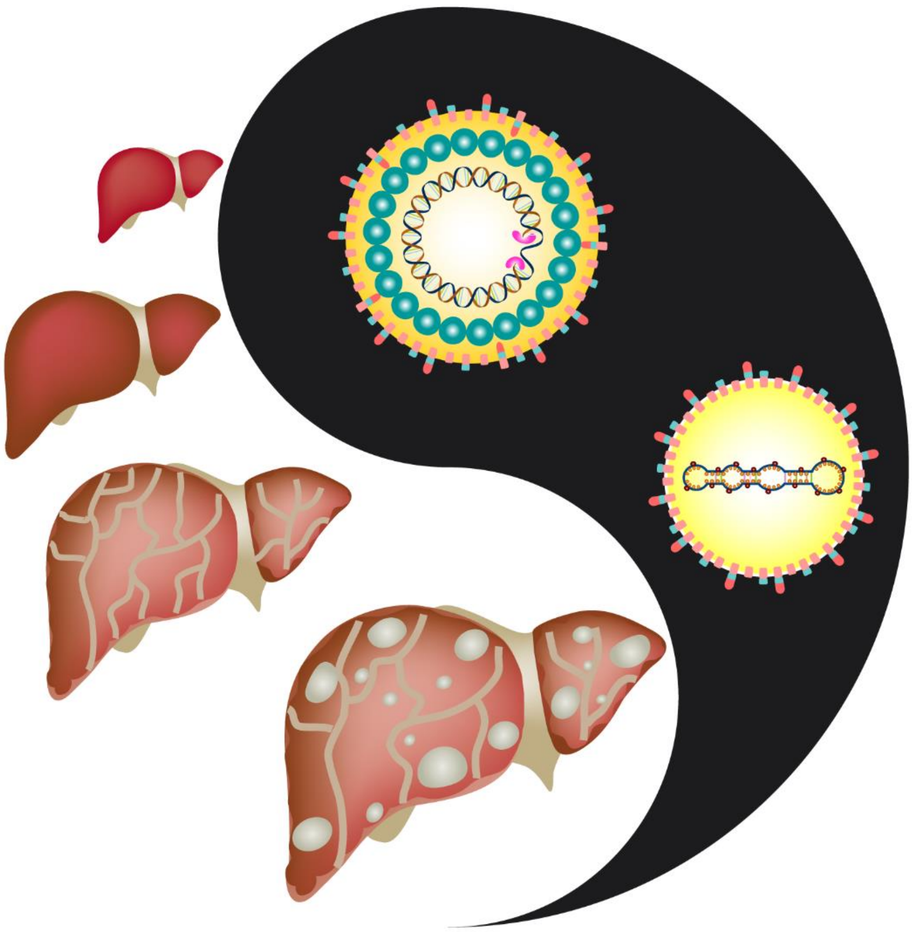
Eliminating Hepatitis D and E:
From Epidemiology to Antiviral Therapy



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Eliminating Hepatitis D and E:

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CONTENTS

Chapter 1

General Introduction and Outline of This Thesis

(Modified from Invited Review by Chinese Medical Journal. 2021.)

Viral hepatitis

Viral hepatitis is a form of liver inflammation caused by the infection with hepatotropic viruses (1, 2). The disease either manifests itself in an acute form following a recent infection and often has a transient presentation but may also progress to a chronic form, the latter typically being defined as a disease persisting for more than six months. Most cases of acute viral hepatitis mainly manifest themselves by a substantial elevation of serum transaminases, which can be spontaneously and quickly normalized. Chronic hepatitis, however, is the result of long-term viral infection and can provoke scar formation (fibrosis) in the liver, while irreversible scaring manifests itself as cirrhosis and may progress to liver cancer. Hepatocellular carcinoma (HCC) is the major type of primary liver cancer, with limited therapeutic options available (1, 2). The most common etiologies for viral hepatitis are hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV) and hepatitis E virus (HEV) (1, 2). Although chronic HBV and HCV infections still contribute the majority (66% and 30%, respectively) of the global death burden of viral hepatitis (3), there are potent antiviral therapies available. In context of the present thesis, it is important to note that HDV is associated with the most severe forms of acute and chronic viral hepatitis upon its co-infection with HBV, and that HEV is the most prevalent cause of acute viral hepatitis worldwide (4-7). Currently, it is being estimated that approximately 257 to 500 million people are living with chronic HBV infection (3, 8, 9). However, which fraction of the HBV-associated disease burden is complicated by HDV infection remains uncertain and answering this question remains highly relevant to the field. Moreover, until now, no licensed antiviral drugs with respect to HEV are available (5, 6) and developing novel therapeutic options is also an important issue in this respect. Thus, in this thesis I aimed to establish the global epidemiology of HDV infection and to develop antiviral agents against HEV infection.

The relevance of answering these questions is further emphasized by that viral hepatitis is a major global public health problem and contributes to a very significant extent to overall mondial morbidity and mortality. Globally, the annual death toll caused by viral hepatitis had increased from 0.89 million in 1990 to 1.34 million in 2015, representing a 51% growth in this time frame (3, 10, 11). The current mortality of viral hepatitis is approximately equal to that inflicted by human immunodeficiency virus (HIV) infection, tuberculosis, or malaria, and it is projected to exceed the combined mortality associated with the above three major diseases by 2040 (3, 12). This means that in the coming decades, viral hepatitis will still be a leading death cause worldwide and remain a significant challenge to public health. In 2016, the World Health Organization (WHO) adopted a strategy aiming at the elimination of viral hepatitis by 2030, in which elimination was defined as a 90% reduction in incidence and a 65% reduction in the number of related deaths from a 2015 baseline (13). Efforts, however, have been focusing on the elimination of HBV and HCV infection, while the attention reserved to HDV and HEV has been limited at best. This lack of adequate public attention is hampering the development of strategies aiming at prevention and control of HDV and HEV infections. The establishment of reliable epidemiology for HDV and the development of effective treatment for HEV holds promise for achieving the global goal of hepatitis elimination successfully by 2030.

Origin and classification of HDV

HDV was first identified both in liver biopsies and in the serum from chronic HBV infected patients experiencing extremely severe hepatitis in the 1970s (4, 14). Subsequent studies found that the genome of HDV was a circular negative single-stranded RNA (ssRNA) composed of approximately 1672-1697 nucleotides (nt), depending on the strain (15, 16). Thus, HDV is the smallest animal virus known and also its biology substantially differentiates it from other animal viruses (17-19). Uniquely, HDV shares common features with some plant subviral agents known as viroids. Hence, the virus is thought to have originated from the recombination of plant viroids with different forms of RNA (20-23). However, this notion has not been supported by direct evidence and thus for now remains a hypothesis. Due to the uncertain origin and its distinct genetic characteristics, the International Committee on Taxonomy of Viruses (ICTV) has classified HDV as the archetypical member of the *Deltaviridae* family, in which it represents the *Deltavirus* genus. The advent of powerful microbiological techniques has now led to the identification of more HDV-like viruses, in birds (24), snakes (25), rodents (26), fish, amphibians, and even invertebrates (27). Thus HDV appears part of a biodiverse virus family and analysis of related viruses may provide new insights into the origin of HDV.

HDV strains are highly divergent, and this adds further to the inconsistent molecular classification of this virus. As a single strand RNA virus, HDV is expected to mutate frequently. The nucleotide divergence between different strains is even higher than 35% (16). Moreover, further heterogeneity may result from recombination. Homologous recombination between different HDV strains has been reported in patients that suffered from multistrain infection and has also been observed in the laboratory upon co-transfection of the genomes of different strains in cell culture systems (28-30). Furthermore, because HDV has a circular negative genome, there are three forms of RNA produced during HDV life cycle, including circular full-length genomic RNA (gRNA, negative) and complementary circular full-length antigenomic RNA (agRNA, positive), as well as a short linear messenger RNA (mRNA, positive) that is only 800 nt in length with a 5'-cap and 3'-polyadenylated tail (31). This feature has led to substantial chaos with respect to registration of HDV sequences in public databases, because biomedical researchers have in the past amplified and sequenced both HDV gRNA and agRNA, occasionally starting deposited sequences at abnormal positions, and submitted the resulting sequences without any form of standardization. This has resulted in the definition of two inconsistent classification systems for HDV strain sequences (15, 16), hampering progress in the field. Proper [molecular] taxonomy is the fundament of biology and the absence of such taxonomy continues to significantly impact HDV research. Hence, it is fair to say that further systematical description of recombinant variants and a framework for molecular classification are both urgently needed to establish the standardization in the HDV field that will help the community to progress.

Life cycle of HDV

Compared to other hepatotropic viruses, HDV has a relatively complicated life cycle, and consequently distinct testing, diagnosis and treatment strategies for this virus are necessary. Because of the high GC content of its nucleotide sequence, the HDV genome can fold into an unbranched, double-stranded, rod-like structure with 74% intra-molecular base-pairing (4, 18, 32). The double stranded-like structure of the HDV genomic RNA is configurationally similar to double-stranded DNA. This similarity enables HDV to hijack and redirect host cellular DNA-dependent RNA polymerases (Pol-II plays the major role while Pol-I and Pol III may also be involved (33)), which treats HDV gRNA as a double-stranded DNA template, to synthesize HDV agRNA via a double-rolling circle mechanism (a strategy normally used by viroids, virusoids, and viroid-like satellite RNAs) (4, 19, 34-38). The *de novo* synthesized positive-sense RNA initially forms as a large linear molecule potentially containing many copies of the genome before undergoing intramolecular cleavage by the autocatalytic self-cleaving ribozyme (an intrinsic sequence of 85 nucleotides integrated in HDV genome) to form linear monomers (39, 40). Subsequently, the resulting cleaved linear products are ligated and closed for form circular antigenomic molecules, the so-called agRNA. These neo-synthesized monomeric antigenomes in turn serve as templates for the production of gRNA via a similar mechanism (4, 19, 34-38).

The HDV genome encodes only one functional open reading frame (ORF) for the translation of two isoforms of hepatitis delta antigen (HDAg), a 24 kDa small-HDAg (S-HDAg) with 195 amino acids and the 27 kDa large-HDAg (L-HDAg) with 214 amino acids (41). Originally, in the nucleoplasm, HDV genomic RNA directly serves as the template for the transcription of mRNA. This unmodified mRNA is then translated into S-HDAg in the cytoplasm (36, 42). By contrast, the L-HDAg is translated from a modified mRNA. During the replication of the HDV antigenome, a fraction of it will undergo an RNA editing event on the amber/W site of the agRNA, mediated by adenosine deaminase acting on RNA 1 (ADAR1) (43-47). Specifically, ADAR1 deaminates adenosine to convert it to inosine (UAG becomes UIG on the agRNA) which is recognized as guanosine by the polymerase and then paired to a cytosine during the next replication cycle (AUC becomes ACC on the neo-synthesized gRNA) (43-47). When this modified gRNA serves as template and is transcribed into mRNA, the stop codon on the amber/W site is converted to a tryptophan codon (UAG is replaced by UGG on the neo-synthesized mRNA). Consequently, translation of the neo-synthesized mRNA proceeds for an additional 19 amino acids and terminates only until frame reading reaches to the downstream stop codon, leading to the production of L-HDAg (43-47). Thus the N-terminal 195 amino acids of the two HDAg isoforms are identical and form several functional domains, including RNA-binding domains, a coiled-coil motif, a helix–loop–helix motif and a nuclear localization sequence, whereas the C-terminus of the L-HDAg is distinct from the expanded 19 amino acids containing a nuclear export signal (NES) and a farnesylation signal (C_{211}XXQ box) that enables a farnesyl lipid group to be added covalently to the cysteine at position 211 by a cellular farnesyltransferase (48-51). To support HDV infection, replication and virion assembly, HDAg proteins undergo several important post-translational modifications. Among these modifications, a phosphorylation at Ser-177 serves to increase agRNA replication by facilitating HDAg RNA-binding activity (52, 53), the acetylation at Lys-72 promotes nuclear localization of HDAg (54), whereas sumoylation at multiple lysine residues enhances the synthesis of gRNA and mRNA but not of agRNA (55). In addition, a methylation at Arg-13 (an RNA-binding domain) facilitates translocation of S-HDAg to the nucleus, an event important in the regulation of agRNA and gRNA replication (56). Moreover, prenylation of the cysteine residue at the C-terminus of L-HDAg is required to allow HDV virion assembly (48, 51). Functionally, the expression of S-HDAg at an early step of replication appears necessary to activate HDV RNA replication and allow accumulation of the replicate in the nucleus. The production of L-HDAg at a later stage, expressed after RNA editing and maturated after an indispensable post-translational farnesylation (which is a type of prenylation), indirectly inhibits HDV RNA replication by acting as a dominant negative inhibitor of S-HDAg (57, 58). Therefore, the relative ratio between S-HDAg and L-HDAg determinates the balance between viral synthesis and virion assembly, the increase of L-HDAg content can induce a tuned progress from replicative to morphogenetic phases of the viral life cycle (57-59). In the nucleus, L-HDAg molecules combine with S-HDAg molecules and the newly synthesized gRNA to form nucleocapsid-like ribonucleoprotein (RNP) complexes, which are exported into the cytoplasm as a consequence of the presence of a nuclear export signal in the C-terminus of L-HDAg (4, 19, 38, 50, 51). Once in the cytoplasm, the farnesyl group that is attached to L-HDAg presumably anchors the RNPs to the endoplasmic reticulum (ER) membrane, where the HBV surface antigens (HBsAg) are synthesized (4, 19, 38, 48, 49, 51, 60). Subsequently, the RNP complex as an inner core of the nascent virus is enveloped by an endoplasmic-reticulum-derived lipid bilayer containing the three HBV surface proteins, small (S-HBsAg), middle (M-HBsAg) and large (L-HBsAg), to create infectious HDV particles, which are 35–37 nm in diameter and can be released in the extracellular fluid following passage through the Golgi complex (4, 19, 38, 51, 61, 62).

Intriguingly, unlike other hepatotropic viruses, HDV is a defective virus. Since the HDV genome does not code for its own RNA-dependent RNA polymerase (RdRp) but only for the HDAg proteins, HDV must rely completely on cellular enzymes for genome replication, and parasitizes on HBV for its propagation (19, 51). Sharing the envelope proteins with HBV, HDV exploits not only the same particle release machinery but also the identical transmission route, especially with regard to cellular entry factors (19, 51). HDV particles circulating in the blood stream are initially captured by heparan sulfate proteoglycans (HSPGs) on the hepatocyte surface (19, 51, 63). This attachment enables HDV closely bind to the identical cellular entry factor of HBV called human sodium/taurocholate cotransporting polypeptide (NTCP) encoded by SLC10A1 (19, 51, 64, 65). NTCP is a bile salt transporter exclusively expressed on the sinusoidal (basolateral) site of parenchymal liver cells (hepatocytes), and this is apparently responsible for the strong liver tropism and host specificity of HBV and HDV (51, 66). Notably, HDV attachment is mediated by both the preS1 region of the L-HBsAg protein as well as the antigenic loop (AGL) of the HBV envelope proteins, while the receptor recognition is via the myristoylated N-terminal receptor-binding site of the preS1 domain of L-HBsAg (19, 51, 63-65).

Due to the indispensable role of HBsAgs in the HDV life cycle, the diagnosis and testing of HDV infection in clinical practice and in epidemiologic studies are mainly performed for subjects who are HBsAg positive (HBV carriers) (67). Nevertheless, it is important to remark that the only contribution of HBV to HDV life cycle is the support for HDV virion assembly, release, and cell entry. In fact, HDV is able to replicate independent from HBV both in HBV uninfected hepatic cells or non-hepatic cells and HDV RNA could survive for at least 6 weeks under such conditions. Furthermore, for HDV to propagate, systems consisting of cells co-expressing HBV envelope proteins and NTCP receptor are sufficient. Hence, the virus can propagate in liver cells of patients in which HBV replication has been totally eliminated but in which the production of HBsAgs is still present, but also in artificial co-transfected cells (51, 64, 65, 68). Furthermore, HBV was previously considered as the exclusive facilitator of HDV propagation, however, this concept has recently been challenged. In an intriguing series of *in vitro* experiments it was demonstrated that HDV RNPs could be artificially packaged into envelopes from several non-HBV related viruses such as hepacivirus, flavivirus and vesiculovirus, allowing egress of HDV RNPs from accordingly infected cells and subsequent entry into cell lines expressing their respective receptors (38, 51, 69-71). All these factors complicate HDV testing, diagnosis and treatment.

Epidemiology of HDV

As compared to HBV and HCV, which were considered the major drivers for global viral hepatitis burden, HDV has been treated stepmotherly since its first identification as a separate disease entity in the 1970s (72). Accordingly, studies on HDV epidemiological behavior have been scare. A first attempt to estimate the regional epidemiological characteristics of HDV was done for South America in 1996, and was based on the data published in the 1980s and 1990s. This study concluded that approximately 5% of HBV carriers in South America had been co-infected with HDV, which would add up to 300,000 people in this region at the time (73). Thereafter, this regional prevalence was somewhat rashly extrapolated by the hepatological disease community to be representative for the global situation, as evident from a publication in 1997 and also in 2010, and this older regional study remained the basis for a estimation of 15–20 million infections with HDV worldwide (74, 75). However, this relatively complacent view was challenged in 2017, by an estimate of 7 million infections in sub-Saharan Africa alone (76). In response, the worldwide number of HDV infections was adjusted upwards to approximately 62–74 million in 2019 (77, 78). These studies marked an overtime resurgence of the awareness and interest in the HDV problem. Unfortunately, the last study was criticized by several research teams regarding methodology and data processing, and thus their result was considered to be an overestimation (79, 80). This ongoing debate regarding the exact global prevalence of HDV might obscure the public health problem posed by HDV infection and in any case it is evident that earlier estimates have been too low and that the exact burden posed by HDV infection requires urgent clarification.

Unfortunately, there are many pitfalls that make it much more difficult to accurately estimate the HDV global burden. First and most importantly, HDV has received relatively little attention over the past decades by the public, health professionals and policy makers alike. Consequently routine diagnosis of HDV has been relatively rare in clinical practice (72). Hence, reliable data about HDV prevalence are extremely limited and updated data is even more scarce (77, 79, 80). This is a particularly prominent problem in many high incidence countries that are the major reservoir of HBV infection. Examples include the mainland of China, India, Russia, Indonesia, Pakistan, Bangladesh, Myanmar, Philippines, Thailand, Vietnam, and many African countries (9). Secondly, there are large heterogeneities in HDV prevalence between different regions and populations. The infection rates of HDV remain extremely high (>20%) among HBV carriers from Mauritania, Somalia, Venezuela, Denmark, Cameroon, Colombia, Niger, Mongolia, whereas it is relatively low (<5%) in Australia, Canada, Singapore, Belgium, Switzerland and Poland (72, 77). The infection rate can also be very high among some risk populations including intravenous drug users (IDUs), HIV-coinfected individuals, HCV-coinfected individuals as well as commercial sex workers. For IUDs, the rates are as high as 80% in some countries (72, 77, 81). By contrast, the prevalence appears much lower in blood donors and asymptomatic HBV carriers. Moreover, immigrants from endemic countries may complicate interpretation of HDV epidemiology in other countries. For example, large numbers of immigrants have imported many HDV cases into the United States of America (USA) and some European countries in recent years (82-85). Last but not least, HDV screening was mainly based on detection of antibodies (IgG and/or IgM) against HDV (anti-HDV), but HDV RNA testing was rarely performed in the majority of epidemiological studies due to the lack of a standardized HDV RNA assay and also because of economic considerations (72, 75). Clearly, anti-HDV IgG indicates resolved HDV infection while anti-HDV IgM or RNA indicates acute or active infection, and normally the positive rate of HDV RNA among anti-HDV positive patients is only about 50%. Therefore, current calculated seroprevalance can only indicate the previous overall exposure of HDV, the accurate ongoing burden cannot be ascertained. Nevertheless, a reliable and reasonable estimate on HDV seroprevalence should help the pubic to recognize the public health challenge caused by HDV infection, provoking the recommended testing for anti-HDV to be modified from current HBsAg-positive carriers who are at high risk for HDV infection to all HBsAg-positive carriers.

Clinical impact of HDV

Compared with HBV mono-infection, co-infection of HBV with HDV usually presents distinct clinical features with respect to symptoms of infection, epidemiological profile and disease progression. Due to the peculiar dependence of HDV life cycle on HBV, HDV can only be transmitted as a dual infection in the presence of a concomitant infection with HBV (19, 67, 86). Two major patterns of dual infection have been described: simultaneous coinfection with HBV and HDV of a susceptible individual; or superinfection with HDV in a chronic HBV carrier. As for simultaneous HBV and HDV coinfection, the most common disease course in adult immunocompetent individuals is that both HBV infection and HDV infection are spontaneously resolved (87-89). In individuals, however, that fail to clear HBV, only a minority can independently clear HDV infection, whereas the majority will progress to chronic dual infection with both viruses (86-89). As most superinfections occur in individuals not capable of clearing HBV, in general clinical outcome of HDV coinfection (in which both viruses are usually cleared) is different from HDV superinfection (which usually leads to persistent infection with both viruses).

Importantly, HDV exposure, either as a coinfection or as a superinfection, is associated with more severe liver injury than HBV mono-infection, even as its clinical manifestation still may involve the full range of possible outcomes, from asymptomatic infection to acute failure and lethality (87-91). It is important to note in this context that the overall prevalence of HDV infection in the context of HBV remains unknown and hence the relative proportion of HDV patients stratified for different severities of liver pathology remains obscure. Generally speaking, chronic hepatitis D (CHD) is considered to be associated with the most severe form of chronic viral hepatitis, with a rapid progression towards fibrosis/cirrhosis and subsequent clinical liver decompensation (38, 88, 89, 92). Indeed, longitudinal studies confirm that a large proportion of CHD patients swiftly progress to cirrhosis and eventually 80% of CHD patients will develop cirrhosis, which is significantly higher than the percentage seen in patients only infected with HBV (91, 93-98). In addition, HDV might also be a direct risk factor for HCC independent of the presence of cirrhosis, because the reported HCC incidence in CHD patients is much higher than that seen in HBV mono-infected patients (95, 98-101). It is intriguing to note that HDV genotype can also influence the outcome of CHD, with genotype 1 and 3 being associated with more severe disease than genotype 2 and 4 (102-106), which, again, highlights the importance of genotype classification to assist the clinical management of HDV infection. Although the notion that an HBV infection complicated by HDV infection is associated with worse outcome is not controversial, statements on the natural course and incidence of HDV are based on a relatively small number of cross-sectional and longitudinal observational studies. Such studies are often provoked by signals that there is outbreak of HDV in a certain region and it is fair to say that we have little insight into the global overall contribution of HDV infection on outcome of HBV infection. Hence there is plenty of discussion and the findings reported are sometimes contradictory (96, 107, 108). Thus, a timely systemic and comprehensive data synthesis is not only necessary to clarify the relation of HDV to HBV-related liver disease, but also necessary to quantify the global disease burden in the context of both HBV/HDV co-infection.

Prevention and treatment of HDV

Despite the fact that HDV had been identified as a separate disease entity already decades ago, there is no satisfactory Food and Drug Administration (FDA)-approved therapy for this potentially devastating disease (4, 37, 71, 89). Professional societal guidelines recommend pegylated IFN (PegIFN) alpha for CHD treatment, which, however, necessitates a lengthy side effect-prone disease and has a low success rate (4, 37, 71, 89). Since HDV lacks its own polymerase, developing antivirals that directly and specifically target viral replication is likely impossible (4, 37, 51). The increasing insight into HDV virology and the life cycle has provided potential targets for therapeutic intervention and accordingly multiple antiviral molecules are currently under development that should allow interfering the viral life cycle (4, 37, 71, 89). These molecules include Myrcludex B (blocks HDV entry into hepatocytes) (109-111), lonafarnib (a prenylation inhibitor that prevents virion assembly) (112, 113), REP 2139 (inhibits HBsAgs release from hepatocytes and interacts with HDAg) (114); and the IFN-lambda (stimulates the host innate immune system) (115). Of note in this context, the lack of a viable cell model remains a major impediment to HDV antiviral development. In fact, only since the discovery of the HBV/HDV cell receptor NTCP between 2012-2014, has it been possible to create the robust NTCP-HBsAg co-expression cell model capable of supporting the entire HDV life cycle (51, 64-66, 110, 116). Nevertheless, HDV virion packaging takes around 9-12 days in such a model due to L-HDAg translation-required RNA editing and subsequent post-translational modifications, which appear to take much longer than what is observed for HCV or HEV (4, 37, 69, 71, 89, 116). Thus, although these novel antivirals hold great promise for a cure for HDV patients, stopping more people from being infected with HDV could be a more cost-effective strategy in the end.

