607, KR452742, KT452735, AB279735, AB279732, AY644670, AY644676, AF268396, AB020564, D00924, KT452685, KT452658, MG181943. (B) For hepadnaviruses, complete genomes were used. Acc. No. of representative sequences were: MH307930 NC024444, FN594748, EU594434, AB032432, FJ798097, FM209516, LT992443, AF193863, FM209514, GU563556, AB194951, AB368296, FJ899779, AB036920, AB516393, AF046996, NC24445, NC004107, AB486012, FJ023659. (C) For hepaciviruses, domains encoding the structural proteins C-E1-E2-p7 were used. Acc. No. of representative sequences were: KC411777, JQ434004, D00944, GU814265, M62321, Y12083, Y13184, D17763, EF108306, KC796074, KC411806, KC796077, U22304, KJ950938, KC551800, KP641127. (D) For deltaviruses, complete S-HDAg coding regions were used. Acc. No. of representative sequences were: X04451, X60193, L22063, AF018077, AJ584848, AJ584847, AJ584844, AJ584849, MH988742, MH824555. (E) For hepeviruses, complete ORF 2 sequences encoding the viral capsid were used. Acc. No. of representative sequences were: AB197673, DQ279091, AB573435, AF082843, AP003430, KJ013415, KJ496143, KT818608, KX387865, AB602441, M73218, M74506, KF951328, KR905549 GU345042, AY535004, JQ001749. Gt, Genotype; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HEV, hepatitis E virus; ORF, open-reading frame; S-HDAg, small form HDV antigen.

compared to the New World. However, divergent HBV and HDV genotypes occur in American natives, namely HBV genotypes F and H and HDV genotype 3.^{187,215} Prior to the novel animal delta-like agents, it was not possible to root the phylogenetic tree of HDV. Now, upon inclusion of the novel snake and duck delta-like agents, surprising similarities are found in the sister relationship of the New World HBV and HDV genotypes

and Old World genotypes (Fig. 4B,D). Notably, the existence of these divergent New World genotypes does not necessarily suggest ancient origins of HBV and HDV in the New World, because these genotypes show only limited genetic diversity compared to the Old World genotypes. It seems plausible that the ancestors of New World HBV and HDV genotypes were introduced to the New World during the peopling of the Americas via

Key point

The direct ancestors of human hepatitis viruses so far remain unknown.