Because HDV can only be transmitted in the context of an HBV infection, HBV immunization for normal persons should also have a protective effect with regard to HDV transmission (117, 118). Hence, HBV vaccine was expected to effectively counteract the spread of HDV infection in the general population, and the implementation of mass vaccination programs was even anticipated to result in the complete elimination of HDV-related disease *per se* (117-122). An HBV vaccine was developed and adopted in the 1980-1990s and its implementation leads to a significant decline in the amount of HBsAg carriers in vaccinated populations. Even if initial vaccination uptake was low on a global scale, by 2018, 108 countries had adopted infant vaccination for HBV and 189 countries had adopted use of the HepB3 vaccine, that involves three doses of hepatitis B vaccine, in their population-wide vaccination programs (123, 124). Thus, it would appear rational to expect the prevalence of HDV infection to go down in conjunction with the universal implementation and increasing coverage of HBV vaccination. Surprisingly, however, both recent regional and global estimates have shown a very substantial burden inflicted by HDV (76-78). Obviously these results require further validation of HDV prevalence and exploration of reasons that have conspired to produce such a result. In the present thesis, I shall investigate HDV global burden and assess the impact of HBV vaccination on the dynamics of HDV prevalence over the past decades. It will appear through my systematic and comprehensive investigations that I can further confirm the unexpectedly substantial HDV burden present in the world, but I can also objectify a favourable impact of HBV vaccination campaigns on HDV prevalence. I shall suggest that the growth of world population is a possible factor that is responsible for the maintenance and increase in HDV global prevalence.

Virology of HEV

HEV was initially reported as the causative agent of a large-scale, waterborne hepatitis epidemic that occurred in India in the late 1970s (125). Definitive confirmation of the nature of the agent and the formal adoption of the associated nomenclature came after a partial cDNA was cloned and sequenced in the 1990 (126). HEV can infect a broad range of species, and these zoonotic, anthropotropic and animal-restricted HEV or HEV-like virus isolates are classified into the family *Hepeviridae*, which is subdivided into the genera *Orthohepevirus* (species *A*-*D*) comprising all mammalian and avian HEV isolates and the genera *Piscihepevirus* comprising the cutthroat trout virus (6, 7). Up to now, eight HEV genotypes have been identified and classified to the species *Orthohepevirus A,* including the main HEV strains that affect humans (127-129). Specifically, HEV genotypes 1 and 2 are restricted to human hosts and are primarily transmitted via the fecal–oral route, while HEV genotypes 3 and 4 have multiple hosts and can be transmitted from zoonotic reservoirs to humans through the consumption of contaminated animal products (6, 7).

HEV is a small, quasi-enveloped, icosahedral virus of about 27–34 nm in diameter (6, 7, 130, 131). The genome of HEV is a 7.2 kb single-stranded, positive-sense RNA that contains three overlapping ORFs (ORF1, ORF2, and ORF3) in addition to a methylguanine-capped 5'-untranslated region (5'UTR) and a polyadenylated 3'UTR, and has thus many features of a eukaryotic mRNA (6, 7, 130-132). Following infection, ORF1 of the uncoated HEV genomic RNA is first translated directly by host ribosomes to produce the nonstructural polyprotein, which contains various functional domains including the methyltransferase (Met), the RNA helicase (Hel), the RNA-dependent RNA polymerase (RdRp), and several non-enzymatic components essential for viral replication (6, 7, 130-133). Following the translation of this ORF1 polyprotein, an antigenomic RNA (negative-sense) intermediate is synthesized from the genomic RNA (positive-sense) by the RdRp, which then serves as a template for the synthesis of full-length genomic RNA (≈7.2 kb) and a shorter subgenomic RNA (≈2.2 kb) that harbors ORF2 and ORF3 (which show significant sequence overlap) (6, 7, 130, 131, 133-135). The newly synthesized full-length genomic RNA either participates in a new round of transcription/translation or is packaged into new progeny virions. Meanwhile, ORF2 is translated into the capsid protein that is necessary for virion assembly (136), cell entry (137, 138), and immunogenicity (139); while ORF3 is translated into a multifunctional phosphoprotein that is essential for virion morphogenesis and release (134, 140, 141).

HEV infection and high-risk populations

Mathematical modelling suggests that HEV leads to 20 million new infections annually in Asia and Africa, where the prevalent HEV strains are genotypes 1 and 2, resulting in 3.4 million cases of acute hepatitis, 70,000 deaths and 3,000 stillbirths (142). Globally, the annual figure for HEV infection-provoked cases of acute hepatitis is approximately 14 million, leading to 300,000 deaths and 5,200 stillbirths (5, 6, 143-145). While major HEV outbreaks are confined to developing countries, a growing number of sporadic cases have been reported from developed countries, where the prevalent HEV strains are genotypes 3 and 4 (5, 6, 146). A recent study has estimated that approximately 12.47% of the global population, corresponding to 939 million individuals, has ever experienced HEV past infection (as deduced from studies investigating the prevalence of anti-HEV IgG), 110 million individuals are experiencing current/recent infection (as deduced from studies on the presence of anti-HEV IgM), and 15 million individuals are experiencing an ongoing infection (extrapolated from studies on the detection of viral RNA) (145).

HEV infection is usually associated with self-limiting acute hepatitis that typically lasts for a few days/weeks (6, 131). Normally, the infection in healthy individuals is asymptomatic. During the outbreaks, adolescents and young adults are vulnerable to contracting HEV infection but are mostly asymptomatic (147). In contrast, disease course in vulnerable populations can be problematic, especially in immunocompromised patients (organ transplant recipients and cancer patients), children under two years of age and pregnant women (6, 143, 147, 148). Organ transplantation patients bear a high risk of developing chronic hepatitis after HEV infection (6, 143, 147, 148). Immunosuppressant use severely suppresses the immune system in transplant recipients, making them vulnerable to a variety of infections. Chronic infection in these patients usually caused by HEV genotype 3 and occasionally by genotype 4 (148-150). Pregnant women acutely infected with HEV genotype 1 and 2 strains can develop severe complications such as acute liver failure, miscarriage, preterm delivery, stillbirth and perinatal mortality, resulting in high mortality that approaches 15-25% (6, 143, 151, 152). Maternal-fetal transmission of the virus can seriously affect fetal/neonatal outcomes, and these include anicteric or icteric hepatitis, hypoglycaemia and neonatal death (5, 153). Considering the higher risk and more severe disease outcome, it is fair to say that these high-risk populations should be given more attention in clinical management, and developing specific antivirals for them remains necessary.

Developing anti-HEV therapy by drug screening and repurposing

For the treatment for symptomatic HEV infection, no FDA-approved medication is currently available. For now only general antiviral drugs including interferon alpha (IFNα), ribavirin or their combination have been used as off-label treatment (5, 6, 131, 153-155). Unfortunately, many patients are not eligible or do not tolerate IFNα or ribavirin treatment. Specifically, use of interferon is limited in transplant recipients due to the associated significant side effects or the induction of organ rejection by the treatment (156). Although ribavirin monotherapy is effective in a large proportion of patients, treatment failure has been reported in a subset of patients (131, 157-159). Most importantly, ribavirin as the most promising anti-HEV therapy is contraindicated in pregnancy due to teratogenicity (160). Therefore, further development of new antiviral therapies against HEV is an urgent clinical need, particularly for immunocompromised patients, young children and pregnant women. However, the development of brand new antivirals typically consumes huge time and money, making it impossible to satisfy HEV immediate therapeutic need in the short term. In this regard, repurposing the existing drugs proven to be safe in humans would be a readily and promising way for developing HEV antivirals (161, 162).

HEV *in vitro* models, based on either infectious clones or subgenomic replicons, have been successfully established which should facilitate efforts aimed at drug screening (162-164). Although HEV infection has mostly been associated with clinical manifestations involving the liver, extrahepatic manifestations including neuronal and renal diseases have been widely reported (5, 165). In apparent agreement, preclinical models for studying HEV have been established with relative ease in both hepatocyte and non-hepatocyte cell lines, and these are now starting to be used for developing anti-HEV therapy (161, 162). These efforts are being facilitated by the replacement of ORF2 gene in HEV replicon clone by a Gaussia luciferase reporter gene in experimental systems, which then allows straightforward monitoring of HEV replication, making it a powerful tool to perform drug screening (161-164). In brief, potential anti-HEV candidates can be first identified in a HEV replicon-based cell culture system by high-throughput screening of a drug library. Subsequently, the selected candidates are further evaluated in models that involve infection with infectious viruses (161, 162). Using this strategy, the laboratory which hosted my PhD. research had prospectively identified several potential anti-HEV candidates from the screening for a library of 94 known safe-in-human broad-spectrum antiviral agents (162). Among these there were several candidates that aroused my interest, including the macrolide antibiotic azithromycin (AZM, a FDA pregnancy category B drug) and ivermectin (an FDA-approved antiparasitic drug). These compounds have been widely used in the clinic and are known to have broad antiviral activities (166-170). In the present thesis I thus decided to first systematically and comprehensively evaluate their activities in inhibiting HEV replication, then to delve deeper into the underlying mechanisms-of-action. My findings on the anti-HEV action of azithromycin and ivermectin, reported in the below, will support the further exploration of repurposing them for treating HEV-infected pregnant women and organ transplantation recipients with chronic hepatitis E.

Scope of this thesis

Viral hepatitis remains a major public health challenge that provokes significant morbidity and mortality. Hepatitis D, as the most severe form of acute and chronic viral hepatitis, is associated with rapid progression to cirrhosis and HCC and urgently requires further investigation aimed at devising rational strategies to counteract disease. Hepatitis E, as the most common form of acute viral hepatitis, is associated with severe disease outcome in high-risk populations. Hence, generation of a clear and quantitative global epidemiology is now required for HDV, and there are urgent needs for novel and specific HEV treatment. In this thesis, I aimed to investigate the molecular classification and epidemiology of HDV, and to further explore the antiviral activities and mechanisms-of-action of two anti-HEV candidates. I firstly systematically analyzed the genetic characteristics and genotype of HDV, and secondly I comprehensively assessed the global prevalence and dynamics of HDV infection. Next, I mainly focused on the repurposing of azithromycin and ivermectin for potential HEV treatment in specific populations and the evidence of their mechanisms of action.

Outline of this thesis

Establishing a standard molecular classification system is crucial for the research and treatment of HDV. HDV genotype plays an important role in the epidemiology, virus evolution, pathogenesis, clinical course and outcome, as well as treatment response. Unfortunately, current HDV molecular classification is inconsistent as two different genotypic systems have been proposed. This may be because of the high genetic diversity in this virus, in turn caused by RNA mutations, whereas recombination between strains in patients suffering from concomitant infection by multiple strains, further increases strain heterogeneity. Addressing the present inconsistencies requires an updated classification system, while proper naming of novel HDV genotypes or subtypes would exceedingly benefit from the presence of standardized criteria. In **Chapter 2**, I systematically retrieved and analyzed a large set of HDV full-length genome sequences. To clarify the molecular classification of HDV and establish an updated system, I firstly standardized HDV sequences to antigenomic RNA employing a concordant reading start site, and the potential recombinants were also identified and appropriately dealt with. Based on this new HDV classification, I then mapped the global distribution of different genotypes and subtypes. Finally, I also proposed standard criteria for future identification of novel genotypes and subtypes and compiled a complete set of reference genomes for each subtype, potentially avoiding further inconsistency in the future. Recent research shows that the public health issue of HDV infection is much greater than before thought. Hence, a reliable estimation of the global HDV burden would help raise public awareness, provoking appropriate strategies for better screening, prevention, and treatment. In **Chapter 3**, I systematically reviewed all the epidemiological and clinical data available in HDV literature, and I was able to define the prevalence, disease progression, and clinical outcome of HDV infection. Based on this meta-analysis, I first quantified HDV prevalence at national, regional and global levels, and analyzed several risk factors associated with HDV transmission. Then, by data synthesis, I characterized the distinct epidemiological profile and infection pattern of HDV, as well as the rapid clinical progression to severe liver diseases. Quantifying the global burden of HDV infection is, however, a real challenge due to insufficient data and many other reasons. Underestimation and overestimation biases can also arise from poor methodology and data processing. Inadequate interpretation of existing data might provoke inconsistent results. Thus, this is a complex issue. Through my work in **Chapter 4**, I hope to provide answers though. To better estimate and predict HDV global burden, I revisited all previous calculations and this allowed me to obtain much better insight into this burden as compared to what was possible before.

Because of the absence of adequate therapy for HDV infection and the dependence on HBV for HDV infection to occur, HBV vaccination has been presented as a cost-effective strategy for preventing HDV infection and transmission. Given the fact that HBV vaccination has been adopted for decades and the coverage is increasing, in **Chapter 5**, I systematically and comprehensively assessed the impact of HBV vaccination on the dynamics of HDV prevalence during this period. To better characterize these dynamic changes, I first divided data into groups *as per* decade, and then measured HDV global burden and infections among different populations accordingly. By comparing the results, I further confirmed HDV substantial burden and the preventive effect of HBV vaccination on HDV prevalence, and I also further highlighted the possible contribution of world population growth to the maintenance of HDV burden.

Developing novel anti-HEV agents is apparently urgent, particularly for the specific treatment in the high-risk populations. Repurposing existing safe medication for treating HEV is thus a promising solution to improve the current situation. Through drug screening, the macrolide antibiotic azithromycin, a commonly used FDA pregnancy category B drug, and the FDA-approved antiparasitic drug ivermectin, have been identified as potential anti-HEV candidates. In **Chapter 6 and 7**, I thus further investigated their antiviral activities against HEV and their underlying mechanisms, respectively. These results support potential repurposing of azithromycin and ivermectin for treating HEV infection.

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Chapter 2

Recombinant identification, molecular classification and proposed reference genomes for hepatitis delta virus

Running Title: Proposed reference genomes for HDV subtypes

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ABSTRACT

Hepatitis delta virus (HDV), as a defective sub-virus that co-infects with hepatitis B virus, imposes an emerging global health burden. However, genetic characteristics and molecular classification of HDV remain under investigated. In this study, we have systematically retrieved and analyzed a large set of HDV full-length genome sequences and identified novel recombinants. Based on phylogenetic and genetic analyses, we have established an updated classification system for HDV when recombinants were excluded. Furthermore, we have mapped the global distribution of different genotypes and subtypes. Finally, we have compiled a complete set of reference genomes for each subtype and proposed criteria for future identification of novel genotypes and subtypes. Of note, the global distribution map indicates that currently available HDV genetic data remains limited, and thus our proposed classification will likely evolve as future epidemiological data will accumulate. These results shall facilitate the future research on the diagnosis, screening, epidemiology, evolution, prevention and clinical management of HDV infection.

KEYWORDS

Hepatitis delta virus; Viral genome; Viral RNA; cRNA; Genetic recombination; Genotype

INTRODUCTION

Hepatitis delta virus (also known as hepatitis D virus, HDV) is a defective sub-virus that requires hepatitis B virus (HBV) surface antigen (HBsAg) for virion assembly. The genome of HDV is a circular negative single-stranded RNA (ssRNA) composed of approximately 1,700 nucleotides (nt) (1). It is considered the smallest RNA genome in all known animal viruses. There are three forms of HDV RNA without any DNA intermediate during viral replication, including circular genomic RNA (negative), circular complementary antigenomic RNA (cRNA, positive), and a short linear polyadenylated antigenomic RNA (positive) (2). This linear form is the messenger RNA (mRNA) template encoding only one functional open reading frame (ORF) for the translation of the hepatitis delta antigen (HDAg) (3). Though HDV RNA is single-stranded, it is capable of undergoing self-cleavage and ligation to generate circular RNA. Due to the high GC content of the nucleotide sequence, HDV RNA can also fold as an unbranched, double-stranded, rod-like structure with over 70% intra-molecular base-pairing (4).

Since the identification of HDV in the 1970s, this peculiar pathogen has been neglected over the past decades (5, 6), and routine diagnosis is rare in clinical practice (7). However, co-infection of HDV with HBV causes the most severe form of acute and chronic viral hepatitis in humans (1). It has been estimated that almost 5% of HBV infected patients have HDV co-infection and up to 80% of these co-infected patients can further progress to cirrhosis (8, 9). This long-term co-infection is associated with more rapid and severe progression to cirrhosis or hepatocellular carcinoma (HCC) than HBV infection alone (7). Worse yet, clustered outbreaks of HDV superinfection periodically occur across the world, imposing an emerging global health burden (1, 7).

As a single-stranded RNA, HDV is expected to evolve rapidly. Previous studies have indicated that HDV genotype plays an important role in pathogenesis and the efficiency of RNA editing can affect its natural history (10). However, the genetic features of HDV remain poorly characterized, and the current molecular classification systems are inconsistent (11, 12). Given that the epidemiology, virus evolution, infection course, clinical outcome, and treatment response are likely associated with the different genotypes or subtypes, we aim to clarify the molecular classification of HDV and to propose standardized reference genomes.

MATERIALS AND METHODS

Sequence download

All HDV full-length genome sequences available before 1 December, 2017 were downloaded from NCBI Nucleotide Database. "Hepatitis delta virus" [Organism] NOT "patent" [title] was used as the search term, and the search results were filtered by sequence length from 1500 nt to 2000 nt. 357 full-length sequences were retrieved from the search records but the final dataset comprised 345 sequences after removing duplication. Information on accession number-strain/isolate-collection date-country/geographic origin, if available, were simultaneously retrieved from the Database.

Sequence alignment and recombinants identification

The original dataset was aligned by ClustalW (1.6) listed in the MEGA (version 7.0.26) using a gap-opening penalty (GOP) of 15 and a gap extension penalty (GEP) of 6.66 (13-15). RNA sequences were standardized to antigenomic cRNA form reading from normal initial site. The standardized dataset was realigned with woodchuck hepatitis B virus (WHV, accession number J04514, WHV8 strain) (16) by ClustalW and went through minimum manual corrections. The well aligned dataset was used to construct the preliminary phylogenetic tree using different algorithm models listed in MEGA. Strains emerged as outlier branches or clustered as peculiar branches located at the crotch of different trees indicating the presence of recombinant sequences. These strains presenting the conflicting signals were regarded as potential recombinants and require further recombination identification. Recombination events were confirmed by Bootscanning analysis performed in Simplot v3.5.1 programs using Kimura 2-parameter with a 160 base pair (bp) window, a 20 bp step increment, and 1,000 bootstrap replicates. The recombination criterion is breakpoint high than >80% of the permuted tree.

Phylogenetic and genetic analyses

The new dataset without potential recombinants was realigned. Model Selection (ML) implemented in MEGA was used to find the best DNA/protein model. The best DNA and protein models were the General Time Reversible (GTR) model and the Jones-Taylor-Thornton (JTT) model, respectively. The Maximum-likelihood (ML) tree was reconstructed using the best model with 5 rate categories (G) and invariable sites (I). The Neighbor-Joining (NJ) tree was reconstructed using p-distance model with Bootstrap method test. Branch support was calculated using 1,000 replicates, and only bootstrap values >70% were showed. Trees were rooted with WHV8 strain.

HDV genotype distribution map was modified according to the free map templates (http://d-maps.com/carte.php?num_car=13180&lang=en) using Inkscape 0.92.2 software. Nucleotide similarities were calculated by the program Sequence Distances implemented in MegAlign software (Lasergene software; DNASTAR), and genetic distances were calculated by MEGA with the Kimura 2-parameter/gamma model and 1000 bootstrap replicates.

RESULTS

Standardization of HDV full-length genome sequences and identification of potential recombinants

357 original full-length sequences were retrieved, but 345 valid sequences were finally included after removing duplications. Our preliminary alignment of original dataset found that only a set of strains isolated from Brazil (accession number from KF786305 to KF786352) were cRNA form (17), and all the others were genomic RNA form. Furthermore, several strains from Turkey (accession number from HQ005364 to HQ005372) showed abnormal initial reading site, starting and ending at site 227 (18). For further phylogenetic and genetic analysis, all original genomic RNA sequences were transformed to cRNA form and these Turkey strains were standardized to read from 1 to 1678.

Through preliminary phylogenetic analysis, 53 strains presenting conflicting signals were screened out for further Bootscanning analysis. 33 out of these 53 strains were finally confirmed that substantial recombination events occurred. Among these, two strains (AB118845 and KF660598) have been previously reported as recombinant (19, 20), and the other 31 strains were newly identified recombinants in this study **(Figure S1 and Figure S2)**. The recombination genotype component and corresponding breakpoint positions were summarized in **Table S1**.

Phylogenetic analysis and updated molecular classification

After removing the recombinant sequences, the phylogenetic trees were constructed using two different models, ML and NJ. The tree topologies obtained with the two models were similar. The ML tree was shown in **Figure 1** and the NJ tree was shown in **Figure S3**. In our

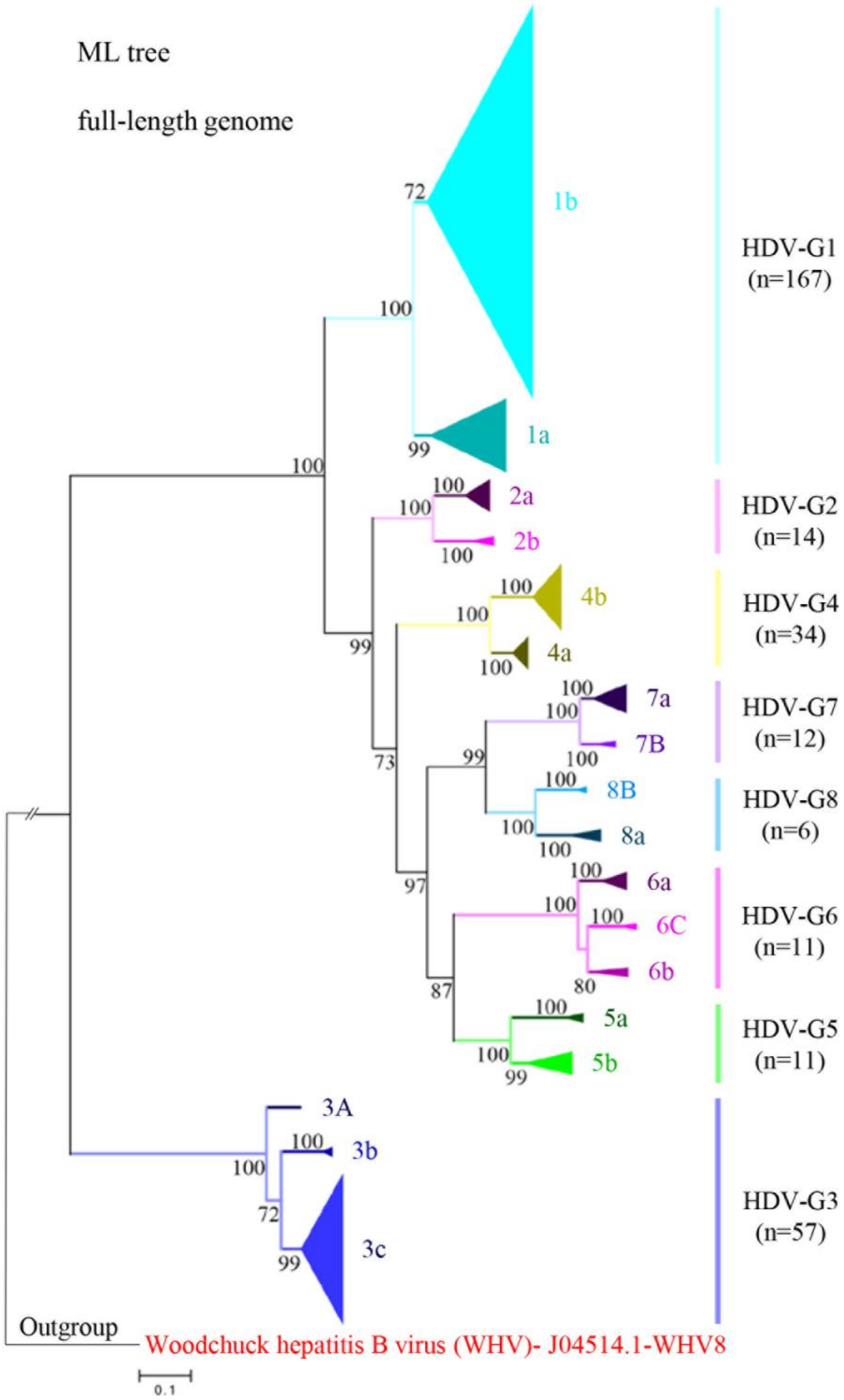

Figure 1. Phylogenetic analysis of 312 HDV full-length antigenomic RNA (cRNA) sequences. The Maximum-likelihood (ML) tree of eight HDV genotypes with subtypes showing the overall classification framework. All original HDV full-length genomic RNA sequences were transformed to cRNA sequences and standardized to read from 1 to 1678. The tree was reconstructed using the best DNA model, General Time Reversible (GTR) model of evolution with 5 rate categories (G) and invariable sites (I). Potential recombinants were excluded from the tree. Branch support was calculated using 1,000 replications, and only bootstrap values >70% are shown. The tree was rooted with woodchuck hepatitis B virus (WHV).

rooted trees, 312 full-length strains were clustered as three big clades and further clearly grouped as eight small solid clades with 100% bootstrap value support. These eight clades were corresponding to eight genotypes, and in line with the classification previously described (12). Notably, genotype 2 and genotype 4 to 8 were consistently clustered as one big clade, and genotype 3 was located more close to the root of the trees.

As shown in the trees, eight genotypes were respectively further grouped into two (a and b) or three (a to c) subtypes **(Figure 1 and Figure S3)**. Genotype 3 and 6 were segregated into three subtypes, and genotype 1, 2, 4, 5, 7 and 8 were only grouped into two subtypes. Referring to the nomenclature used for HCV (21), capital in the nomenclature of HDV subtypes was used to indicate the unconfirmed status due to the available sequences less than three. Furthermore, we performed the same phylogenetic analysis using subgenomic HDAg coding gene fragment (600 nt) and the corresponding amino acid sequences. However, though some genotypes could be classified faithfully by using subgenomic fragment or HDAg amino acid, the classification of many other genotypes and subtypes were variable **(Figure S4A and S4B)**.

To further support our classification system, we compared the nucleotide similarities and genetic distances between different groups **(Figure 2 and Figure S5)**. Comparative genetic analyses showed that HDV genotype 3 was distantly separated from other genotypes. The intergenotypic nucleotide similarities and genetic distances between genotype 3 and other genotypes were 61.79-63.97% and 0.334-0.355, respectively, outside the ranges of intergenotypic nucleotide similarities and genetic distances between other genotypes (69.64-79.96%, 0.207-0.285). In addition, HDV genotype 1 showed higher divergence than other genotypes. The nucleotide similarity of intersubtype between subtype 1a and 1b was 82.58%, which was lower than the intersubtype nucleotide similarity range within other genotypes (85.82-90.49%). The genetic distance of intersubtype between subtype 1a and 1b was 0.162, higher than the range within other genotypes (0.094-0.139).

Diversity of HDV genotype distribution

The global distribution of HDV genotype varies geographically **(Figure 3)**. Genotype 1 is common globally; genotype 2 and 4 are mainly in Asia; genotype 3 is in South America; whereas genotype 5-8 are in Africa. It is noteworthy that several genotypes or subtypes concurrently prevail in most endemic regions, such as Asia and Africa. Particularly, HDV strains circulating in Africa exhibit extremely high genotypic diversity.

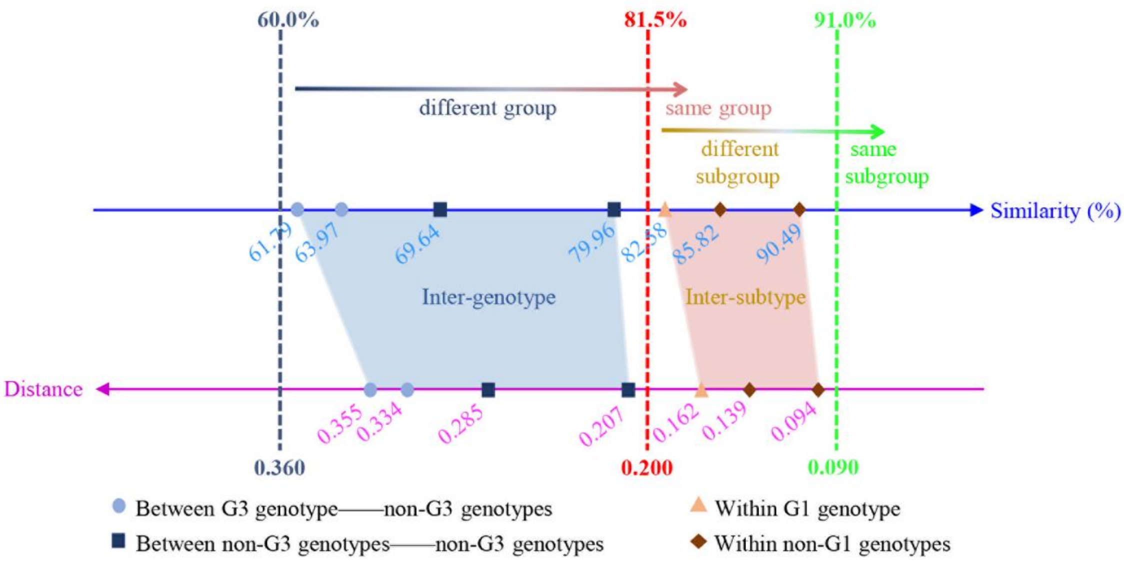

Figure 2. Genetic analysis of 312 HDV full-length antigenomic RNA (cRNA) sequences. Cartoon representation of the identification criteria of HDV novel genotype or subtype. The comparisons of mean intergenotypic and intersubtypic nucleotide similarity and genetic distance were based on 312 HDV full-length cRNA sequences. Axes show the percentage similarity and genetic distance, respectively. The blue dash line indicates the lowest range of intergenotypic nucleotide similarity and genetic distance, the red dash line indicates the cutoff range between genotype and subtype, and the green dash line indicates the highest range of intersubtypic nucleotide similarity and genetic distance. The detailed results of calculation were shown in Figure S5A and S5B.

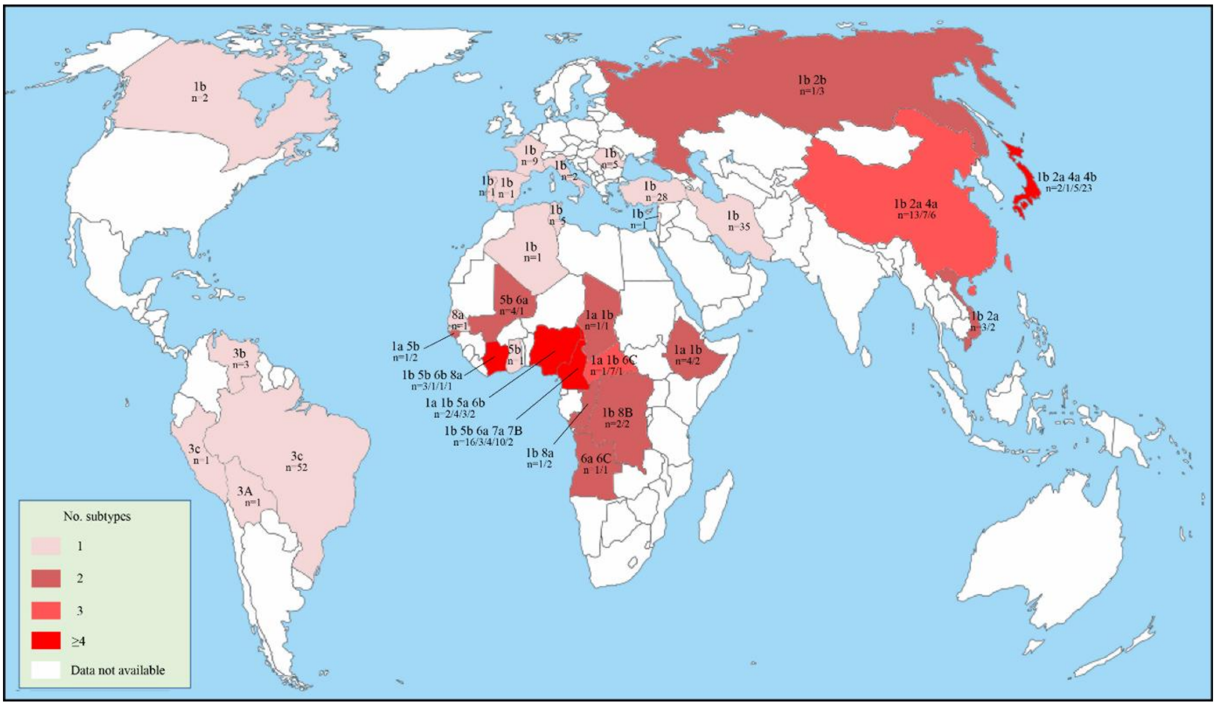

Figure 3. Worldwide distribution of HDV genotypes and subtypes. Data was based on HDV full-length genomic sequences. This map was modified according to the free map templates (http://d-maps.com/carte.php?num_car=13180&lang=en) using Inkscape 0.92.2 software.

Proposed criteria for identifying novel HDV genotype or subtype

Based on our phylogenetic and genetic analysis, we hereby proposed the identification criteria for novel HDV genotype and subtype. Firstly, standardized HDV full-length antigenomic sequence is recommended to be used. Secondly, potential recombination should be excluded and phylogenetically cluster together as a solid group or subgroup is essentially required. Finally, phylogenetic grouping should be supported by nucleotide similarity and genetic distance of intergenotype or intersubtype. The demarcation of a novel genotype is the intergenotypic nucleotide similarity at the range of 60.0%-81.5% and the corresponding intergenotypic genetic distance at the range of 0.200-0.360. For novel subtype, the intersubtypic nucleotide similarity and the corresponding intersubtypic genetic distance are at the range of 81.5%-91.0% and 0.090-0.200, respectively **(Figure 2)**.

Proposed reference genomes for HDV subtypes

In order to facilitate the communication between researchers and help clarify the epidemiology of HDV, we proposed a standard reference set of full-length genome sequences. These reference sequences were selected according to the following detailed criteria. Firstly, only full-length genome sequences were considered, and subgenomic fragments or potential full-length recombinants were eliminated. Secondly, to ensure the clarity of the strain origin and to minimize disruption of previous prototype notification, priority was given to the sequences with full information, but previous noted prototype strains (12) were also taken into consideration at the same time. Thirdly, if there was no sequence with complete information, priority was given to the prototype strain(s), if prototype strain(s) was/were noted. Fourthly, if no sequence was available with complete information or noted as prototype strain for a subtype, or more than one sequences were available for a subtype, priority was firstly given to the sequence with earlier collection date, then to the sequence earlier submitted to GenBank. If the collection or submission dates were identical, the sequence with lowest alphabetic/numeric accession number was proposed.

Finally, 21 full-length genome sequences were proposed as the references for eighteen HDV subtypes **(Table 1)**. We proposed one reference sequence for the each subtype of genotype 2 to 8. But 5 reference sequences were proposed for two subtypes of genotype 1. This is because HDV genotype 1 showed more introgenotypic divergence than other genotypes. As for 1a, a strain of ETH2170 (accession no. KY463677) collected in 2013 was noted as the prototype strain of genotype 1 (12). However, another 1a strain named 36011-NIE1150 (accession no. JX888100) was collected in 2006, which was earlier than the prototype strain. Thus, both strains were proposed as the reference sequences of 1a. For 1b, 141 full-length genome sequences were available to date. Because it was previously further classified as three subtypes (12), thus three sequences were proposed to these three previously assigned subtypes. Notably, all the proposed reference sequences were RNA form and only a few of them have collection date available.

Table 1. Reference sequences for HDV subtypes

Genotype	Subtype [a]	Accession no.	Strain/Isolate [b]	Date [c]	Country [d]
1	1a	JX888100	36011- NIE1150	**2006**	Nigeria
	1a	KY463677	**ETH2170**	**2013**	Ethiopia
	1b	JX888098	24187-NIE	**2004**	Nigeria
	1b	KJ744242	D34	**2008**	Iran
	1b	KJ744255	D66	**2003**	Iran
2	2a	X60193	**7/18/83**	2005	Japan
	2b	AJ309879	Yakut26	2016	Russia
3	3A	LT604954	dFr6727	2017	Bolivia
	3b	AB037947	VnzD8375	2001	Venezuela
	3c	KC590319	Brazil-1	**2011**	Brazil
4	4a	AF018077	**TW-2b (Taiwan isolate)**	1998	China
	4b	AB118818	Miyako (JA-M2)	**2006**	Japan
5	5a	JX888103	36102-NIE875	2006	Nigeria
	5b	AM183331	dFr2005	2007	GW
6	6a	AJ584847	**dFr48**	2005	Cameroon
	6b	JX888102	36036-NIE464	**2006**	Nigeria
	6C	AM183332	dFr2139	2007	CAR
7	7a	AJ584844	**dFr-45**	2005	Cameroon
	7B	AM183333	dFr2158	2017	Cameroon
8	8a	AJ584849	**dFr644**	2005	CGO
	8B	LT594488	dFr7707	2017	COD

[a] Capital standing for the unconfirmed status due to the available sequences less than 3.
[b] Previous designated prototype strains are indicated in bold.
[c] Sequences with clear collection date are highlighted in bold, sequences with collection date missed and replaced by Genbank release date are shown in italic.
[d] GW- Guinea-Bissau; CAR-Central African Republic; CGO-Republic of the Congo; COD-Democratic Republic of the Congo

DISCUSSION

With the introduction of HBV vaccine, although the prevalence of HDV has declined in some sporadic areas, the global prevalence of HDV is still high and even increasing in most of the endemic areas, such as Central and Northern Africa, the Amazon Basin, Eastern and Mediterranean Europe, the Middle East and parts of Asia (5, 7, 22, 23). In sub-Saharan Africa, the estimated prevalence of anti-HDV has exceeded the global prevalence and around 7 million people are infected by HDV (7). HBsAg-positive patients with HDV co-infection showed higher risk to progress to liver fibrosis or HCC compared to asymptomatic controls (7, 22, 24). Thus, for better management or prevention of HDV infection, diagnosis and screening for high-risk populations are recommended (5, 7). This in turn requires a unified HDV genotype classification system. Due to the confusion as a negative and circular genome, both genomic and antigenomic RNA forms of HDV genome sequences have been submitted to the Genbank database by different research groups and a substantial set of sequences were even read from abnormal initial site (17, 18). Furthermore, two different genotype classification systems have been previously proposed (11, 12). Thus, it is urgent to clarify these inconsistencies, in order to facilitate the future research in this field.

Previous studies have demonstrated that HDV homologous recombination may occur both in nature (patient with mixed infection) and the laboratory (cotransfection in cell culture system) (25-27). Through phylogenetic and Bootscaning analysis, we confirmed two previous reported recombinants and identified 31 new potential recombinants. The two recombinants, one intra-genotypic recombination (AB118845, 4a/4b) (20) and the other inter-genotypic recombination (KF660598, 2a/1b) (19), have the same recombination pattern with only one crossover. However, the other 31 newly identified recombinants, have another predominant pattern with two crossovers. The formation of different recombination patterns may be associated with the distinct replication mechanisms of HDV genome (27-29). Recombination events were detected among several genotypes, but more frequent in genotype 1 and 5. For genotype 1, it may be explained by its global distribution **(Figure 3)** (12). Although genotype 5 is mainly present in Western Africa, the high recombination frequency may be associated with the intergenotypic evolutional relationship and the African origin (12, 30). Analysis of the recombination junctions has indicated that recombination events occurred at four regions throughout the whole genome. Among these regions, nt 694-872 at genomic RNA corresponding to nt 807-985 at antigenomic RNA is the hotspot of HDV RNA recombination. This genome region serves as the pseudoknot ribozyme domain of HDV genome. It is identical with the hotspot fragment "D" previously shown in the HDV-1/HDV-4 recombination map (28). A model has been proposed to illustrate the mechanism of HDV recombination, which is via a viral-RNA-structure-promoted template-switching mechanism driven by the host RNA polymerase (28), although further validation is required.

For molecular classification of HDV, eight clades have been proposed a decade ago, but recently designated as eight genotypes (12). In contrast, a latest study has proposed to group the eight HDV genotypes into three large genogroups by grouping clade 2 and clade 4 to 6 as one (11). In our study, we have applied the ML and NJ models to reconstruct phylogenetic trees based on standardized full-length antigenomic sequences excluding the potential recombinants. Indeed, all the HDV strains were clustered as three big clades, but further grouped into eight groups with high bootstrap value support. Even though the three big clades shared some characteristics as described, three genotype classification has neglected HDV genotypic divergence. Besides, this classification system only divided HDV strains as genotype, but not further into subtypes. Thus, we agree with the classification system of eight genotypes (12). However, the subtype classification of genotype 1, 3 and 6 in our system showed clear differences, compared to the previous study (12). We grouped genotype 1 into two subtypes, 1a and 1b. Because the previously classified strains of the HDV-1b, HDV-1c and HDV-1d subtypes (12) do not always cluster as independent groups but rather as one big branch supported by over 70% Bootstrap value in our trees **(Figure 1 and Figure S3)**. Genotype 3 was segregated into 3a, 3b and 3c three subtypes by including more sequences; whereas this was not clear in the previous study due to the lacking of sufficient sequences (12). We classified genotype 6 into three subtypes; whereas only two were previously defined (12). Consequently, 312 strains were further classified as eight genotypes with eighteen subtypes in our updated classification system supported by high Bootstrap values, nucleotide identity and genetic distance. Of note, neither HDAg gene fragments nor amino acid sequences can classify HDV strains into subtypes faithfully **(Figure S4)**, because many genotypic characteristics are located outside of the HDAg gene (11, 12).

Among these eight genotypes, genotype 1 is the most predominant with highest divergence. This may be resulted from prolonged wide-spread transmission **(Figure 3)** (12). Genotype 3 is located close to the root of phylogenetic tree, showing distantly nucleotide similarity and genetic distance with other genotypes. Given that genotype 3 was only found in South America **(Figure 3)**, it is plausible whether this genotype is an independent lineage or the early HDV progenitor. The exact evolutional relationship between genotype 3 and other genotypes requires further investigate. However, the current genetic data is still insufficient to address this question **(Figure 3)** (5, 7). Importantly, based on our phylogenetic and genetic results, we have proposed detailed criteria for identifying novel genotype or subtype.

Finally, we have compiled a complete set of reference genome sequences for HDV subtypes. The main criteria were based on previous study of hepatitis E virus reference genomes (31) and the unique features of HDV. We have proposed one reference sequence for each subtype of genotype 2 to 8. But five reference sequences were proposed for the two subtypes of genotype 1, because of the huge divergent. When generating these 21 reference sequences, we found that the majority HDV full-length sequences have missing information, in particular the collection date. Thus, we strongly recommend researchers to provide the essential information, when submitting their sequence data to the online database.

In summary, we have systematically retrieved and analyzed a large set of HDV full-length genome sequences and identified novel recombinants. Based on phylogenetic and genetic analyses, we have established an updated classification system for HDV when recombinants were excluded. Furthermore, we have mapped the global distribution of different genotypes and subtypes. Finally, we have compiled a complete set of reference genomes for each subtype and proposed criteria for future identification of novel genotypes and subtypes. Of note, our global distribution map indicates that currently available HDV genetic data remains limited, and thus the proposed classification will likely evolve as future epidemiological data will accumulate. Overall, these results shall facilitate the future research on the diagnosis, screening, epidemiology, evolution, prevention and clinical management of HDV infection.

ABBREVIATIONS

HDV, hepatitis delta virus/hepatitis D virus; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; ssRNA, single-stranded RNA; nts, nucleotides; cRNA, complementary RNA; mRNA, messenger RNA; ORF, open reading frame; HDAg, hepatitis delta antigen; HCC, hepatocellular carcinoma; bp, base pair; WHV, woodchuck hepatitis B virus; ML, Maximum-likelihood; NJ, Neighbor-Joining; GOP, gap-opening penalty; GEP, gap extension penalty; GTR, General Time Reversible; JTT, Jones-Taylor-Thornton.

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SUPPLEMENTARY METHODS AND RESULTS

Supplementary Figures

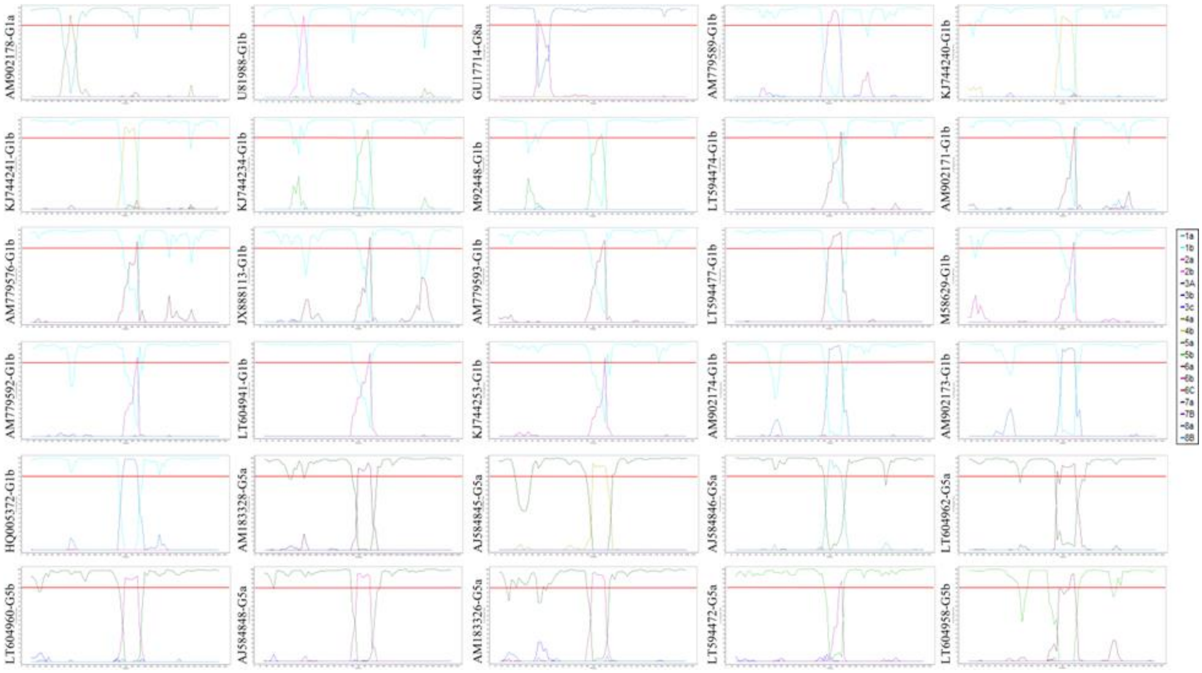


Figure S1. Bootscan analysis of 30 HDV full-length recombinants. A 600 bp window, a 20 bp step increment, and 1,000 bootstrap replicates were used. The query sequence was confirmed as recombinant when the similarity with the "parental" reference sequences across the whole genome was over 80% of the permuted trees. The red line indicates the 80% threshold used to denote significance between genotypes. Breakpoint positions were numbered to the aligned cRNA dataset, and the corresponding original genomic positions were summarized in Table S1. HDV genotype 1, 2, 4, 5 and 8 could recombine with other genotypes or subtypes; however, recombinants formed more frequently with genotype 1 and 5. In terms of genomic breakpoint positions, nt 577-650, 694-872, 1154-1340 and 1370 were the four RNA regions that recombination events occurred throughout the whole genome, and the corresponding antigenomic RNA positions were nt 1029-1133, 807-985, 339-525 and 309, respectively. Among these regions, nt 694-872 at genomic RNA or corresponding to nt 807-985 at antigenomic RNA was the hotspot for recombination.

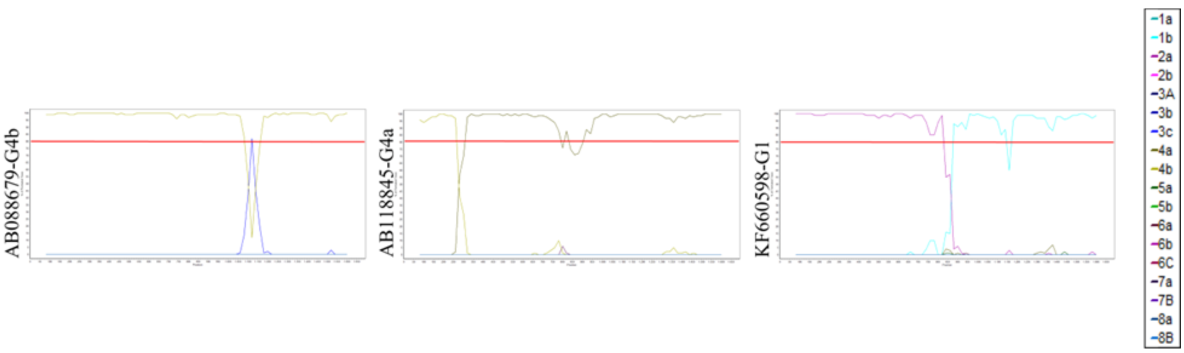


Figure S2. Bootscan analysis of one newly detected and two previous reported HDV full-length recombinants. AB088679 strain was newly detected in this study. AB118845 and KF66598 strains were identified previously. A 600 bp window, a 20 bp step increment, and 1,000 bootstrap replicates were used. The query sequence was confirmed as recombinant when the similarity with the "parental" reference sequences across the whole genome was over 80% of the permuted trees. The red line indicates the 80% threshold used to denote significance between genotypes. Breakpoint positions were numbered to the aligned cRNA dataset, and the corresponding original genomic positions were summarized in Table S1. Two patterns of HDV recombination were showed. One category, such as the two recombinants of AB118845 and KF660598, has only one crossover. The second category including the other 31 recombinants has two crossovers.