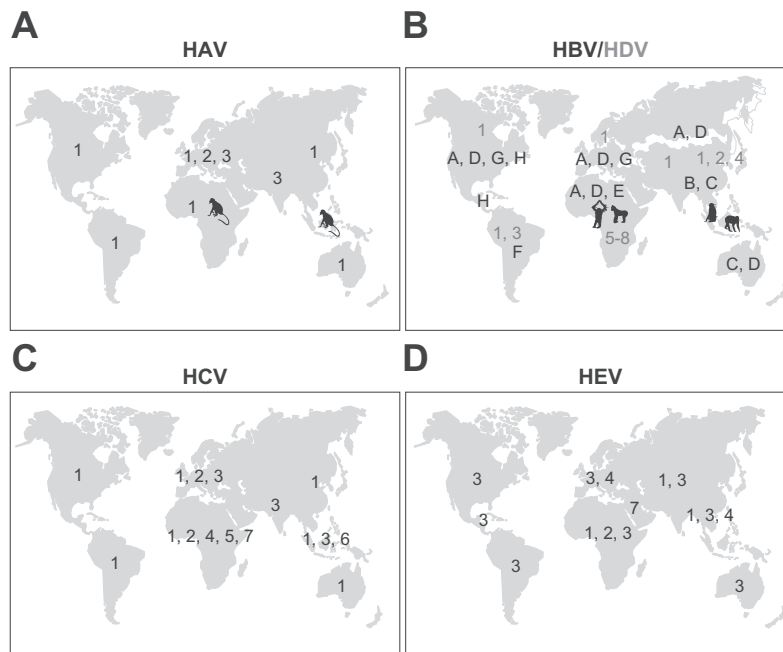


Fig. 5. Global distribution of hepatitis virus genotypes. Distribution of the most prevalent genotypes were adapted and simplified from²⁴⁶ for HAV, from^{247,248} for HBV, from^{249,250} for HCV, from⁸⁷ for HDV and from¹⁸⁵ for HEV. NHP genotypes are shown as pictograms and represent HAV genotype 5 in Africa, HAV genotypes 4 and 6 in Asia,²⁴⁶ Chimpanzee HBV and Gorilla HBV in Africa and Orangutan HBV and Gibbon HBV genotypes in Asia.^{119,251} HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HEV, hepatitis E virus; NHP, non-human primate.

the Beringian land bridge approximately 15–23 thousand years ago,²¹⁶ before then going extinct in the Old World.³³

The evolutionary ancestors of human hepatitis viruses are unknown. Presumably, a zoonotic introduction into humans or their primate precursors occurred non-recently in the Old World. Further evidence elucidating the genealogy of human hepatitis viruses can be expected in the coming years.

Concluding remarks

Major progress has been made in the study of human hepatitis viruses during the last 60 years. However, crucial aspects of hepatitis virus evolution and pathogenesis remain poorly understood. At the same time, properties we took for certain have been called into question, such as the predominant association of hepatitis viruses with human hosts, the determinants of hepatotropism and the distinction of typically either enveloped or non-enveloped viruses. In this review, we discussed pathogenesis, evolutionary origins and zoonotic risks of human hepatitis viruses in light of recent discoveries of a plethora of animal homologues.

It is anticipated that the newly discovered animal viruses will help to answer crucial questions regarding the pathogenesis of hepatitis viruses, e.g., whether the existence of quasi-enveloped particles of HAV and HEV is evolutionarily conserved, and which viral determinants are involved in oncogenesis and the potential to cause chronic

infections. Although hepatotropism is not fully understood even for the well-investigated HAV, HCV and HEV, the recent detection of HDV-like agents in animals apparently showing both a broad organ tropism and a lack of detectable helper viruses emphasises the need to investigate the bases underlying the unique biological properties of human hepatitis viruses.^{47,48}

The evolutionary origins of human hepatitis viruses have remained enigmatic. The recent discoveries of highly diverse animal homologues hint at the evolutionary origins of all human hepatitis viruses in non-human hosts. These origins may be rather ancient, and involve old vertebrate lineages such as reptiles, amphibians, birds, fish and potentially even arthropods^{197–200,217} (Fig. 6A). Among mammals, small mammals such as bats and rodents harbour particularly diversified hepatitis viruses. One may speculate that arthropod-borne hepatitis virus precursors may have been passed to insectivorous small mammals via the blood-borne route or by ingestion of insects.^{25,217} These hosts hypothetically acted as a point of entry for ancient viruses carried by arthropods or lower vertebrates into mammals. In small mammals, viruses may then have diversified and eventually have been transmitted directly or via intermediate hosts to humans.^{29,119} It is also conceivable that several independent introductions of hepatitis viruses into different vertebrate lineages from arthropods, lower vertebrates and yet unknown sources have occurred (Fig. 6B). Presumably, hepatitis virus evolution combined both

Key point

Animal homologues of hepatitis B and C viruses provide novel opportunities for *in vivo* studies of viral pathogenesis.