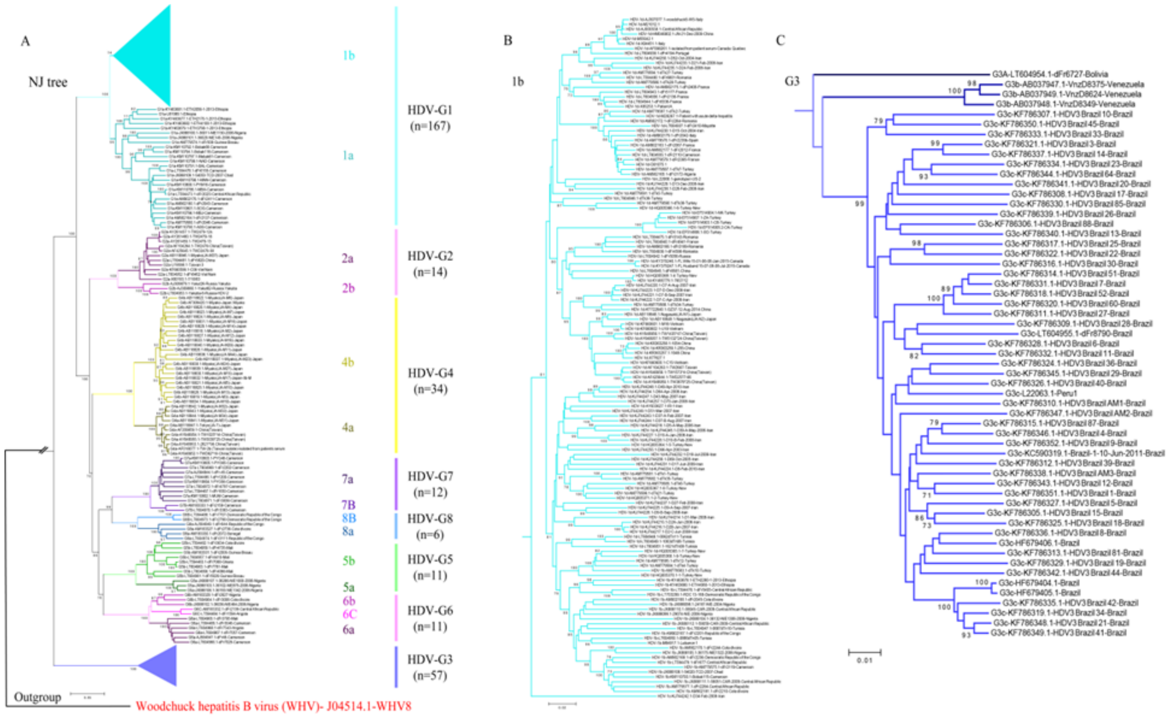


Figure S3. The Neighbor-Joining (NJ) tree of 312 HDV full-length antigenomic RNA (cRNA) sequences. (A) The overall framework of HDV NJ tree; **(B)** the topology of 1b branch indicating previous designated three subtypes; **(C)** the topology of genotype 3 clade. All original HDV full-length genomic RNA sequences were transformed to cRNA sequences and standardized to read from 1 to 1678. The tree was reconstructed using p-distance model with Bootstrap method test. Potential recombinants were ruled out from the tree. Branch support was calculated using 1,000 replicates, and only bootstrap values >70% are shown. The tree was rooted with woodchuck hepatitis B virus (WHV).

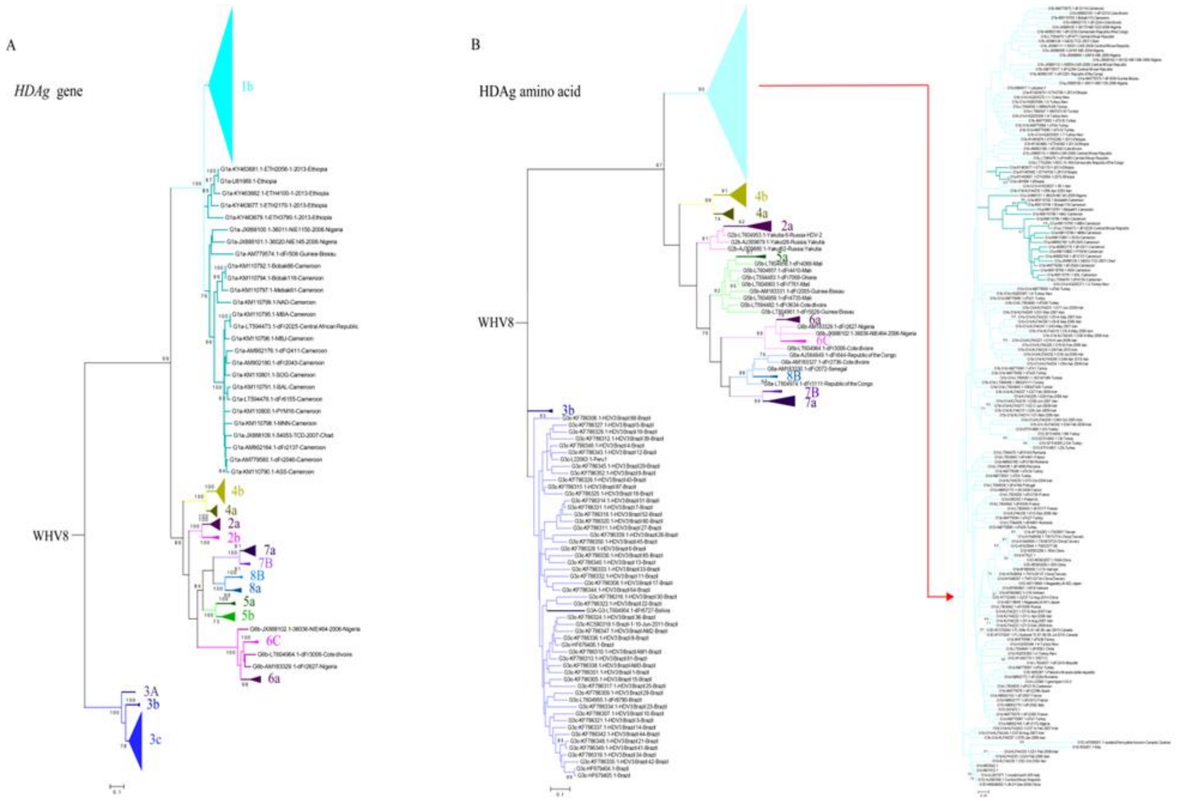


Figure S4. Maximum-likelihood (ML) trees of HDV subgenomic sequences. (A) HDAg coding gene fragment; **(B)** HDAg amino acid. The General Time Reversible (GTR) model and the Jones-Taylor-Thornton (JTT) model were used as the best DNA and protein model, respectively, with 5 rate categories (G) and invariable sites (I). Potential recombinants were ruled out from the trees. Branch support was calculated using 1,000 replicates, and only bootstrap values >70% are shown. The tree was rooted with woodchuck hepatitis B virus (WHV). The branches identical with the full-length classification results were compressed.

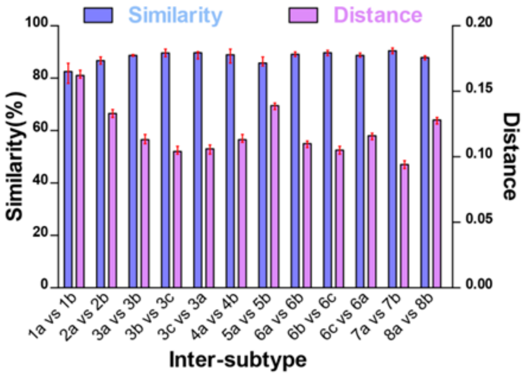
A

Intergenotypic nucleotide similarities and genetic distances

Genotype	G1	G2	G3	G4	G5	G6	G7	G8	Introgenotype Distance(S.E.)
G1		0.251	0.338	0.266	0.274	0.272	0.285	0.278	0.135(0.004)
G2	72.16		0.336	0.220	0.230	0.241	0.260	0.243	0.076(0.004)
G3	63.97	63.42		0.334	0.349	0.352	0.353	0.355	0.073(0.003)
G4	71.56	78.58	63.42		0.246	0.253	0.252	0.245	0.079(0.004)
G5	70.51	75.79	62.20	74.76		0.238	0.246	0.232	0.109(0.004)
G6	70.29	74.83	61.94	74.24	76.07		0.267	0.258	0.092(0.004)
G7	69.64	73.48	62.09	74.56	75.59	73.92		0.207	0.076(0.004)
G8	70.13	74.72	61.79	74.75	76.57	74.29	79.96		0.098(0.005)
Introgenotype Similarity (%)	85.7(77.3-99.5)	92.47(85.4-99.5)	92.32(87.4-99.7)	92.45(85.8-99.5)	88.52(84.0-98.4)	90.93(88.0-99.6)	92.60(89.4-99.4)	90.71(87.0-97.6)	

B

Intersubtypic nucleotide similarities and genetic distances



Subtype	1a	1b	2a	2b	3A	3b	3c	4a	4b
Similarity (%)	89.86	86.77	95.78	90.80	NA	97.93	92.75	97.26	95.08
Distance	0.103	0.126	0.044	0.050	NA	0.018	0.069	0.030	0.037
Subtype	5a	5b	6a	6b	6C	7a	7B	8a	8B
Similarity (%)	90.20	91.44	94.58	92.87	96.60	93.49	94.90	93.38	97.62
Distance	0.091	0.083	0.065	0.073	0.033	0.068	0.045	0.069	0.024

Figure S5. The detailed results of calculated nucleotide similarity and genetic distance. (A) Intergenotypic nucleotide similarities and genetic distances distance; **(B)** Intersubtypic nucleotide similarities and genetic distances. The comparisons were based on 312 HDV full-length cRNA sequences. Nucleotide similarities were calculated by the program Sequence Distances implemented in MegAlign software, and genetic distances were calculated by MEGA with the Kimura 2-parameter/gamma model and 1000 bootstrap replicates.

Supplementary Table

Table S1. The genotypes component of HDV recombinants

Accession NO.	Recombined genotype		Recombination event (position)	
	Major	Minor	RNA	cRNA
AB118845	**4a**	**4b**	**1370**	**309**
KF660598	**2a/1b**	**1b/2a**	**827**	**852**
AM902178	1a	4a	1203-1340	339-476
U81988	1b	2a	1203-1300	379-476
GU177114	8a	6b	1154-1300	379-525
AM779589	1b	2a	743-872	807-936
KJ744240	1b	4b	743-872	807-936
KJ744241	1b	4b	743-872	807-936

KJ744234	1b	5b	743-872	807-936
M92448	1b	5b	743-872	807-936
LT594474	1b	6a	743-827	852-936
AM902171	1b	6a	743-790	889-936
AM779576	1b	6a	743-872	807-936
JX888113	1b	6a	743-827	852-936
AM779593	1b	6a	694-872	807-985
LT594477	1b	6a	694-872	807-985
M58629	1b	6a	694-872	807-985
AM779592	1b	6b	743-827	852-936
LT604941	1b	6b	694-872	807-985
KJ744253	1b	6b	743-790	889-936
AM902174	1b	8a	743-872	807-936
AM902173	1b	8a	743-872	807-936
HQ005372	1b	8a	743-872	807-936
AM183328	5a	2a	743-872	807-936
AJ584845	5a	4b	724-844	835-955
AJ584846	5a	1b	694-872	807-985
LT604962	5a	6a	743-872	807-936
LT604960	5a	2a	743-872	807-936
AJ584848	5a	2a	743-872	807-936
AM183326	5a	2a	743-872	807-936
LT594472	5b	2a	743-872	807-936
LT604958	5b	6a	694-872	807-985
AB088679	4b	3c	577-650	1029-1133

The designation of subtypes was according to our updated classification system. Two previous reported recombinants were indicated in bold.

Chapter 3

Estimating the global prevalence, disease progression and clinical outcome of hepatitis delta virus infection

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ABSTRACT

Background: Hepatitis delta virus (HDV) co-infects with hepatitis B virus (HBV) causing the most severe form of viral hepatitis. However, its exact global disease burden remains largely obscure. We aim to establish the global epidemiology, infection mode-stratified disease progression and clinical outcome of HDV infection.

Methods: We conducted a meta-analysis with a random-effects model, and performed data synthesis.

Results: The pooled prevalence of HDV is 0.80% (95% CI 0.63-1.00) among the general population and 13.02% (95% CI 11.96-14.11) among HBV carriers, corresponding to 48-60 million infections globally. Among HBV patients with fulminant hepatitis, cirrhosis or hepatocellular carcinoma, HDV prevalence is 26.75% (95% CI 19.84-34.29), 25.77% (95% CI 20.62-31.27), and 19.80% (95% CI 10.97-30.45), respectively. The odds ratio (OR) of HDV infection among HBV patients with chronic liver disease compared to asymptomatic controls is 4.55 (95% CI 3.65-5.67). HDV co-infected patients are more likely to develop cirrhosis than HBV mono-infected patients with OR of 3.84 (95% CI 1.79-8.24). Overall, HDV infection progresses to cirrhosis within 5 years, and to hepatocellular carcinoma within 10 years in average.

Conclusions: Findings suggest that HDV poses a heavy global burden with rapid progression to severe liver diseases, urging effective strategies for screening, prevention and treatment.

KEYWORDS

Hepatitis delta virus; Epidemiology; Disease progression; Cirrhosis; Hepatocellular carcinoma

INTRODUCTION

Hepatitis delta virus (also known as hepatitis D virus, HDV) is a defective sub-virus that requires hepatitis B virus (HBV) surface antigens (HBsAgs) to propagate. Following its discovery in the 1970s, HDV has been largely neglected over the past decades, and establishing HDV status has been relatively uncommon in routine clinical practice. Early reported global prevalence of HDV was estimated at 15-20 million infections, corresponding to about 5% of HBV carriers (1). This relatively complacent view on the HDV public health problem was challenged in 2017, when a study targeting sub-Saharan Africa estimated the presence of seven million infections in this specific region alone (2). Indeed, a subsequent study in 2018 estimated the world-wide number of HDV infections at about 62-72 million (3) and this number was recently upwardly revised to 74 million (4). Thus, the public health problem posed by HDV infection appears much bigger than initially assumed. However, there is ongoing debate regarding the exact global prevalence of HDV (5, 6), and regional estimates remain largely lacking.

Globally, viral hepatitis causes around 1.34 million deaths annually, with 66% of the deaths attributed to HBV infection (7). Which fraction, however, of the HBV-associated mortality involves disease complicated by HDV infection remains uncertain. Despite being a defective virus, HDV is generally associated with the most severe forms of acute and chronic viral hepatitis in humans. Patients infected with both HDV and HBV display apparently dramatically accelerated progression to cirrhosis and development of hepatocellular carcinoma compared to those patients displaying HBV infection alone (8-10). It is thus likely that HBV complicated by HDV infection is associated with alternative disease progression, treatment response and patient outcome when compared to non-HDV complicated HBV infection, but quantitative data on the contribution of HDV infection on outcome of HBV infection are largely lacking (11-13). Interestingly, HDV infection can occur either via simultaneous coinfection with HBV of a susceptible individual or through superinfection of an HBV carrier (14). These two transmission modes may also lead to distinct clinical outcome but again systematic analysis of such an effect has not been performed (14). By performing a systematic review, meta-analysis and additional data synthesis, we aimed to generate a high-confidence estimate of the global prevalence of HDV infection and its relation to outcome HBV infection in the context of both HBV/HDV coinfection as well as of HDV superinfection in an existing HBV infection.

MATERIALS AND METHODS

Literature search and selection criteria

For this systematic review and meta-analysis, we searched EMBASE, Medline Ovid, Cochrane Database, and China Knowledge Resource Integrated database for cross-sectional and longitudinal observational studies measuring the prevalence and outcome of HDV infection, published in English and Chinese languages from database inception to February 2019. The prevalence of HDV was defined by the detection of HDV antibodies (anti-HDV IgG and/or anti-HDV IgM) using immunoassay, supplemented by the additional detection of delta antigen and HDV RNA. Study subjects were classified either as general population or HBsAg positive carriers, and for further sub-analysis groups were divided into blood donors, population at large (general group), intravenous drug users (IDUs), people with high-risk sexual activity, HIV patients, HCV patients, blood transfusion recipients, mixed patients, patients with liver disease and asymptomatic HBV carriers, as per cohort information. HBV patients with liver disease were divided into different categories: acute hepatitis (AH), fulminant hepatitis (FH), chronic hepatitis (CH), liver cirrhosis, and hepatocellular carcinoma (HCC).

Data collection and processing

Non-redundant records were initially screened by title and abstract according to the selection criteria independently performed by Z.M and S.L. The selected results were cross-checked to resolve discrepancies, and the remaining disagreements were discussed with J.L and Q.P and resolved by consensus. Subsequently, the selected records were subjected full-text assessment, and data were extracted from the primary literature independently by Z.M and S.L. Discrepancies were identified and resolved by discussing or arbitrage by J.L and Q.P. For exclusion of potential duplicate data from the same geographical location, consensus by the investigational team was achieved. Authors from primary studies were contacted for clarification if required.

The quality of the studies included was assessed by a scoring system (15, 16), which was independently performed by two investigators (Z.M and S.L) and reviewed by the other investigators (J.L and Q.P). Then, sensitivity analyses were performed to assess the effects of study quality and data source. Our study was done in accordance with the Preferred Reporting

Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and Guidelines for Accurate and Transparent Health Estimates Reporting (GATHER) statements (17, 18).

Statistical analysis

The Metaprop module in the R-3.4.2 statistical software package was used for meta-analysis. The pooled prevalence was calculated by the DerSimonian-Laird random-effects model with Freeman-Tukey double arcsine transformation (19, 20). The 95% confidence interval (CI) was estimated using Wilson score method. Odds ratios were pooled with DerSimonian-Laird random-effects model. To avoid small sample bias in the random effects model, we excluded studies with fewer than 100 subjects for the general population and 20 for HBsAg positive carriers. Detailed information regarding materials, methods and related references as well as additional discussion are provided in the online Supplementary Data file.

RESULTS

Estimates of HDV prevalence at national, regional and global levels

Our search returned 3,518 records, and 634 of these met the inclusion criteria **(Figure 1)**. In total, 332,155 individuals of the general populations from 48 countries and regions and 271,629 HBsAg positive carriers from 83 countries and regions were included **(Supplementary Figure 1)**. For estimating the global prevalence, we calculated that the pooled prevalence of HDV is 0.80% (95% CI 0.63-1.00) in the general population and 13.02% (95% CI 11.96-14.11) among HBsAg positive carriers, corresponding to 48-60 million infections worldwide **(Figure 2)**. China, India and Nigeria are the leading countries in this respect **(Figure 2 and Supplementary Table 1)**. Regionally, HDV is highly prevalent in central Asia, eastern Europe, tropical and central Latin America, as well as central and west sub-Saharan Africa **(Table 1)**. Asia (44.41%-56.55%) followed by Africa (22.30%-38.37%) are predominant with respect to global HDV burden. HDV infection is especially prevalent in low-income and lower-middle income countries, but concomitantly data from these resource-limited countries are relatively limited **(Table 1 and Supplementary Figure 2)**.

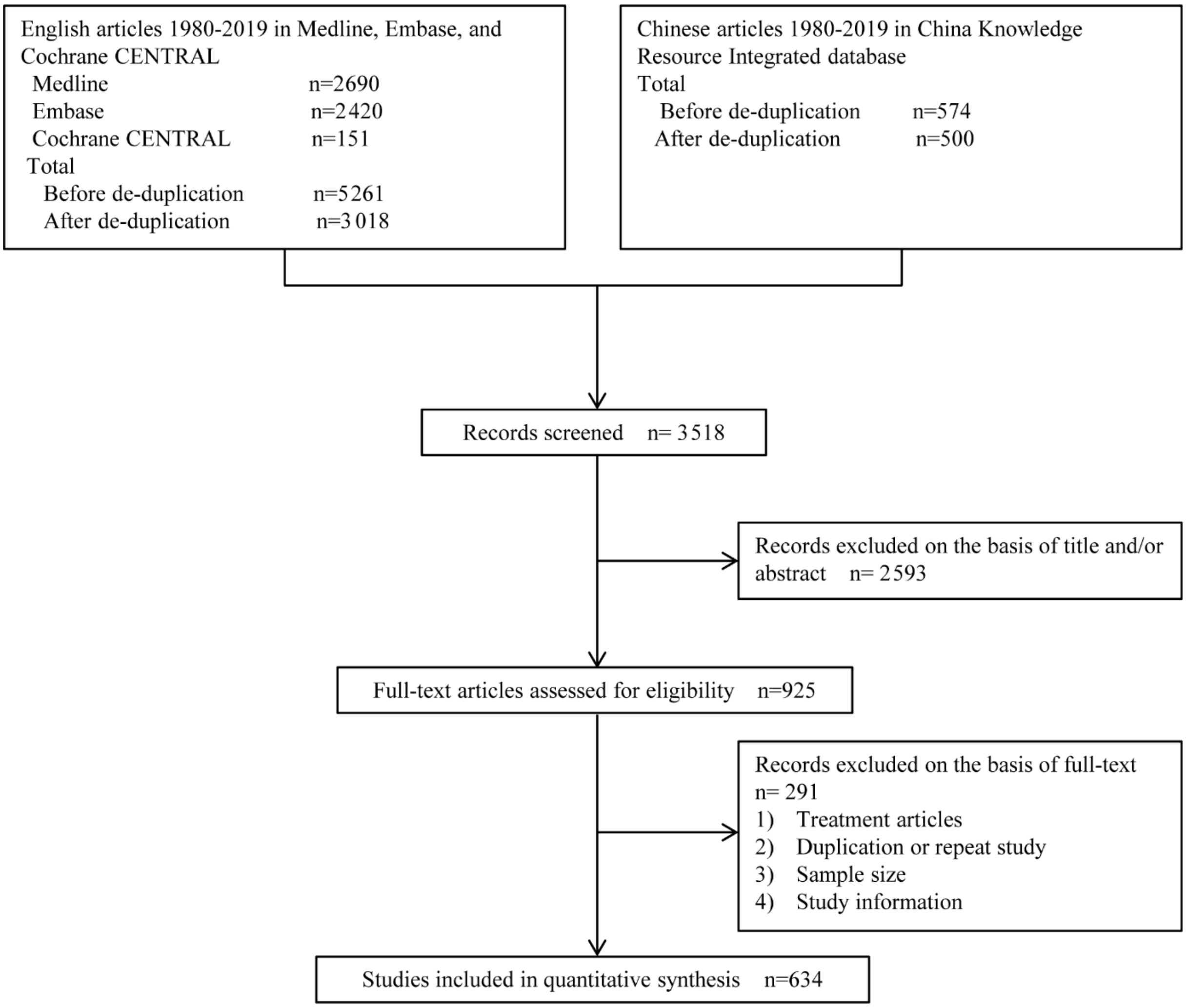

Figure 1. Flowchart of study selection.

Analysis of risk factors for HDV transmission

Further analysis of our data showed that the prevalence of HDV is high among IDUs but low among blood donors **(Supplementary Table 2)**. IDU, HIV and HCV are the remain risk factors for HDV transmission observed in HBsAg positive carriers with respective ORs of 15.44 (95% CI 8.68-27.49), 2.99 (95% CI 1.84-4.88), and 3.05 (95% CI 1.19-7.86), relative to controls **(Supplementary Table 2)**. There is no significant difference for the prevalence of HDV in males (14.95%; 95% CI 12.43-17.67) versus females (14.18%; 95% CI 11.49-17.10) among HBsAg positive carriers, with an OR of 1.05 (95% CI 0.91-1.21) **(Supplementary Figure 3-5)**.

Table 1. Estimates of HDV infection prevalence by GBD, WHO or World Bank region

Population, thousand	General population			HBsAg+ carrier		
		HDV prevalence (CI: 95%)[a]	HDV population, thousand	HBsAg population, thousand (prevalence)	HDV prevalence (CI: 95%)[a]	HDV population, thousand
GBD region						
Asia Pacific, high income	182909	0.65% (0.02-2.12)[1]	1189 (37-3878)	2561 (1.40%)	5.55% (2.86-9.06)	142 (73-232)
Asia, central	90018	8.31% (4.15-13.73)[1]	7481 (3736-12359)	5851 (6.50%)	51.27% (33.69-68.69)	3000 (1971-4019)
Asia, east	1455952	0.69% (0.24-1.36)[1]	10046 (3494-19801)	106284 (7.30%)	10.16% (8.50-11.95)[1]	10799 (9034-12701)
Asia, south	1712556	0.36% (0.02-1.12)	6165 (343-19181)	53089 (3.10%)	17.53% (12.08-23.74)	9307 (6413-12603)
Asia, southeast	660824	0.26% (0.05-0.61)	1718 (330-4031)	56831 (8.60%)	6.62% (2.13-13.33)	3762 (1210-7576)
Australasia	29909			359 (1.20%)	5.13% (3.94-6.46)[1]	18 (14-23)
Europe, central	113765	0.09% (0.00-0.38)	102 (0-432)	2275 (2.00%)	5.64% (1.82-11.38)	128 (41-259)
Europe, eastern	211400	1.40% (0.71-2.32)[1]	2960 (1501-4904)	4439 (2.10%)	29.15% (14.70-46.19)	1294 (653-2051)
Europe, western	400667	0.25% (0.11-0.47)	1002 (441-1883)	2404 (0.60%)	14.72% (13.11-16.40)	354 (315-394)
Latin America, Andean	56667			227 (0.40%)	65.52% (55.26-75.09)[1]	149 (125-170)
Latin America, central	225750	1.76% (1.06-2.62)	3973 (2393-5915)	1355 (0.60%)	40.57% (18.57-64.80)	550 (252-878)
Latin America, tropical	196250	1.13% (0.24-2.66)[1]	2218 (471-5220)	1178 (0.60%)	12.86% (6.21-21.47)[1]	151 (73-253)
Latin America, southern	55000	0.48% (0.00-3.95)[1]	264 (0-2173)	110 (0.20%)	2.91% (0.89-6.03)[1]	3 (1-7)
North Africa and Middle East	501333	0.35% (0.14-0.65)	1755 (702-3259)	13035 (2.60%)	8.58% (7.07-10.21)	1118 (922-1331)
North America, high income	368667	0.20% (0.15-0.26)	737 (553-959)	1106 (0.30%)	13.01% (8.54-18.25)	144 (94-202)
Oceania	11065	4.04% (3.53-4.58)	447 (391-507)	1217 (11.00%)	44.22% (13.58-77.58)	538 (165-944)
Sub-Saharan Africa, central	120941	1.32% (0.68-2.16)	1596 (822-2612)	15239 (12.60%)	26.18% (14.81-39.46)	3989 (2257-6013)
Sub-Saharan Africa, east	436157	1.03% (0.34-2.09)	4492 (1483-9116)	34456 (7.90%)	11.6% (6.78-17.51)	3997 (2336-6033)
Sub-Saharan Africa, southern	80671	0.00% (0.00-0.02)[1]	0 (0-16)	12423 (15.40%)	11.41% (0.00-43.96)	1417 (0-5461)
Sub-Saharan Africa, west	399653	1.38% (0.84-2.03)	5515 (3357-8113)	47159 (11.80%)	16.55% (11.56-22.24)	7805 (5452-10488)
WHO region						
AFRO	1085639	1.02% (0.61-1.52)	1107 (6622-16502)	103136 (9.50%)	15.29% (11.16-19.93)	15769 (11510-20555)
EMRO	707500	0.7% (0.34-1.20)	4953 (2406-8490)	21225 (3.00%)	12.56% (9.56-15.91)	2666 (2029-3377)
EURO	901625	0.23% (0.12-0.36)	2074 (1082-3246)	18033 (2.00%)	13.81% (12.38-15.31)	2490 (2232-2761)
PAHO	990250	0.92% (0.46-1.52)	9110 (4555-15052)	5942 (0.60%)	14.82% (10.96-19.16)	881 (651-1138)

SEARO	1969943	0.17% (0.01-0.50)	3349 (197-9850)	78798 (4.00%)	8.98% (4.95-14.07)	7076 (3900-11087)
WPRO	1906526	1.47% (0.77-2.40)	28026 (14680-45757)	135363 (7.10%)	11.14% (9.59-12.78)	15079 (12981-17299)
World Bank region						
High income	1145222	0.30% (0.17-0.47)	3436 (1947-5383)	12597 (1.10%)	12.38% (10.91-13.93)	1560 (1374-1755)
Upper-middle income	2670725	0.59% (0.38-0.85)	15757 (10149-22701)	128195 (4.80%)	11.04% (9.71-12.44)	14153 (12448-15947)
Lower-middle income	2974795	1.73% (0.98-2.70)	51464 (29153-80319)	157664 (5.30%)	18.39% (14.67-22.42)	28994 (23129-35348)
Low income	704758	1.02% (0.54-1.64)	7189 (3806-11558)	57085 (8.10%)	14.46% (10.10-19.44)	8255 (5766-11097)
Global	7486974	0.80% (0.63-1.00)	59896 (47168-74870)	366862 (4.90%)	13.02% (11.96-14.11)	47765 (43877-51764)

[a] Regional data of HDV infection available from only one country is marked (1); HDV prevalence equal to 0.00% standing for negative HDV infection among samples; blank means no HDV infection data is available among general population. GBD=Global Burden of Diseases. WHO=World Health Organization. AFRO=Regional Office for Africa. EMRO=Eastern Mediterranean Regional Office. EURO=Regional Office for Europe. PAHO=Pan American Health Organization. SEARO=South-East Asia Regional Office. WPRO=Western Pacific Regional Office.

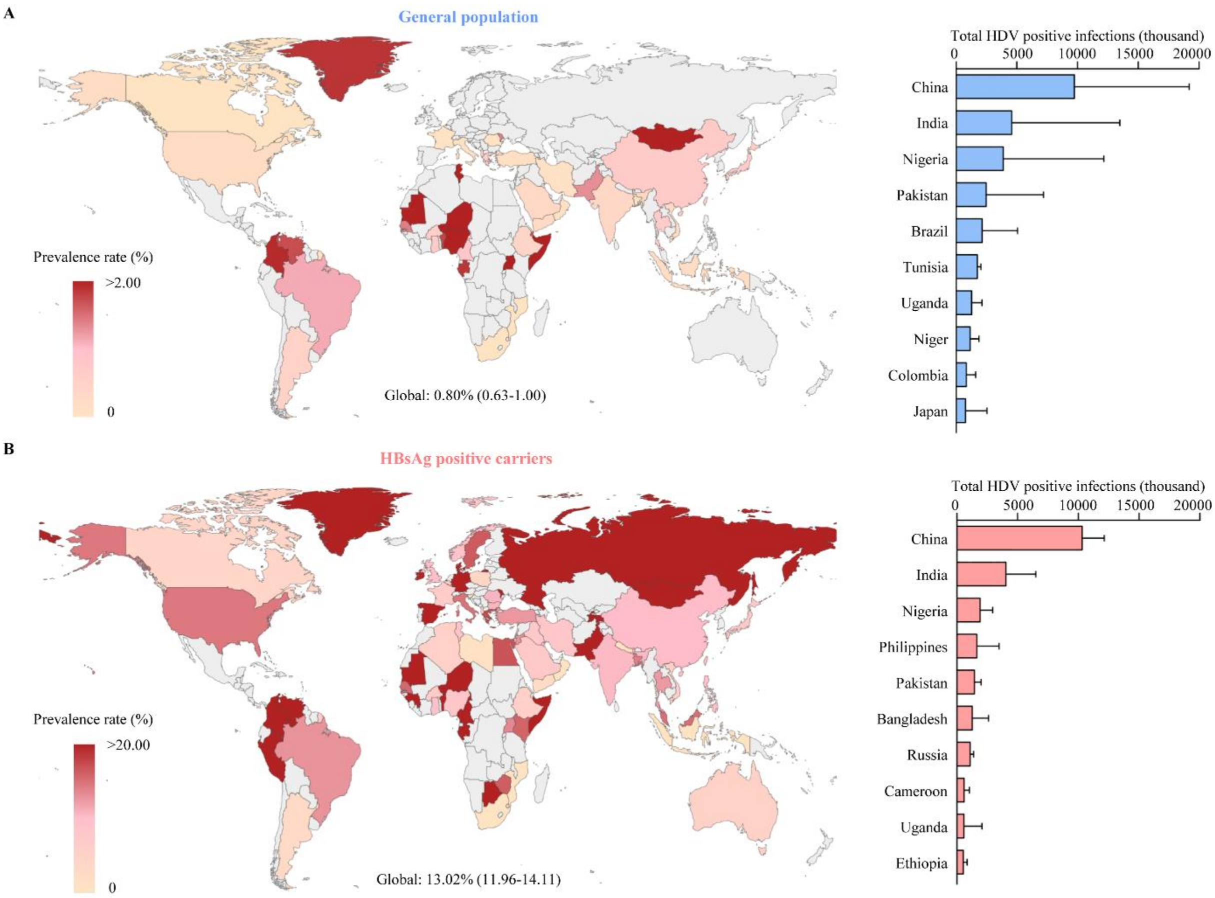

Figure 2. Global prevalence of HDV infection. (A) General population; **(B)** HBsAg-positive carriers. Blank means HDV pooled prevalence is not applicable due to lacking HDV epidemiological data. First ten counties for the estimates of HDV burden were listed respectively. Hepatitis delta virus, HDV; hepatitis B virus surface antigens, HBsAgs.

HDV infection presents a distinct epidemiological profile among HBV patients

There are hardly data that comprehensively capture as how and to what extent HDV contributes to severe liver diseases. Interestingly, the prevalence of HDV infection in HBsAg positive patients is very distinct between different forms of liver pathology. Among acute HBV patients, HDV infection is much higher in FH (26.75%; 95% CI 19.84-34.29) as compared to less symptomatic cases of AH (11.70%; 95% CI 8.90-14.81) **(Figure 3A)**. In chronic HBV patients, HDV infection rates are low in ASC (3.96%; 95% CI 3.13-4.88), but are high in CH (16.75%; 95% CI 14.00-19.69), cirrhosis (25.77%; 95% CI 20.62-31.27), and HCC (19.80%; 95% CI 10.97-30.45) **(Figure 3B)**. Comparison of symptomatic chronic HBV patients with asymptomatic controls of the same population yielded an OR for HDV infection of 3.56 (95% CI 2.72-4.65), 6.75 (95% CI 4.42-10.30), and 5.61 (95% CI 2.60-12.09) for CH, cirrhosis, and HCC, respectively **(Figure 3C)**. The pooled OR of these severe liver diseases is 4.55 (95% CI 3.65-5.67) and thus HDV infection is significantly linked to more serious pathology in HBV patients.

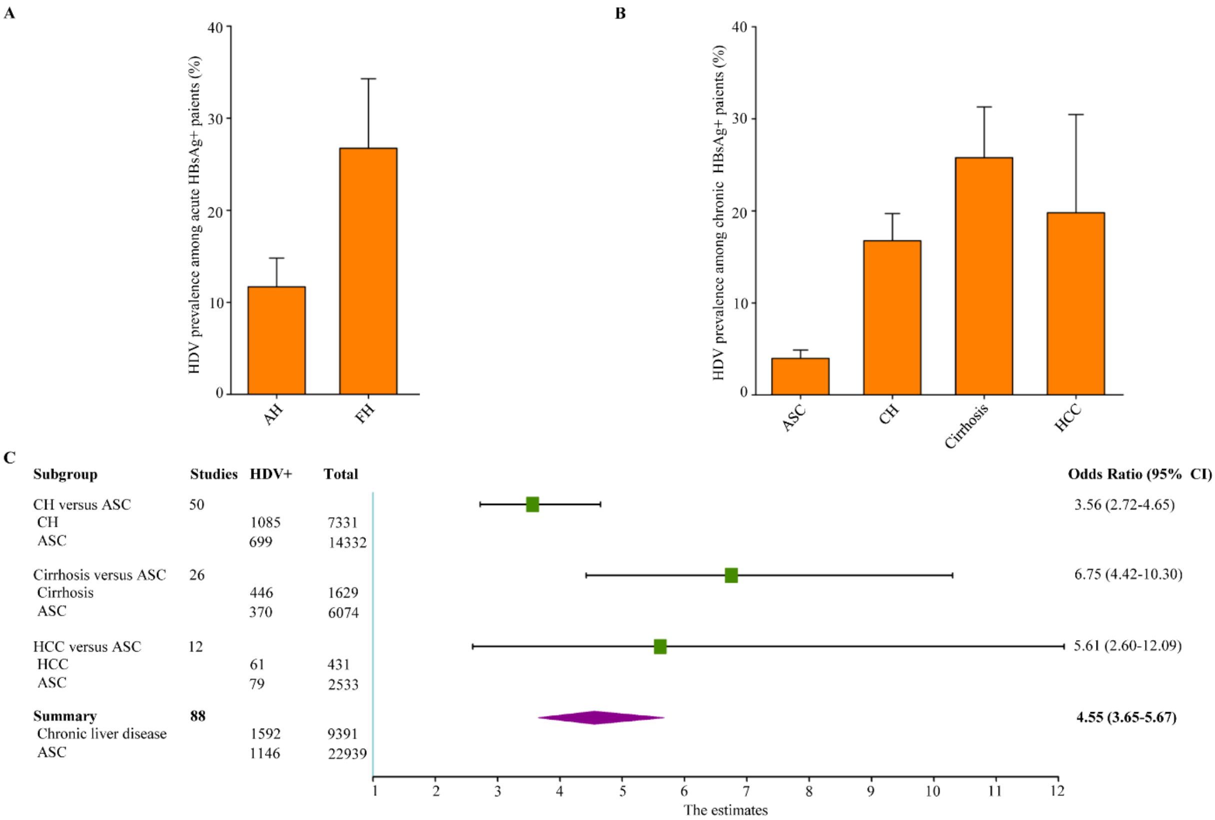

Figure 3. The epidemiological profile of HDV infection. (A) Prevalence of HDV among acute HBV patients. **(B)** Prevalence of HDV among chronic HBV patients. **(C)** Forest plot of hepatitis D virus prevalence among patients with chronic liver diseases compared with asymptomatic controls. Data are pooled from a random-effects model. Hepatitis delta virus, HDV; Asymptomatic carrier, ASC; Acute hepatitis, AH; Fulminant hepatitis, FH; Chronic hepatitis, CH; Hepatocellular carcinoma, HCC; Confidence interval, CI.

Different infection patterns of HDV infection result in distinct outcomes

Two major HDV infection patterns, coinfection and superinfection, provoke different outcomes **(Figure 4A)**. The majority of HBV-HDV co-infected patients spontaneously recover from HDV infection (80.96%; 95% CI 48.71-98.91), but only a minor proportion of superinfected patients recover (30.35%; 95% CI 12.05-52.70). In contrast, only a relatively small proportion of co-infected patients develop chronic disease (10.45%; 95% CI 4.49-18.52), but a substantial proportion of superinfected patients progress to chronic disease (77.38%; 95% CI 55.09-93.54). The OR to recover or become chronically infected following HDV coinfection are 5.05 (95% CI 1.45-17.56) and 0.05 (95% CI 0.01-0.27), respectively, relative to HDV superinfection. Stratification according to the pattern of viral infection reveals that most patients are HDV dominant (69.28%; range 56.30-85.25) or HBV-HDV codominant (27.56%;

range 14.52-40.60), with only a small fraction of patients being HBV dominant (3.16%; range 3.10-3.23). Thus, patients with HBV infection will clearly benefit from measures that prevent further HDV infection.

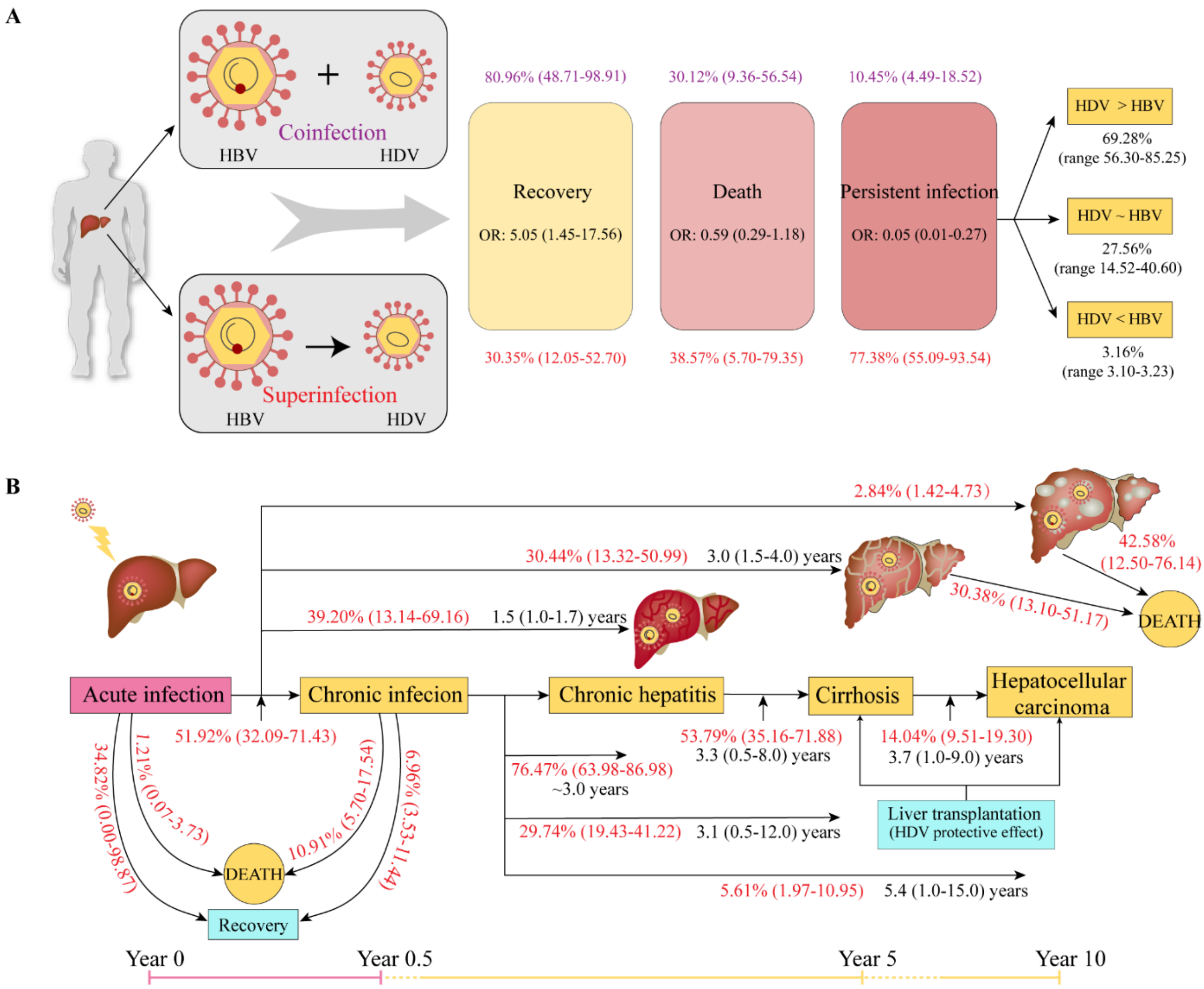

Figure 4. Schematic diagram of HDV infection and disease progression. (A) HDV infection patterns. Coinfection is that HDV and HBV simultaneously infect an individual or HDV infect the individual at the early stage after HBV infection. The essential diagnostic marker of this pattern is positive HBsAg and high-titer of anti-HBc IgM antibodies. Superinfection is that HDV infects the individual who has already established HBV infection or is a chronic HBV carrier (HBsAg positive). Anti-HBc IgM antibodies are absent in this pattern. HDV>HBV, HDV replication dominant; HDV~HBV, HDV and HBV codominant; HDV<HBV, HBV replication dominant. **(B)** Clinical progression of HDV infection. Pooled probability was shown with 95% CI unless specifically indicated. Time was shown as mean (range). The total percentage exceed 100% after data synthesis. Hepatitis delta virus, HDV; Hepatitis B virus, HBV; hepatitis B virus surface antigens, HBsAgs; Odds ratio, OR.

HDV infection leads to rapid progression to severe liver diseases

We observe that HDV infection predisposes to rapid progression into severe liver diseases **(Figure 4B)**. Upon acute infection, 39.20% (95% CI 13.14-69.16) of HDV-infected patients develop CH within a mean of 1.5 years (range 1.0-1.7) and 30.44% (95% CI 13.32-50.99) to cirrhosis within 3 years (mean, range 1.5-4.0). For established chronic infection, 76.47% (95% CI 63.98-86.98) of HDV-infected patients develop CH within a mean of 3 years, and 29.74% (95% CI 19.43-41.22) to cirrhosis within 3.1 years (mean, range 0.5-12.0). With respect to patients with CH, 53.79% (95% CI 35.16-71.88) of the patients with CH will progress to cirrhosis within a mean of 3.3 years (range 0.5-8.0), and 14.04% (95% CI 9.51-19.30) of the cirrhotic patients will progress to HCC within a mean of 3.7 years (range 1.0-9.0). In general, HDV infection progresses to cirrhosis averagely within 5 years, and to HCC within 10 years in average.

Compared to HBV mono-infection, double infection with HDV results in more severe clinical outcome. Among double-infected patients, only 14.99% (95% CI 2.87-34.22) are asymptomatic but 38.85% (95% CI 31.57-46.39) are cirrhotic **(Figure 5A)**. In contrast, from the HBV mono-infected patients, 14.36% (95% CI 10.04-19.30) is cirrhotic, whereas 57.2% (95% CI 26.10-85.42) is asymptomatic. For double-infected patients, the odds ratios for being asymptomatic, or having a diagnosis of cirrhosis, HCC or mortality are 0.12 (95% CI 0.06-0.21), 3.90 (95% CI 2.94-5.18), 1.97 (95% CI 1.02-3.78), and 2.05 (95% CI 1.18-3.56), respectively, relative to HBV mono-infected patients **(Figure 5B)**. The observed probability for cirrhosis development is much higher among double-infected patients (40.50%; 95% CI 22.09-60.43) than HBV mono-infected patients (14.22%; 95% CI 8.46-21.17), with an OR of 3.84 (95% CI 1.79-8.24) **(Figure 5C)**. Interestingly, the positive rates of either HBeAg and HBV DNA in serum of double-infected patients are lower than those observed in HBV mono-infected patients with respective ORs of 0.74 (95% CI 0.06-0.93) and 0.47 (95% CI 0.30-0.74) **(Figure 5D)**.

Quality and sensitivity analyses

In our quality and sensitivity analyses **(Supplementary Table 3 and 4, Supplementary Figure 6-11)**, the exclusion of low-scoring studies or the data from literature of Chinese language only showed minor effects on the estimates of the overall prevalence of HDV infection both among the general population and HBsAg positive population. However, the exclusion of these Chinese studies published in the Chinese language decreased the pooled prevalence in China of the general population from 0.69% to 0.48%, probably due to the influence by an extremely large negative cohort study from Hong Kong, but increased the rate of HBsAg positive individuals from 10.16% to 14.37%. Additionally, we noted significant heterogeneity within our meta-analysis.

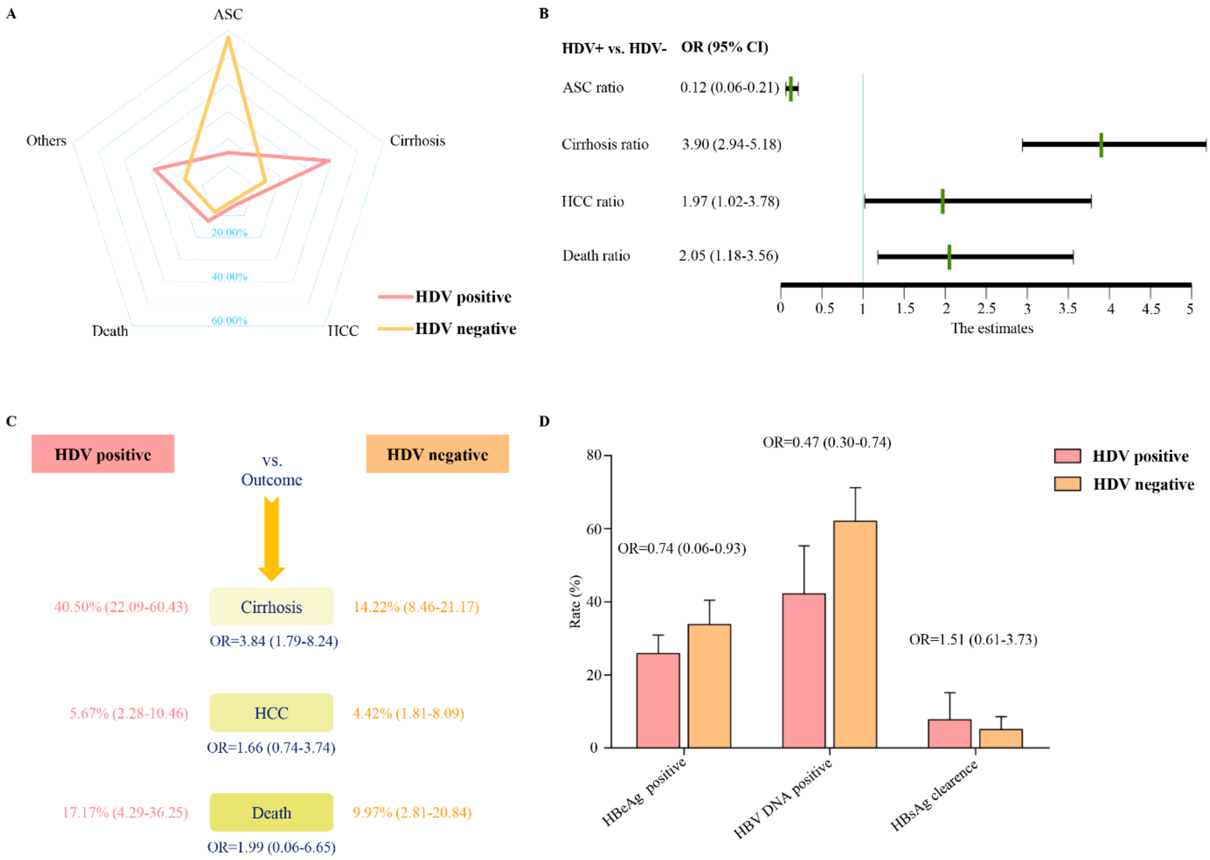

Figure 5. Clinical outcome comparison between HDV positive and negative patients. (A) Radar chart represents the composition of liver diseases among patients. **(B)** Forest plot of liver disease ratio among HDV positive patients compared with HDV negative patients. Data are pooled from a random-effects model. **(C)** The development of liver diseases among HDV positive patients compared with HDV negative patients. **(D)** The suppressive effect of HDV on HBV replication. Hepatitis B e antigen (HBeAg) and HBV DNA are the indicators of HBV replication. Asymptomatic carrier, ASC; Hepatocellular carcinoma, HCC; Odds ratio, OR; Confidence interval, CI; Hepatitis B e antigen, HBeAg.