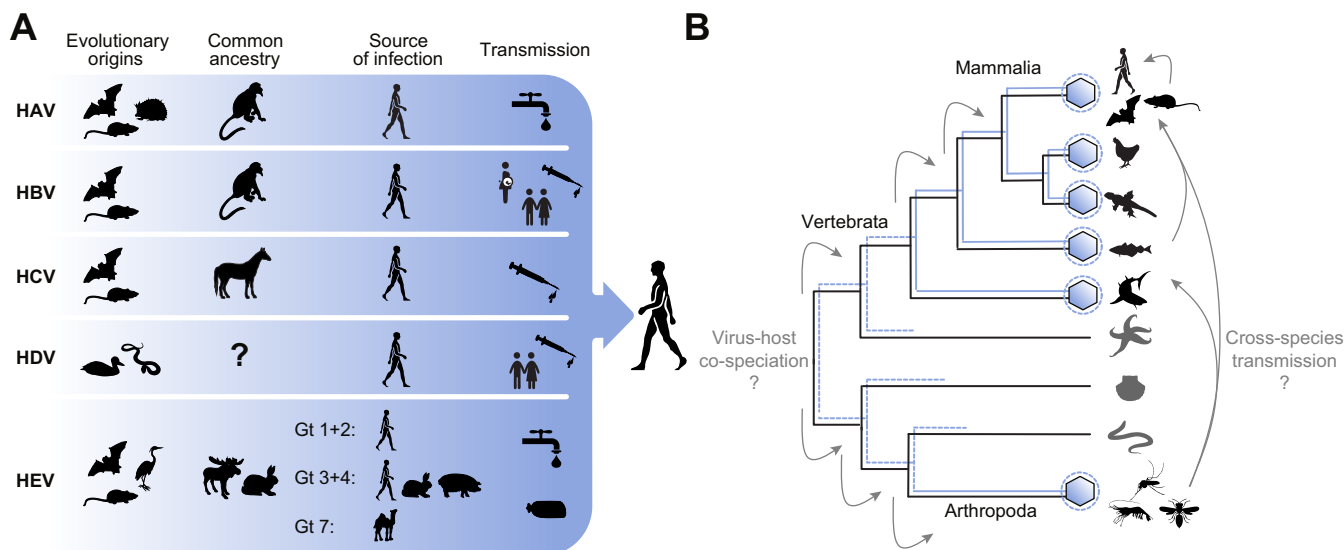


Fig. 6. Evolutionary origins of hepatitis viruses. (A) Evolutionary origins, ancestors and sources of human infections. Animal pictograms represent host taxa. (B) Hypotheses for the evolution of hepatitis-like viruses among diverse animals. Black lines, simplified bilateria phylogeny from²⁵² including selected taxa. Black pictograms, animal taxa comprising hosts of hepatitis viruses and related viruses. Grey pictograms, representatives of selected animal taxa. From bottom to top: phylum Nematoda, superphylum Lophotrochozoa, phylum Echinodermata. Blue lines: hypothesised virus evolution. Gt, genotype; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HEV, hepatitis E virus.

long-term virus-host associations and cross-species transmission events, such as those projected for other viruses.^{29,34,36,119,203,218} Future discoveries of viruses will identify missing links in hepatitis virus evolution and may identify direct ancestors of human hepatitis viruses.

Finally, there is an increasing drive towards the elimination of viral hepatitis B and C as aspired by the World Health Organization.¹ Notably, a prerequisite for the eradication of hepatitis viruses in humans is the absence of an animal reservoir. The discovery of hepatitis virus homologues in non-human hosts might imply that reintroductions into the human population after successful eradication and cessation of vaccination in the case of HBV may be possible. This is best illustrated by the ability of the surface proteins of the bat-borne TBHBV to confer viral entry into human hepatocytes, hinting at the zoonotic potential of this bat hepadnavirus.³⁰

In summary, the last decades have yielded unprecedented insight into the evolution of human hepatitis viruses, refuting their conceptualisation as predominantly human pathogens in all cases. Beyond enhancing our understanding of viral evolution, the diverse animal homologues of human hepatitis viruses allow conceptualisation

of new animal models for preclinical testing of urgently needed therapeutics for chronic hepatitis B and C, and for determining the many unknowns of hepatitis virus pathogenesis.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

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Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2018.11.010>.

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