DISCUSSION

In the present study we estimate that there are 48 to 60 million cases of HDV infection in HBV-infected individuals worldwide, yielding a global prevalence of 0.80% in the general population and 13.02% in HBsAg-positive carriers. A recent study that reported a global prevalence of 0.98% (3), but our study provides a more accurate estimate **(Supplementary Data)**, and is in line with the recently postulated global prevalence of 0.82% (6). The discrepancy with earlier studies can largely be attributed to the stratification for different populations and the exclusion of non-representative populations (e.g., IDUs, HIV patients, and patients with liver diseases). This avoids overestimation as was the criticism made with regard to the previous studies (5, 6).

The substantial global burden of HDV infection is fostered by several factors. Although it was previously identified as the satellite virus of HBV, a recent experimental study has demonstrated that HBV-unrelated viruses can also act as helper viruses for HDV transmission, such as HCV (21). Interestingly, we observe a high prevalence rate and a three-times increase in the odds for HDV infection among HBV-HCV double-infected patients. These results appear to support the experimental findings that HCV may assist the assembly and secretion of HDV infectious particles in patients, but requires further confirmatory investigation (21). Moreover, our study shows that the prevalence of HDV is extremely high among HBV-positive IDUs. Thus our study fits well with previous work showing the importance of injection drug use in driving HDV transmission (2, 3). Notably, previous study reported that IDUs represent a large reservoir of HDV burden (7). Indeed, we observe a fifteen-times increase in the odds for HDV infection in HBV-positive IDUs as compared with HBV-positive non-drug using counterparts. However, we estimate that only approximately 1.24%-1.56% and 1.04%-1.31% of the HDV burden can be attributed to users of intravenous drugs (743,000 cases) and HIV exposure (624,000 cases), respectively (16, 22). Thus strategies aimed at reducing HDV transmission by IDUs are mainly effective in reducing HDV prevalence because they prevent contagion of the population at large.

The prevalence of HDV varied substantially between geographical regions. With respect to the general population, in 18 countries the prevalence is over 1% and over half of the countries involved are from Africa while Latin America also has a fair number of high prevalence countries. Particularly, HDV infection rates highly prevail in Tunisia (15.33%), Mongolia (8.31%), and Niger (5.04%). Among HBsAg-positive carriers, the prevalence of HDV in 13 countries is excess of 30%, while in 10 countries is between 20%-30%, and in 23 countries is between 10%-20%. Consistent with previous observations, central Asia, eastern Europe, tropical and central Latin America, as well as central and west sub-Saharan Africa are high-endemic areas of HDV infection (3). Our findings show that Asia (44.41%-56.55%) and Africa (22.30%-38.37%) constitute the largest populations hit by HDV infections. Notably, Asia and Africa are the large reservoirs for HBV infection and accordingly are also the worst-hit areas with respect to HDV burden (23).

Regarding the estimation at national level, potential bias may be present in particular countries. Because of the limited sample number, there could be overestimation in the general population from these countries such as Colombia (1703), Nigeria (1419), Pakistan (2076), Tunisia (750) and Uganda (358), compared with the estimations among HBsAg positive carriers **(Supplementary Table 1)**. Besides, the limited origin of the samples among general population may also lead to overestimation in country like Brazil **(Supplementary Data)**. Finally, the national estimations of HDV prevalence are balanced by the estimations among general population and HBsAg positive carriers **(Supplementary Data)**. Our results show that China, India and Nigeria are the top three countries with respect to the number of HDV-infected individuals.

The importance of highlighting the high global prevalence of HDV infection is illustrated by the neglect in screening for HDV. Indeed, there is a paucity of studies about HDV prevalence in low-income and lower-middle-income countries. Such countries account for 50% of the world population, 60% of HBV burden but 70.34%-75.34% of HDV burden (23). Also in view of the observed propensity for serious liver disease in HDV superinfected individuals observed in the present study, a global health need emerges for effective prevention especially aimed at these countries.

The implementation of a global HBV vaccination program may be a cost-effective approach in this respect. Previous studies and mathematical modelling suggest that a HBV vaccination coverage above 80% is sufficient for eventual eradication of both HBV and HDV infection (3, 24). Early childhood HBV vaccination coverage, however, is still low (globally only 39 % in 2015), especially in African and South-East Asia (7), and it is estimated that over 100 million people are annually de novo infected with HBV (25, 26). Thus more efforts in this respect are necessary.

An important finding of our study is the dichotomy in outcome between simultaneous coinfection with HBV/HDV and a later HDV superinfection. The majority of the HBV-HDV co-infected patients spontaneously recover, whereas a substantial proportion of superinfected patients progress to chronic disease **(Supplementary Data)**. The implication of this result is that treatment of HBV carriers is important, as to prevent later chronic HDV infection. Unfortunately, only 10% of HBV infections are diagnosed, and only 5% receive antiviral therapy, also because of the relatively high costs associated with HBV-directed anti-viral therapy (23). We find clinical evidence that HDV and HBV actively interact with each other, resulting in three replicative patterns, but most patients are HDV dominant **(Supplementary Data)**. Mechanistically, this may be partially explained by a previous experimental study that HDV replication can suppress HBV replication by interfering with HBV mRNA synthesis and stability (11). Furthermore, HDV infection can induce the production of both type I and type III interferons (IFN-β and IFN-λ), which both inhibit HBV infection whereas HDV is resistant to self-induced innate immune responses (27, 28).

HDV infection is associated with progression to severe liver disease but, intriguingly, different liver diseases associated with HBV infection show a distinct relationship to HDV status. Among acute HBV patients, although vast majority of the data were collected from the studies published before year 2000, HDV infection is much higher in FH (26.75%) as compared to that in less symptomatic cases of acute hepatitis (11.70%) **(Figure 3A)** (29-31). The figure that HDV infection is much more often observed in more severe symptomatic cases than less symptomatic cases is identical among chronic HBV patients, and this is also well supported by the pooled odds ratio **(Figure 3)**. Together, HDV prevalence is particularly high in symptomatic HBV patients, especially patients with fulminant hepatitis and cirrhosis, than less symptomatic or asymptomatic cases. Over half (52%) of the patients suffering from acute HDV infection develop chronicity, and the majority (76%) of these chronically infected patients progress to CH. In turn, half (54%) of the CH patients progress to cirrhosis within three to five years (10, 32-34), and thus disease progression is much more aggressive in patients with HDV infection as compared to those suffering from HCV or HBV infection alone (35, 36). These results may correspond to previous findings that HDV replication synergistically activates hepatitis B virus X (HBx)-mediated TGF-β and c-Jun signaling cascades, both linked to fibrosis **(Supplementary Data)** (37, 38). Counterintuitively, however, protective effects has been associated with an HDV-positive status on the outcome of liver transplantation for cirrhosis or HCC (39).

There are several limitations of our study. First, we failed to collect sufficient data regarding antiviral treatment. Second, we mainly included publications in English, but also included the literatures published in Chinese language. This improves results because China bears a large part of the global HBV burden, but available English publications are mainly from Taiwan, and the prevalence of HDV may be different from the mainland and Taiwan. Thirdly, HDV is currently classified into eight genotypes (40) and different genotypes maybe lead to distinct clinical outcomes, but we did not include this aspect in the analysis because available data is limited. Fourthly, we performed the estimates both among the general population and HBsAg positive population. Interpretation of results relating to the latter is directly influenced by the HBV burden reference, but this itself is uncertain with estimates ranging from 250 million to 500 million (41). We employed the most frequently cited reference burden of 367 million and estimated the infection of HDV as 48 million, but HDV estimates range from 32 million to 61 million when refer to different HBV estimates **(Supplementary Figure 12)**. Finally, the interpretation of our estimates may be affected by the study quality, data source and study population included, resulting in variations that may increase the heterogeneity in our analysis **(Supplementary Data)**. Thus, our current estimates will likely evolve as more high-quality epidemiological data come available.

In summary, we now provide a high-confidence estimate of global HDV prevalence, although our results also show the need for high-quality epidemiological surveys for HDV in low-income and lower-middle-income countries. Our results quantify the effect of HDV infection in the context of HBV infection and highlight the risk of HDV superinfection in this context. Overall, our study shows that the global HDV burden is substantial, whereas its association to rapid progression to severe liver disease calls for more efforts with respect to screening, prevention and treatment.

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SUPPLEMENTARY METHODS AND RESULTS

Methods

1. Search term and records

(1) embase.com

('Hepatitis delta virus'/mj OR 'delta agent hepatitis'/mj OR 'hepatitis delta antigen'/mj OR ((Hepatitis NEAR/3 (delta)) OR 'Hepatitis d' OjR hdv OR hdag):ab,ti) AND ('prevalence'/de OR prevalence/de OR 'epidemic'/de OR epidemiology/de OR epidemiology:lnk OR 'epidemiological data'/de OR 'molecular epidemiology'/de OR 'endemic disease'/de OR 'infection'/de OR 'mixed infection'/de OR 'concurrent infection'/de OR 'Human immunodeficiency virus infection'/de OR 'virus infection'/de OR 'virus hepatitis'/de OR 'genotype'/de OR 'genomics'/de OR 'molecular genetics'/de OR 'genetics'/de OR 'clinical outcome'/de OR 'liver cirrhosis'/de OR 'liver cell carcinoma'/de OR therapy/de OR 'alpha interferon'/de OR interferon/de OR 'peginterferon'/de OR 'peginterferon alpha'/de OR 'recombinant alpha2b interferon'/de OR 'recombinant alpha interferon'/de OR 'recombinant alpha interferon A'/de OR 'recombinant alpha interferon AD'/de OR 'recombinant alpha2 interferon'/de OR 'recombinant alpha2a interferon'/de OR 'recombinant alpha2b interferon'/de OR 'recombinant alpha2c interferon'/de OR 'alpha interferon'/de OR 'alpha interferon A'/de OR 'alpha interferon A-D'/de OR 'alpha interferon C'/de OR 'alpha interferon derivative'/de OR 'alpha1 interferon'/de OR 'alpha2 interferon'/de OR 'alpha2a interferon'/de OR 'alpha2b interferon'/de OR 'alpha2b interferon plus ribavirin'/de OR 'alpha2c interferon'/de OR 'alphan1 interferon'/de OR 'alphan3 interferon'/de OR 'peginterferon alfacon 2'/de OR 'peginterferon alpha'/de OR 'peginterferon alpha2a'/de OR 'peginterferon alpha2b'/de OR (prevalen* OR seroprevalen* OR epidemic* OR outbreak* OR epidemiolog* OR endemic* OR infect* OR coinfect* OR genotype* OR genomic* OR genetic* OR (clinical* NEAR/3 outcome) OR cirrhosis OR (('liver cell' OR Hepatocellula*) NEAR/3 carcinom*) OR therap* OR treat* OR interferon* OR peginterferon*):ab,ti) NOT ([animals]/lim NOT [humans]/lim) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) AND [english]/lim

(2) Medline Ovid

(* Hepatitis Delta Virus/ OR Hepatitis delta Antigens/ OR ((Hepatitis ADJ3 (delta)) OR Hepatitis d OR hdv OR hdag).ab,ti.) AND (Prevalence/ OR Seroepidemiologic Studies/ OR Epidemics/ OR

Epidemiology/ OR epidemiology.fs. OR Molecular Epidemiology/ OR Infection/ OR genotype/ OR genomics/ OR molecular biology/ OR Genetics/ OR Liver Cirrhosis/ OR Carcinoma, Hepatocellular/ OR therapeutics/ OR exp Interferon-alpha/ OR Interferons/ OR (prevalen* OR seroprevalen* OR epidemic* OR outbreak* OR epidemiolog* OR endemic* OR infect* OR coinfect* OR genotype* OR genomic* OR genetic* OR (clinical* ADJ3 outcome) OR cirrhosis OR ((liver cell OR Hepatocellula*) ADJ3 carcinom*) OR therap* OR treat* OR interferon* OR peginterferon*).ab,ti.) NOT (exp animals/ NOT humans/) NOT (letter* OR news OR comment* OR editorial* OR congres* OR abstract* OR book* OR chapter* OR dissertation abstract*).pt. AND english.la.

(3) Cochrane CENTRAL

(((Hepatitis NEAR/3 (delta)) OR Hepatitis-d OR hdv OR hdag):ab,ti) AND ((prevalen* OR seroprevalen* OR epidemic* OR outbreak* OR epidemiolog* OR endemic* OR infect* OR coinfect* OR genotype* OR genomic* OR genetic* OR (clinical* NEAR/3 outcome) OR cirrhosis OR ((liver-cell OR Hepatocellula*) NEAR/3 carcinom*) OR therap* OR treat* OR interferon* OR peginterferon*):ab,ti)

(4) China Knowledge Resource Integrated databases

“hepatitis delta virus”

Summary

Databases	**References**	**De-duplicated**
embase.com	2690	2657
Medline Ovid	2420	268
Cochrane CENTRAL	151	93
China Knowledge Resource Integrated databases	574	500
Total	**5835**	**3518**

We conducted a systematic review and meta-analysis of the published studies for the prevalence and clinical outcome of HDV infection from EMBASE, Medline Ovid, and Cochrane Database. The first search was designed and conducted by an experienced medical librarian on May 1st, 2018, and only targeted English publications without time restriction. But we then found that the available English publications of China were mainly from Taiwan, and the prevalence of HDV may be different from the mainland and Taiwan. Thus, additional search was performed (by Z.M.) in China Knowledge Resource Integrated database for the studies published in Chinese language with term “hepatitis delta virus”.

Abstracts from conferences were included in our search. All search records were downloaded and duplicate records were removed. Finally, the reference lists of included articles were manually searched. All publications were updated to February 2019.

2. Definition

(1) Hepatitis D virus prevalence: The antibodies to delta antigen are regarded as diagnostic markers used for routine HDV screening (immunoassay), whereas the nucleic acid is an indicator of active infection and measurement of treatment response (1). HDV prevalence was represented by the detection of serological markers, mostly were the total antibodies to delta antigen, or anti-HDV IgG and anti-HDV IgM using immunoassay, and sometimes may be supplemented by the additional detection of HDAg and HDV RNA.

(2) Coinfection and superinfection: Coinfection is HDV and HBV simultaneously infect an individual or HDV infects the individual at the early stage after HBV infection. The crucial diagnostic marker of this pattern is represented by positive HBsAg and high-titer of IgM anti-HBc antibodies. Superinfection is HDV infection in the individual who has already established chronic HBV infection (i.e. HBsAg positive carrier), and anti-HBc IgM antibodies are absent in this pattern. The diagnosis of acute delta hepatitis was determined by the presence of serum delta antigen (HDAg) and/or IgM or total anti-HD.

3. Studies selection

Inclusion was restricted to human epidemiological studies in which testing for HDV serological markers was performed and longitudinal observational studies that reported outcomes of HDV. Exclusion was studies that solely tested for the presence of HDV RNA and studies with samples from liver tissue (for prevalence estimate only) as well as other studies (virology research, reviews and meta-analyses) containing no primary data. Abstracts from conferences were excluded unless clear information and result were reported.

4. Subjects grouping

Level one: population

1-1 General population

1-2 HBsAg positive carriers

Level two: group

2-1 Blood donors (volunteers and commercial donors)

2-2 General group (Children, students, pregnant women, rural and urban residents, villagers and in/out-patients who hadn't been further diagnosed)

2-3 Intravenous drug users (IDUs)

2-4 People with high-risk sexual activity (commercial sex workers; men with histories of sexual contact with sex workers; men who have sex with men)

2-5 HIV patients

2-6 HCV patients (anti-HCV positive)

2-7 People with blood transfusion

2-8 Mixed patients (haemophilia, hemodialysis, transplant, refugees and immigrants)

2-9 Patients who had been clinically and/or histopathologically diagnosed with liver disease (defined as acute hepatitis, AH; fulminant hepatitis, FH; chronic hepatitis, CH; liver cirrhosis, LC; hepatocellular carcinoma, HCC)

2-10 Asymptomatic HBV carriers

Level three: subgroup from HBV patients with liver disease

3-1 Acute hepatitis, AH

3-2 Fulminant hepatitis, FH

3-3 Chronic hepatitis, CH (including chronic persistent hepatitis and chronic active hepatitis)

3-4 Cirrhosis (compensated or decompensated)

3-5 Hepatocellular carcinoma, HCC

Study subjects were classified either as general population (HBV infection status is unknown) or HBsAg positive carriers (HBV infection status is known), and further divided into different groups. In general, 1-1 General population including groups 2-1 to 2-5, and groups 2-7 to 2-9; 1-2 HBsAg positive carriers including groups 2-1 to 2-10. Group 2-9 Patients who had been clinically and/or histopathologically diagnosed as specific liver disease including subgroups 3-1 to 3-5 (here is specially HBV positive). For level two grouping, we excluded studies done solely in patients with treatment and studies done solely in population with several risk factors exposure concomitantly, for example, IDU-HIV, IDU-liver disease, and HIV-liver disease. But for level three, HBV patients diagnosed with liver diseases, superimposed or combined diagnosis were classified into both subgroups, for example, cirrhosis with HCC were grouped as cirrhosis and HCC separately.

5. Data extraction items (column header)

Reference number

Reference year

Study type: retrospective or not

Publication date

Sample enrolment date

Sample type: sample from patients' sera, autopsy, or liver tissue (biopsy samples or formalin-fixed and paraffin-embedded liver biopsy samples)

Country

Region

Assay methods

Manufacturer

Level one: 1-1 General population and 1-2 HBsAg carriers

Level two: 2-1 to 2-9 (see above)

Level three: 3-1 to 3-5 (see above)

Samples-General population

Samples-HBsAg positive

HDV positive

HDV RNA positive

HDV genotype

Comparison

Control samples-HBsAg positive

Control-HDV positive

Male samples-HBsAg positive

Male-HDV positive

Female samples-HBsAg positive

Female -HDV positive

In order to standardize the extracted data, a template sheet including above research items deemed relevant was created using Microsoft Excel. Then, the investigators added the results to the sheet per template style. Except above listed items, for longitudinal observational studies we also recorded other information such as observation time and outcome of the progression. All extracted data were stratified according to the population and group.

6. Score system for quality assessment of the studies

For quality assessment, we developed an scoring system (2, 3). Because we had excluded the studies only tested HDV RNA and studies with samples from liver tissue (biopsy samples or formalin-fixed and paraffin-embedded liver biopsy samples). Thus all the studies remained were received a score of ten as a default, then a score deduction was made according to the following four items: Retrospective study (-2); Sample year unknown (-3); Assay methods not reported (-3); Manufacturer not stated (-2). After this done, each study got a final score ranging from two to ten, finally only high-quality studies with score above seven (including seven) were included for meta-analysis. We performed sensitivity analyses to assess the effects of study quality and data source. Firstly, we excluded all the studies with score bellow ten to see the effect of low-quality studies. Besides, we also excluded the studies published in Chinese language to see the effect of Chinese publications. Finally, we excluded all the data of China to see the effect of China.

7. The estimates of HDV prevalence among general population and HBsAg positive carriers

Because HDV co-infects with HBV, this provides two ways to estimate the HDV burden, either based on general population or HBsAg positive carriers. However, each way has its disadvantages.

(1) Minor adjustment for estimating HDV prevalence among general population. For general population, because HDV infection is extremely low among general group but high among risk groups, such as IDU, HIV patients and patients with liver disease, the inclusion of all groups will lead to overestimate (4-7). To solve it, only representative populations composed by general group (72%) and blood donors (28%) were included. However, the available epidemiological data of HDV were very limited for many countries. For some countries, there were only one or two studies available. To avoid bias, we added the data either from the risk groups (HIV patients, mixed patients, or patients with liver disease) or from the low-quality studies to the countries that with only one or two studies included. Importantly, this minor adjustment did not make a difference for the global pooled prevalence.

(2) The selection of the reference of HBV burden for estimating HDV burden among HBsAg positive carriers. For HBsAg positive carriers, unlike that of general population, the prevalence of HDV among HBsAg positive carriers is much higher. Thus the inclusion of other risk groups shall not lead to bias. On the contrary, only all inclusion could faithfully restore the actual burden of HDV, or the pooled results shall greatly underestimate the burden. Therefore, all the groups were included for estimating HDV burden among HBsAg positive carriers. And most importantly, over 70% participants were composed by general group, blood donors, and asymptomatic carriers, whereas patients with liver disease and the other high risk groups (IDU, HIV patients, HCV patients, mixed patients, people with sexual activity and people with blood transfusion) only accounting for about 21% and 9%, respectively, which is very similar to the actual situation. However, the reported HBV burden varies widely from 250 million to 500 million (8). The improper reference of HBV burden will lead to HDV burden estimate bias. To solve this, according to previous research about the situation of HBV global burden estimates cited in published literature and combining the recently published estimate of HBV burden, we finally selected the most convincing and frequently cited data as the reference of HBV burden, that is 367 million (8, 9).

Discussion

1. The accurate estimation of HDV burden and the heterogeneity

HDV infection is an ever forgotten issue, and its global prevalence has not yet been adequately defined. A recent global study assumed that the global burden of HDV had substantially increased from previous estimate of 15-20 million to current 62-72 million (5). Most recently, the updated estimate even climbs up to 74 million when combined the data published between January 2017 to February 2019 (4). However, our results show that 48-60 million people are infected with HDV worldwide. The discordance might be attributable to the underestimate of the early study but overestimate of the recent study. The early estimates were retrieved from a narrative review, which underestimated the prevalence rate based on the limited data published between the 1980s and 1990s (10). On the contrary, the recent study overestimated the prevalence due to the main limitations regarding methodology and the data source based on, as criticized on the previous studies (6, 7).

In the present study, we employed two methodological approaches to estimate HDV prevalence based on general population and HBsAg positive carriers. For the general population, to avoid overestimation that might be caused by high risk patients (e.g., HIV patients, IDUs, and patients with live diseases), we only included the representative populations (composed by 72% general group and 28% blood donors) to pool the prevalence, followed by minor adjustment. As a consequence, the prevalence decreased from 1.32% to 0.80%, parallels with the more recent assumption (0.82%) (7). Additionally, the minor adjustment eliminated some national bias to some extent. A good example is that the prevalence in Nigeria greatly decreased from 6.81% to 2.09%, which finally leading to Nigeria as the third-ranked country with heavy HDV burden, ranking behind China and India which are the major reservoirs of HBV infection (9). However, for HBsAg positive carriers, we included all the groups reflecting the actual composition among HBV-positive populations and chose a proper HBV burden reference of 367 million to faithfully restore the burden of HDV (9).

The limited number and origin of the samples may cause bias in national estimation. Because of the limited sample number, overestimation may present in the general population from these countries such as Colombia (1703), Nigeria (1419), Pakistan (2076), Tunisia (750) and Uganda (358), compared with the estimations among HBV carriers (Supplementary Table 1). Besides, the limited origin of the samples among general population may also lead to overestimation in country like Brazil. Among the general population in Brazil, 27.8% of the samples are from western region (State of Acre), 31.8% from central Amazon region (The State of Amazonas, Brazilian Amazon, western Amazon and Amazonas), and 40.4% from eastern region (Maranhão). No data is available from the big cities in the southeast of the country such as Sao Paolo or Rio. However, the situation is better among HBV carriers with more data source come to available. Among HBV carriers, the samples were collected from almost the whole country, and especially more data are available from southeast of the country such as Maranhão, Sao Paulo, Mato Grosso do Sul and Mato Grosso. Thus, the national estimation of HDV infection is balanced by the estimations among general population and HBV carriers. For example, the infection of HDV in Brazil is around 147000 to 2147000 although with wide range (Supplementary Table 1). For more accurate estimate, future epidemiological studies are required in Brazil, especially among general population. Thus, comparing with the recent studies of reporting the global prevalence of HDV, our study presents a more accurate estimate that verified by two ways.

Estimating the global prevalence of HDV infection is a difficult task, because the lack (or insufficient) of data around the world. We applied relatively more strict inclusion and exclusion criteria which may lead to considerable heterogeneity. In this study, the heterogeneity is mainly from variations in study quality, data source and study population. Firstly, although we have performed quality assessment for all the studies, we finally included relatively high-quality studies with score above seven (including seven) due to the lack of "absolute" high-quality studies with score ten. Secondly, we only included publications in English for other countries, but included the literatures published in English and in Chinese language for China. This greatly enriched the available data of China thus avoid the estimate bias of HDV prevalence in China. However, for other non-English countries, the same work could not be done because of language barrier and resource limitation. Thirdly, because the huge difference of HDV prevalence among general population and HBV carriers, we proposed different strategies regarding the inclusion of population for estimating HDV prevalence among general population and HBV carriers. Especially for the general population, only representative populations composed by general group (72%) and blood donors (28%) were included but followed by minor adjustment, which then included some data either from the risk groups (HIV patients, mixed patients, or patients with liver disease) or from the low-quality studies. Thus, we employed a random-effects model to calculate the pooled HDV prevalence due to the heterogeneity arising from these variations, and excluded the small sample bias. As a result, our conducts not only showed minor effects on overall prevalence estimates, but also eliminated some national bias to certain extent, such as China and Nigeria, however, increased the heterogeneity at the same time. Most importantly, some estimates were balanced by the independent estimates among general population and HBV carriers. These approaches have helped us to generate more accurate estimate of HDV global prevalence than the previous studies.

2. The distinct outcomes result from the different infection patterns of HDV

HDV can spread either via coinfection or superinfection with HBV, which may lead to distinct outcomes (11). Coinfection mostly leads to the eradication of both agents, whereas the majority of patients with HDV superinfection evolve to persistent infection and hepatitis. For coinfection, the simultaneous or primary acute infection of both HBV and HDV may trigger the strong immune response of the host to defend the infection (12). As a result, both viruses fail to establish fruitful infection and are cleared by the host (13). However, for superinfection, the prior infection of HBV has led to the impairment and/or exhaustion of the immune system, which then is incapable to defeat the subsequent infection of HDV (14). On the other hand, chronic HBV infection is always nonproductive but has continuous HBsAg translation concomitantly. Therefore, HDV takes this great advantage for the production of infectious particles to invade new hepatocytes thus establish stable infection.

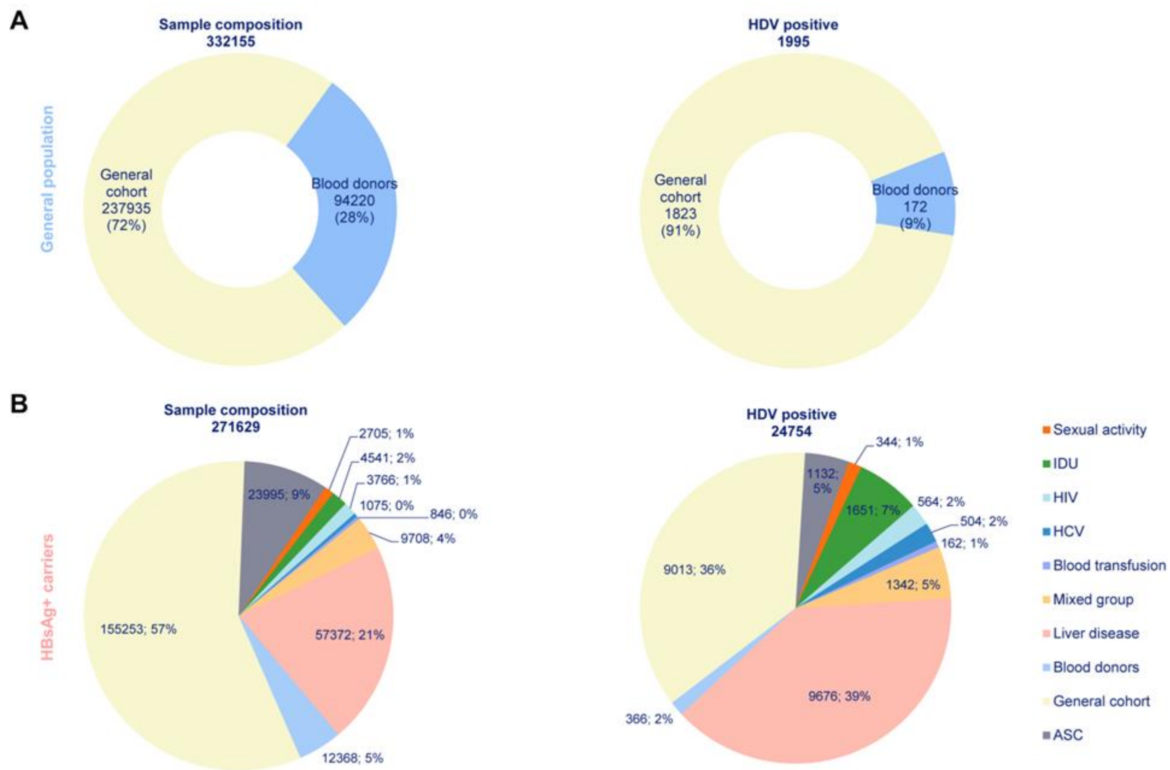
3. The suppressive effect of HDV on HBV

When chronic infection established in patients, during the long term course, the infection activity of HBV and HDV present as three patterns. The majority is HDV replication dominant, a small proportion is HBV-HDV codominant, whereas only minority is HBV replication dominant. One reason leading to this outcome is that chronic infection of HBV is inactive, but HDV is not. Because HDV infection can induce the production of both type I and type III interferons (IFN-β and IFN-λ), as well as interferon-stimulated genes (ISGs) (12). These interferons and ISGs subsequently inhabit HBV replication. However, HDV is resistant to self-induced innate immune responses (12, 13). Another reason is that HDV viral proteins can suppress HBV replication. Previous in vitro studies showed that both S- and L-HDAg proteins can repress HBV enhancers 1 and 2. S-HDAg strongly inhibits HBV mRNA synthesis or stability, whereas L-HDAg can transactivate the IFN-α-inducible MxA gene to inhibit HBV replication (14). Such effects are also reflected by the outcomes of the interaction between HBV and HDV. The positivity of HBeAg and HBV DNA (indicators of HBV replication) among HDV positive patients are lower compared with those of HDV negative, well-supported by the protective ORs.

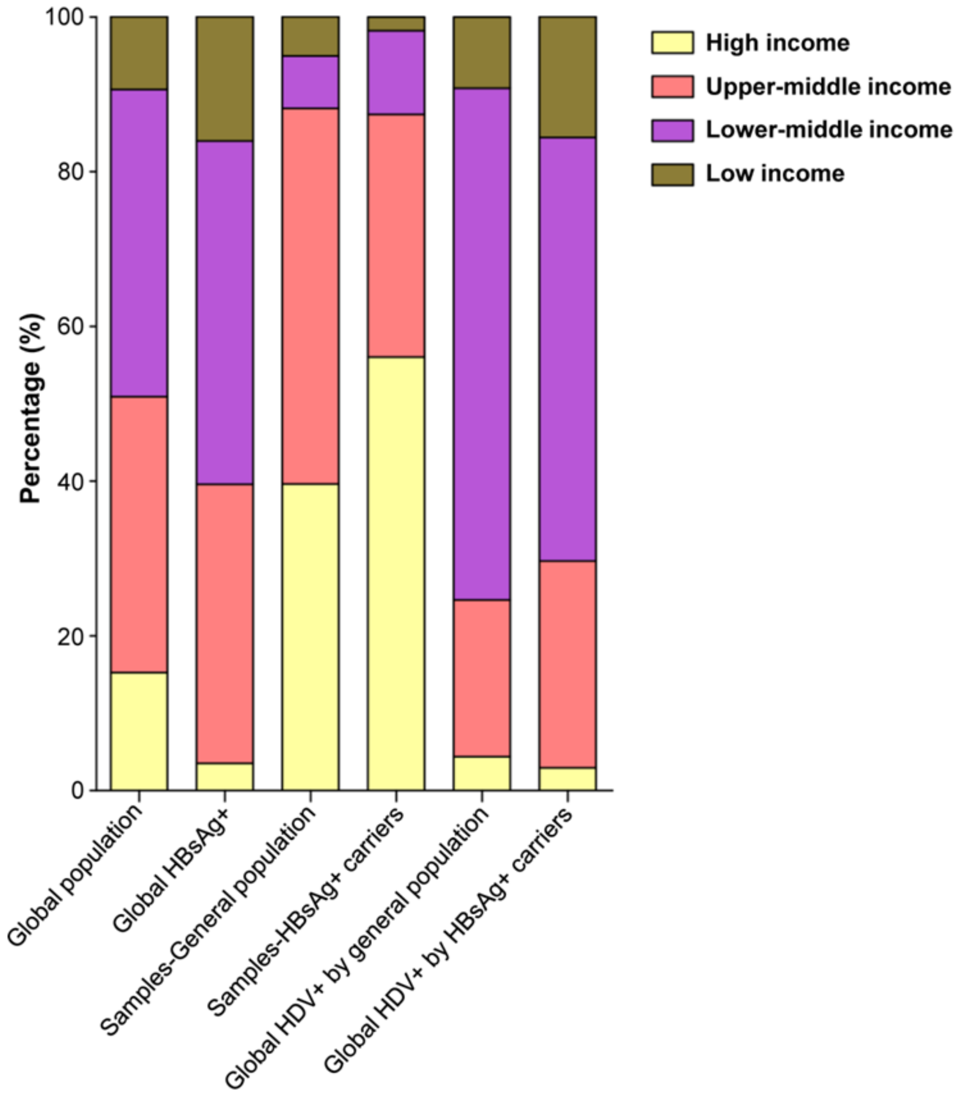
4. The rapid development of liver diseases after HDV infection

HDV infection is a risk for the development of liver diseases. Firstly, among acute patients, HDV infection rate is extremely high in patients with fulminant hepatitis compared with that of acute hepatitis. This may be explained by the cytopathic effect of HDV replication. HDV has been shown to impair cell proliferation, to induce cell cycle arrest or even cell death (14). Recent study also showed that HDV S-HDAg could inhibit glutathione S-transferase P1 (GSTP1) expression by directly binding to GSTP1 mRNA, leading to accumulation of cellular reactive oxygen species (ROS), and finally increased cellular apoptosis (15). Thus, hepatic cell death induced by HDV infection may contributes to fulminant hepatitis at the acute stage. Secondly, among chronic HBV patients, the infection rate of HDV is low in asymptomatic carriers, but high in symptomatic patients, especially patients with cirrhosis and HCC. Furthermore, HDV-HBV co-infected patients are more likely to develop cirrhosis than HBV mono-infected patients (40.50% vs. 14.22%). This results in more cirrhotic patients than asymptomatic carriers among HDV co-infected population (14.99% vs. 38.85%), conversely, more asymptomatic carriers than cirrhotic patients among HBV mono-infected population (57.20% vs. 14.36%). HDV replication has been reported to increase TGF-β and c-Jun induced signal transductions by the isoprenylation of L-HDAg. Notably, TGF-β is a fibrogenic cytokine that plays a major regulatory role in liver fibrosis and cirrhosis (16, 17). Particularly, L-HDAg can synergistically activate hepatitis B virus X (HBx)-mediated TGF-β and c-Jun signaling cascades, which may then lead to the acceleration of fibrogenesis (17). This may partially explain that why HDV infected patients rapidly progress to cirrhosis mostly within five years, which is more rapid than HCV or HBV infection alone (18, 19). Taken together, these results show that HDV replication is strongly associated with the development of more severe liver diseases, especially the rapid progression to cirrhosis.

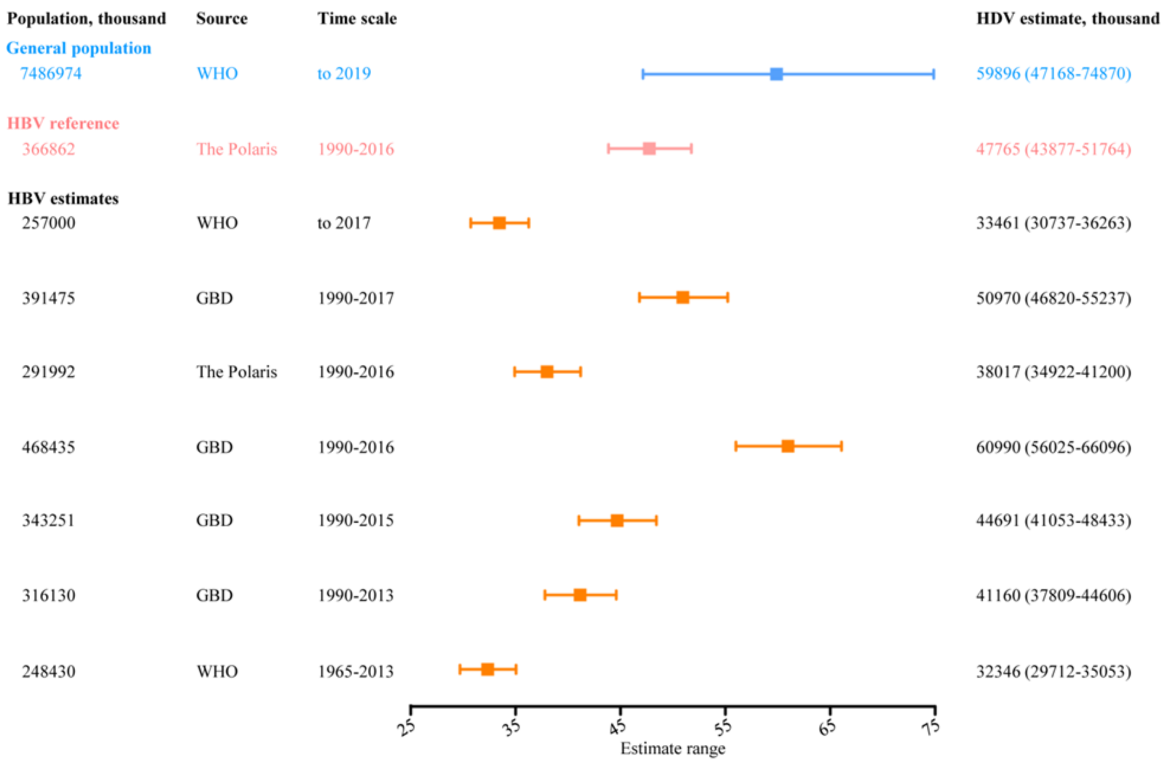
Supplementary Figures



Supplementary Figure 1. Dataset composition of general population (A) and HBsAg positive carriers (B) for the pooled prevalence of HDV.



Supplementary Figure 2. Data source analysis among World Bank region.



Supplementary Figure 12. HDV estimate refer to different HBV burden reference (9, 20-25). World Health Organization, WHO; Global Burden of Diseases, GBD.

Supplementary Figures 3-11 and Supplementary Tables 1-4 are provided in the online Supplementary Data file (https://academic.oup.com/jid/article/221/10/1677/5645271).

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Chapter 4

Revisiting the estimation of hepatitis D global prevalence

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TO THE EDITOR

We read the meta-analysis on global prevalence of hepatitis D virus (HDV) by Stockdale et al. with interest (1, 2). They estimated HDV prevalence as 4.5% among the general hepatitis B virus (HBV) carriers and 16.4% among carriers attending hepatology clinics. They only translated the rate of 4.5% as the prevalence of 0.16% in the global population corresponding to 12 million people worldwide. This topic has been extensively studied and emphasized historically by different research teams **(Table 1)**. But the authors have largely avoided to present their study in this existing context. Here we have several concerns regarding their study design and findings (3).

Firstly, the authors only included data from 1998 to 2019. Omission of studies prior to 1998 would result in great loss of valuable information (2). The authors may justify their exclusion of old studies, but it would be better to objectively present the evolving dynamics of HDV burden by including those data.

Secondly, they only included studies reporting total or IgG anti-HDV antibody, but excluded studies detecting markers for acute infection or viral replication including anti-HDV IgM antibody, viral antigen or viral RNA as an initial test. Anti-HDV IgG antibody indicates previous exposure of HDV, while the other markers indicate acute or active infection. Therefore, all these markers were used to define HDV infection (1, 2, 4). Unnecessary exclusion of studies would further aggravate the already shortage of available data on HDV epidemiology

Most importantly, the included populations are insufficient to represent the global population. In total, they included 24,024 general HBV carriers and 88,201 hepatology clinic populations. These probably only represent 20% of the global population. Particularly, less than half of the general HBV carriers are actually from the general community, but over one third of them are composed by blood donors. The prevalence of HDV in blood donors is expected to be very low, but would be much higher among risk carriers, such as intravenous drug users and carriers with liver diseases (1, 2). Thus the low prevalence data from general HBV carriers are insufficient to represent HDV overall prevalence.

Furthermore, the general HBV carriers are not the major reservoir of HDV burden. HBV carriers attending hepatology clinics are more likely have liver diseases. HDV prevalence in this population would be much higher compared to the general or asymptomatic HBV carriers (1). Moreover, most of HDV positive patients are symptomatic, and thus prone to attending hepatology clinics (1). Therefore, HBV carriers with liver diseases are the large contributor to HDV burden. This study interpreted HDV prevalence among HBV population by only using data from the general HBV carriers, thereby likely underestimating the prevalence rate. In fact, their estimates of 4.5% and 16.4% could only specifically reflect the prevalence of HDV among general HBV carriers and carriers with liver diseases, respectively.

Table 1. Historical estimates of regional and global prevalence and burden of HDV infection

Study [a]	**Time**	**HDV prevalence** [b]				**Estimate (million, M)**
		General population	**HBV carriers**			
			Liver disease	**General**	**Mixed**	
J R Torres R.1996 [9]	To 1996				5.00%	300,000
Stephanos.1997 [8]	To 1996				5.00%	15 M
Heiner et al.2010 [7]	To 2010					15-20 M
Stockdale et al.2017 [6]	1995-2016	0.70%	15.03%	8.39%		7 M
Chen et al.2018 [2]	1977-2016	0.98%	12.60%	10.58%	14.57%	62-72 M
Stockdale et al.2020a [4]	1977-2016	0.82%				61 M
Shen et al.2020 [5]	1977-2018	1.00%		10.07%		74 M
Miao et al.2019 [1]	To 2019	0.80%	17.93%		13.02%	48-60 M
Stockdale et al.2020b [3]	1998-2019	0.16%	16.40%	4.50%		12 M
Proposed	**To 2019**	**~0.70%**	**16.40%**	**4.50%**	**13.02%**	**~50 M**

[a] Two are regional estimates, J R Torres R.1996 targeting South America and Stockdale et al.2017 targeting Saharan Africa, others are global estimates; 1-9, reference.
[b] HDV prevalence is defined either among general population or among HBV carriers, and the latter can be further categorized into general carriers without risk factors, carriers with liver diseases (nearly equivalent to hepatology clinic populations), and mixed carriers (all types of HBV carriers). HDV, hepatitis D virus; HBV, hepatitis B virus.

Taken together, inclusion of limited studies and selection of the low prevalence population for calculation could have caused their underestimation of HDV global prevalence (3). To better estimate HDV burden, we revisited the previous studies on this topic **(Table 1)** (1-9). We re-estimated HDV prevalence using two approaches based on the general population or HBV carriers, respectively. Among the general population, we calculated the overlap interval of the lower bound of these previous meta-analyses, but excluded the current Stockdale study due to their bias (3). This resulted in a prevalence rate of approximately 0.7% corresponding to 50 million of the global population. For HDV prevalence among HBV carriers, we recommend the previous estimation as 13.02%; because that study has comprehensively included different categories of HBV carriers (1, 10). This corresponds to 32-61 million HDV infections depending on the referred HBV burden estimates ranging from 250 to 500 million (1).

In summary, we have emphasized the critical concerns of the current Stockdale study (3), and revisited the estimation of HDV burden. We estimate that the global prevalence of HDV infection is approximately 50 million with prevalence of 0.7% among the general population and 13% among HBV carriers. Nevertheless, we acknowledge estimating the global burden of HDV infection is a difficult task. Only when more high quality epidemiology studies become available in the future, the burden could be more precisely estimated.

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Chapter 5

The dynamics of hepatitis delta virus prevalence and its potential association with hepatitis B virus vaccination

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ABSTRACT

Background and Aim: Intuitively, implementation of hepatitis B virus (HBV) vaccination since 1990s should have made impact on the prevalence of its satellite virus, hepatitis delta virus (HDV). However, the global dynamics of HDV prevalence and the impact by HBV vaccination thereon remain largely unknown.

Methods: We conducted a systematic review and meta-analysis with a random-effects model to pool the dynamic prevalence of HDV infection and estimated the effects of HBV vaccine.

Results: Our analysis included 361 articles reporting data of 116,130 general individuals, 83,167 non-risk and 74,127 risk HBV carriers. From 1981-1990 to 2011-2019, HDV prevalence decreased from 0.86% to 0.44% among the general population but increased from 3.46% to 5.55% among non-risk HBV carriers. HDV prevalence decreased among risk HBV carriers except carriers with liver disease. Since 1991, the growth of world population contributed to 10-20 million HDV cases, but the implementation of HBV vaccination program has prevented approximate 12 million new cases by now. Overall, HDV global prevalence increased from 45 to 53 million infections over the past four decades.

Conclusion: Our findings suggest that although HBV vaccine has substantially prevented HDV infection, current global burden remains substantial. More effective interventions are required to contain HDV transmission.

HIGHLIGHTS

- The effect of HBV vaccine implementation on the prevalence of HDV remains largely unknown.
- This study estimated a prevention of 12 million HDV cases by HBV vaccination in general population.
- HDV prevalence was increased in HBV carriers.
- The global burden of HDV remains substantial requiring enhanced awareness and interventions.

MAIN TEXT

Hepatitis delta virus (also named hepatitis D virus, HDV), the satellite virus of hepatitis B virus (HBV), exacerbates the disease burden associated with HBV infection. An early study, based on data published in the 1980s and 1990s, estimated that there were 15 million HDV infections globally, which would have represented 5% of all chronic HBV carriers at the time (1). In 2018, a study reported approximately 62-72 million HDV infections worldwide, and this number was subsequently adjusted to an even higher estimate of 74 million (2, 3). However, this estimate was challenged and arguments were brought forward that this number constituted an over-estimation (4, 5). To clarify this controversy, we have recently re-estimated the global prevalence as 48-60 million HDV infections, corresponding to 0.8% of the general population, or 13% of all HBV carriers (6). Very recently, an estimate of approximately 50 million HDV infections or a global prevalence of 0.7% in the general population was proposed (7, 8). Nevertheless, the dynamics of HDV epidemiology over the past decades remains largely obscure.

In this respect, especially the effect of HBV vaccination on HDV prevalence requires further investigation. The HBV vaccine has been available since early 1981, but the initial coverage was relatively low. By 2015, however, 180 countries included three doses of hepatitis B (HepB3) vaccine in their population-wide vaccination programs, averting 210 million new chronic HBV infections (9). Because HDV uses the HBV envelope proteins and co-infects with HBV, the implementation of HBV vaccination is expected to affect HDV infection rates as well, but the exact impact of HBV vaccination in this respect remains largely unknown. The present study investigated the global dynamics of HDV prevalence during the past four decades and assessed the impact of HBV vaccination by systematic review and meta-analysis of the contemporary body of biomedical literature.

Our search returned 3,734 records, and 361 articles met the inclusion criteria **(Fig. 1 and Supplementary Tables 1 and 2)**. Based on risk of bias assessment, we included studies with low risk scores (score > 7). We categorized the participants as general population, general HBV carriers (termed as “non-risk HBV carriers”), and HBV carriers with specific risk factors (termed as “risk HBV carriers”) including intravenous drug users (IDUs), HIV patients, mixed population and patients with liver disease **(see more details in supplementary file)**. In total, 116,130 individuals from the general population, 83,167 non-risk HBV carriers and 74,127 risk HBV carriers were finally included **(Supplementary Tables 1 to 5)**. No publication bias was observed as assessed by funnel plot and Egger's test (p>0.05, **Supplementary Fig. 1**). In order to investigate the global dynamics of HDV prevalence and its potential associations with HBV vaccination, data of HBV and HDV infections were divided into four continuous groups corresponding to the past four decades (from 1981-1990 to 2011-2019) according to the sample collection time **(Fig. 2 and Supplementary Fig. 2 to 4)**.

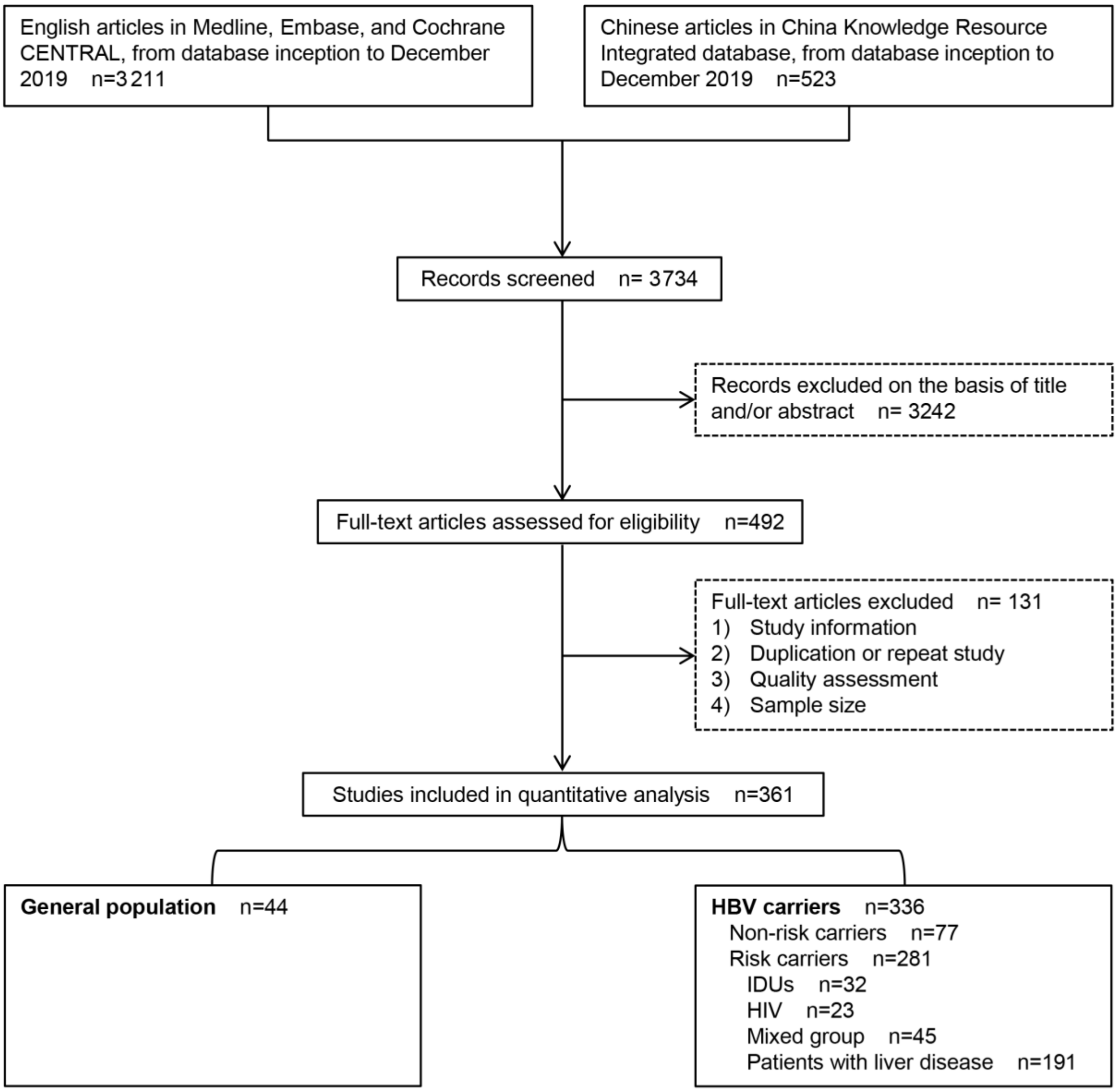

Figure 1. Flowchart of study selection. IDUs, intravenous drug users; HIV, human immunodeficiency virus.

We found, accompanied with the continuous increase of HepB3 coverage from 1981-1990 to 2011-2019, the global prevalence of HBV infection among the general population sharply

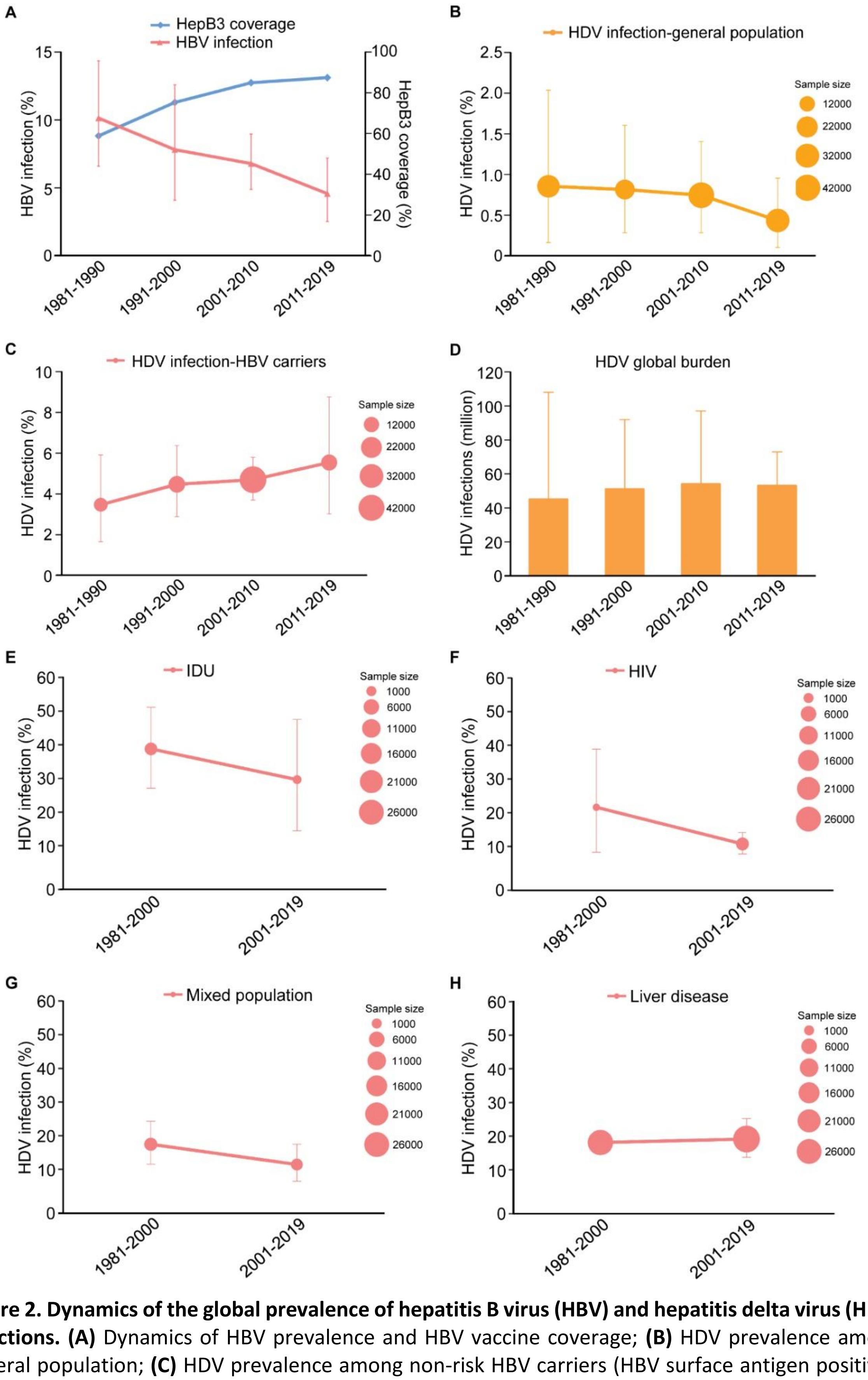

Figure 2. Dynamics of the global prevalence of hepatitis B virus (HBV) and hepatitis delta virus (HDV) infections. (A) Dynamics of HBV prevalence and HBV vaccine coverage; **(B)** HDV prevalence among general population; **(C)** HDV prevalence among non-risk HBV carriers (HBV surface antigen positive); **(D)** Global dynamics of HDV prevalence; Global dynamics of HDV prevalence among risk HBV carriers including IDU **(E)**, HIV **(F)**, mixed population related to other risk factors **(G)** and carriers with liver

disease **(H)**. IDU, intravenous drug user; HIV, human immunodeficiency virus; HDV, hepatitis delta virus; HBV, hepatitis B virus; HepB3, three doses of hepatitis B vaccine.

decreased from 10.15% (95% CI, 6.61-14.35) to 4.58% (95% CI, 2.53-7.19) **(Fig. 2A)**. The decreased HBV prevalence appears to be associated with the increase in HBV vaccine coverage (r2=0.8942, p=0.0544) **(Supplementary Figure 5A)**. During the same period, the global prevalence of HDV infection among general population moderately decreased from the initial decade in which prevalence was 0.86% (95% CI, 0.17-2.04) to the third decade in which prevalence was 0.75% (95% CI, 0.29-1.41). In the last decade the decline in HDV prevalence accelerated substantially to a prevalence rate of 0.44% (95% CI, 0.11-0.96) **(Fig. 2B)**. This decrease in HDV prevalence among the general population appears to be associated with increased coverage and implementation of HBV vaccination programs (r2=0.8675, p=0.0686) and decreased HBV prevalence (r2=0.8210, p=0.0987), although these associations did not reach statistical significance **(Supplementary Fig. 5B and 5C)**. However, HDV prevalence among non-risk HBV carriers increased from 3.46% (95% CI, 1.63-5.92) to 5.55% (95% CI, 3.01-8.80) over the past four decades **(Fig. 2C)**. Thus HBV vaccination programs appear to have substantial impact on the epidemiology of HDV infection.

At the start of the study period, when HBV vaccination was still relatively uncommon, the global estimate of HDV infection was 45 (95% CI, 9-107) million **(Fig. 2D)**. This number was further increased and peaked during 2001-2010 with about 54 (95% CI, 31–97) million HDV infections. But during the recent decade, the number slightly decreased again to 53 (95% CI, 32-73) million. These results show that HDV global burden was sustained over the past decades, even if the prevalence of HBV and HDV infections was decreased among general population following the implementation of HBV vaccination. Compared to 1980-1990, the global population has increased by 44% (from 5.3 to 7.6 billion), and this is estimated to yield about 10 to 20 million HDV cases. Relating results to population growth, our findings suggest that HBV vaccination has prevented approximately 12 million new cases of HDV infection.

For risk HBV carriers, the available data on HDV prevalence are very limited. Thus we resorted to a dichotomal analysis in which data covering two decades (1981-2000 versus 2001-2019) were contrasted **(Supplementary Fig. 6 to 9)**. The pooled prevalence of HDV among IDUs, HIV patients and the mixed population were 38.66% (95% CI, 26.99-51.01), 21.67% (95% CI, 8.30-38.90) and 20.55% (95% CI, 15.44-26.16) during the first 20 years (1981-2000), which then decreased to 29.53% (95% CI, 14.31-47.40), 10.78% (95% CI, 7.80-14.16), 10.70% (95% CI, 6.23-16.14) in the recent 20 years (2001-2019), respectively **(Fig. 2E-G)**. Strikingly, HDV prevalence among HBV carriers with symptomatic liver diseases changed upwardly from 18.02% (95% CI, 15.59-20.58) during the first period to 19.05% (95% CI, 13.61-25.15) in the second period **(Fig. 2H)**.

Table 1. The dynamics of HDV prevalence among non-risk HBV carriers by World Bank Region

World Bank Region [a]	Sample	HDV positive	HDV prevalence (CI: 95%)
1981-2000			
High income	10893	647	3.70% (2.15-5.64)
Upper-middle income	11010	419	3.46% (2.16-5.04)
Lower-middle income	1126	114	7.72% (0.05-25.58)
Total	23029	1180	N/A
2001-2019			
High income	26641	1192	3.75% (2.99-4.58)
Upper-middle income	26935	1787	5.80% (3.87-8.08)
Lower-middle income	4859	413	8.00% (3.17-14.72)
Total	58435	3392	N/A

[a] Data from the Low-Income Economies were not available. HDV, hepatitis delta virus; HBV, hepatitis B virus; CI, confidence interval; N/A, not applicable

Similarly, the available national data of HDV infection among non-risk HBV carriers are also limited, and again we resorted to contrasting two larger temporal cohorts . As shown in **Supplementary Fig. 10**, the comparison of the data between 1981-2000 to that available from 2001-2019 shows that HDV prevalence from Pakistan (from 8.86% to 20.08%), United States of America (from 14.24% to 23.55%), Germany (from 5.27% to 6.64%), France (from 0.90% to 2.21%), Iran (from 2.50% to 3.15%), and China (from 4.07% to 4.54%) all increased. In contrast, HDV prevalence decreased in the United Kingdom (from 6.84% to 5.86%), Vietnam (from 2.33% to 1.26%), Italy (from 5.56% to 3.49%) and Australia (from 6.64% to 4.31%). Regionally, HDV prevalence in the Upper-Middle-Income Economies increased (from 3.46% to 5.80%, an increase of 67%), but remained largely unchanged in High-Income Economies (from 3.70% to 3.75%, an increase of 1%) and the Lower-Middle-Income Economies (from 7.72% to 8.00%, an increase of 3.6%) **(Table 1)**. Unfortunately, data from the Low-Income Economies were not available in this study.

Of note, our study has some limitations. Firstly, the available data on HDV global prevalence are very limited, and most included studies had small sample sizes. Secondly, although no publication bias (funnel plot and Egger's test $p>0.05$) was observed, we found considerable inter-study heterogeneity (I2 > 50%). Thirdly, the diagnosis of HDV infection for most included studies was measured by enzyme immunoassay for HDV antibodies, and only few studies show data with confirmation through detecting viral RNA.

In summary, HDV prevalence among general population declined by 50%, but increased by 60% among non-risk HBV carriers over the past four decades. Implementation of HBV vaccination has already prevented around 12 million new HDV cases worldwide, but the global burden of HDV prevalence remains substantial, partially due to the growth of world population. Our findings call for enhanced awareness, screening and interventions if HDV eradication is to be achieved.

ABBREVIATIONS

HDV, hepatitis D/delta virus; HBV, hepatitis B virus; HBsAg, HBV surface antigen; WHO, World Health Organization; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HCC, hepatocellular carcinoma; HepB3, the three doses of hepatitis B vaccine; PRISMA, preferred reporting items for systematic reviews and meta-analyses; IDUs, intravenous drug users; CI, confidence interval.

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SUPPLEMENTARY METHODS AND RESULTS

Methods

1. Search term and records

(1) embase.com

('Hepatitis delta virus'/mj OR 'delta agent hepatitis'/mj OR 'hepatitis delta antigen'/mj OR ((Hepatitis NEAR/3 (delta)) OR 'Hepatitis d' OjR hdv OR hdag):ab,ti) AND ('prevalence'/de OR prevalence/de OR 'epidemic'/de OR epidemiology/de OR epidemiology: lnk OR 'epidemiological data'/de OR 'molecular epidemiology'/de OR 'endemic disease'/de OR 'infection'/de OR 'mixed infection'/de OR 'concurrent infection'/de OR 'Human immunodeficiency virus infection'/de OR 'virus infection'/de OR 'virus hepatitis'/de OR 'genotype'/de OR 'genomics'/de OR 'molecular genetics'/de OR 'genetics'/de OR 'clinical outcome'/de OR 'liver cirrhosis'/de OR 'liver cell carcinoma'/de OR therapy/de OR 'alpha interferon'/de OR interferon/de OR 'peginterferon'/de OR 'peginterferon alpha'/de OR 'recombinant alpha2b interferon'/de OR 'recombinant alpha interferon'/de OR 'recombinant alpha interferon A'/de OR 'recombinant alpha interferon AD'/de OR 'recombinant alpha2 interferon'/de OR 'recombinant alpha2a interferon'/de OR 'recombinant alpha2b interferon'/de OR 'recombinant alpha2c interferon'/de OR 'alpha interferon'/de OR 'alpha interferon A'/de OR 'alpha interferon A-D'/de OR 'alpha interferon C'/de OR 'alpha interferon derivative'/de OR 'alpha1 interferon'/de OR 'alpha2 interferon'/de OR 'alpha2a interferon'/de OR 'alpha2b interferon'/de OR 'alpha2b interferon plus ribavirin'/de OR 'alpha2c interferon'/de OR 'alphan1 interferon'/de OR 'alphan3 interferon'/de OR 'peginterferon alfacon 2'/de OR 'peginterferon alpha'/de OR 'peginterferon alpha2a'/de OR 'peginterferon alpha2b'/de OR (prevalen* OR seroprevalen* OR epidemic* OR outbreak* OR epidemiolog* OR endemic* OR infect* OR coinfect* OR genotype* OR genomic* OR genetic* OR (clinical* NEAR/3 outcome) OR cirrhosis OR (('liver cell' OR Hepatocellula*) NEAR/3 carcinom*) OR therap* OR treat* OR interferon* OR peginterferon*):ab,ti) NOT ([animals]/lim NOT [humans]/lim) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) AND [english]/lim

(2) Medline Ovid

(* Hepatitis Delta Virus/ OR Hepatitis delta Antigens/ OR ((Hepatitis ADJ3 (delta)) OR Hepatitis d OR hdv OR hdag).ab,ti.) AND (Prevalence/ OR Seroepidemiologic Studies/ OR Epidemics/ OR

Epidemiology/ OR epidemiology.fs. OR Molecular Epidemiology/ OR Infection/ OR genotype/ OR genomics/ OR molecular biology/ OR Genetics/ OR Liver Cirrhosis/ OR Carcinoma, Hepatocellular/ OR therapeutics/ OR exp Interferon-alpha/ OR Interferons/ OR (prevalen* OR seroprevalen* OR epidemic* OR outbreak* OR epidemiolog* OR endemic* OR infect* OR coinfect* OR genotype* OR genomic* OR genetic* OR (clinical* ADJ3 outcome) OR cirrhosis OR ((liver cell OR Hepatocellula*) ADJ3 carcinom*) OR therap* OR treat* OR interferon* OR peginterferon*).ab,ti.) NOT (exp animals/ NOT humans/) NOT (letter* OR news OR comment* OR editorial* OR congres* OR abstract* OR book* OR chapter* OR dissertation abstract*).pt. AND english.la.

(3) Cochrane CENTRAL

(((Hepatitis NEAR/3 (delta)) OR Hepatitis-d OR hdv OR hdag):ab,ti) AND ((prevalen* OR seroprevalen* OR epidemic* OR outbreak* OR epidemiolog* OR endemic* OR infect* OR coinfect* OR genotype* OR genomic* OR genetic* OR (clinical* NEAR/3 outcome) OR cirrhosis OR ((liver-cell OR Hepatocellula*) NEAR/3 carcinom*) OR therap* OR treat* OR interferon* OR peginterferon*):ab,ti)

(4) China Knowledge Resource Integrated databases

“hepatitis delta virus”

Summary

We conducted a systematic review and meta-analysis for the published studies on the prevalence of HDV infection from EMBASE, Medline Ovid, and Cochrane Database. The search of English publications was designed and conducted by an experienced medical information specialist (Wichor M. Bramer, Department of Medical Library, Erasmus MC-University Medical Center). The additional search of Chinese publications was performed by Z. Miao in China Knowledge Resource Integrated database with Chinese term “hepatitis delta virus”. Abstracts from conferences were included in our search. All search records were downloaded and duplicate records were removed. Our search had no time restriction, and all publications were updated to December 2019. Our search returned 3 724 non-redundant records in total, 492 of them were eligible for full-text assessment after the screening of title and/or abstract, and 361 studies were finally included in quantitative analysis.

2. Study selection and data processing

(1) Definition of hepatitis delta virus (HDV) prevalence: HDV prevalence was defined as the positive detection of HDV antibody and/or RNA. HDV antibodies (anti-HDV IgG and anti-HDV IgM) were detected by enzyme immunoassay, HDV RNA was detected by polymerase chain reaction.

(2) Definition of hepatitis B virus (HBV) prevalence: HBV prevalence was defined as the positive detection of HBV surface antigen.

(3) Study subjects grouping: Based on the detection for HBV infection status of the study cohort, study subjects were classified as either general population (unknown HBV infection status) or HBV carriers (confirmed HBV infection by positive HBsAg). General population was composed by blood donors and the general cohort without any risk factors. HBV carriers were categorized as non-risk carriers and risk carriers, non-risk carriers were composed by the general carriers, blood donors and asymptomatic carriers, risk carriers were further group as intravenous drug users (IDUs), HIV patients, Mixed population and patients with liver disease. General cohort/carriers including children, students, pregnant women, rural and urban residents, villagers and in/out-patients without severe symptoms. Mixed population are related to other risk factors like haemophilia, hemodialysis, transplant, refugees and immigrants. Carriers with liver diseases were clinically and/or histopathologically diagnosed as acute hepatitis, fulminant hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Data were grouped into four groups (1981-1990, 1991-2000, 2001-2010 and 2011-2019) or two groups (1981-2000 and 2001-2019) that cover the past four decades according to sample time.

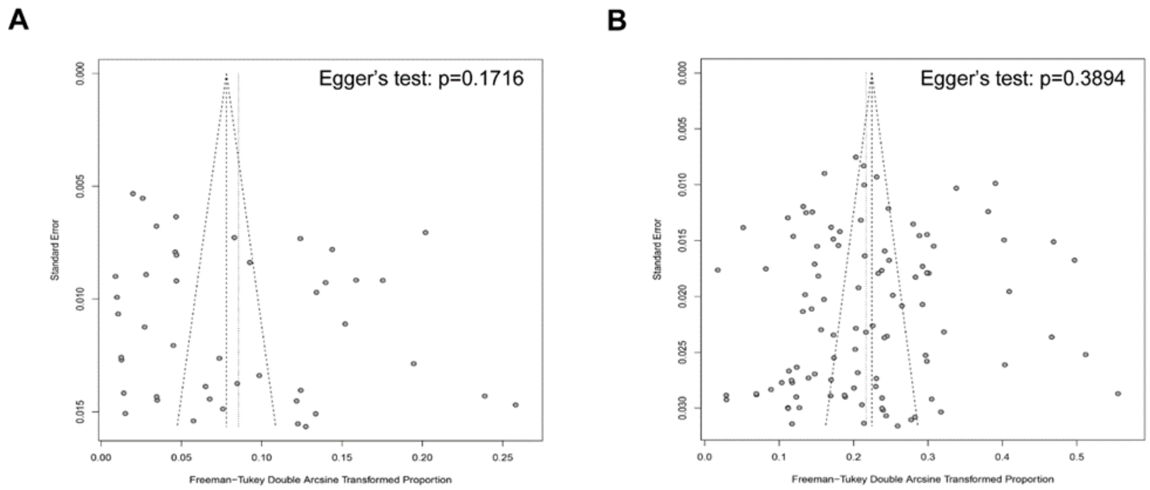
(4) Study selection and data extraction: Data extraction included study type (retrospective or perspective), the geographical information (nation and region), assay methods (detection methods for HDV antibody and/or RNA), population setting (study cohort information), recruitment time, sample size, and prevalence of HBV and HDV infections (1). Only the studies that reported HDV prevalence and had the availability to the above mentioned information were included. Studies like case reports, comments, letters, abstracts, systematic reviews/meta-analyses that provided no primary data were excluded. Duplicate data from the same location were excluded. Additional exclusions were performed based on the following selection regarding sample size and risk of bias.

HDV was an ever forgotten pathogen, and because the neglect over the past decades, the lack of prevalence data around the world was significantly observed. Besides, in our previous HDV burden estimate study, we observed a significant polarization regarding the sample size reported in HDV studies (1). In total, 115 (100%) studies including 335 270 individuals were reported, in which the sample size reported in 64 (56%) studies were below 1 000, accounting for only about 9% of the total sample, however, there were 9 (8%) studies from a single site or country reported very large sample size (over 10 000) and taking about 55% of the total sample. To avoid small sample bias in the random effects model and eliminate the effect of large sample, we only included studies with sample size range 1000-10000 for general population, 250-5000 for non-risk HBV carriers, and a minimum of 20 for risk HBV carriers. What is more, we performed Newcastle-Ottawa Scale (a specific item regarding sample time was added to this system to make full score reach 10) to assess risk of bias, and only studies with low risk of bias (score>7) were finally included (2, 3, 4). We evaluated the publication bias by funnel plot and Egger's test, and performed I2 statistical analysis to access the inter-study heterogeneity (5, 6). Although our selection aggravated the shortage of HDV available data, the estimate bias caused by small sample and the effect of large sample were greatly eliminated, which in turn ensured the congruence of our subgroup analyses and finally generated the more credible results.

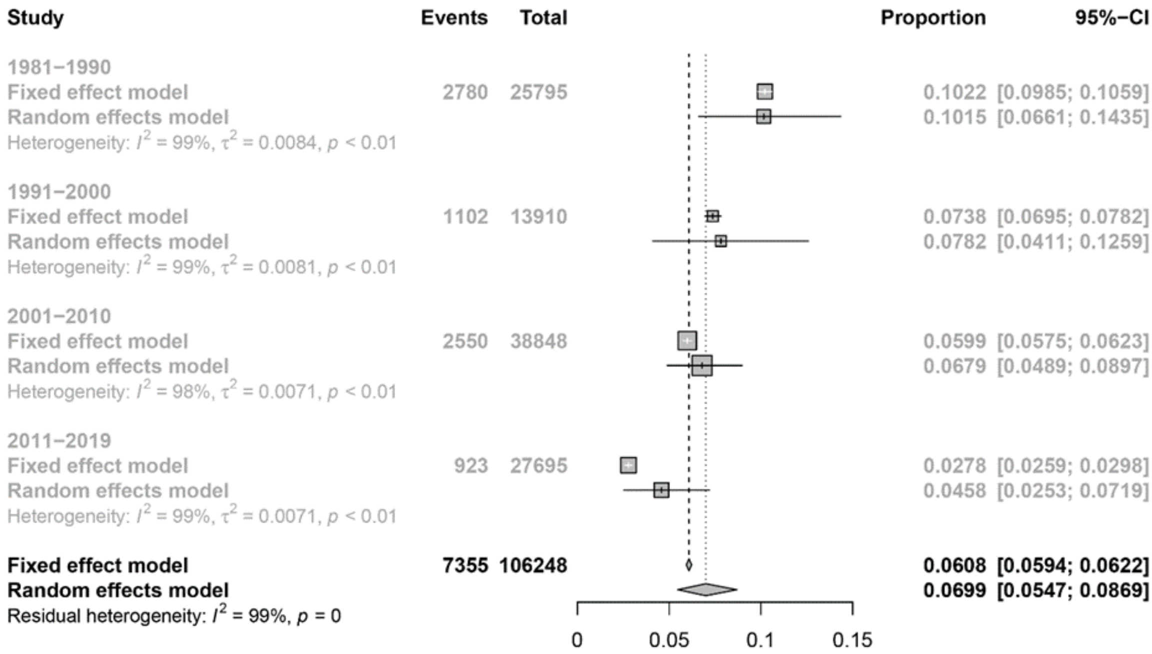
Statistical analysis

To calculate the pooled prevalence, DerSimonian-Laird random-effects model with Freeman-Tukey double arcsine transformation was used. The 95% confidence interval (CI) was estimated by Wilson score method. To avoid small sample bias in the random effects model and eliminate the effect of large sample that from a single site or country, we only included studies with sample size range 1000-10000 for general population, 250-5000 for non-risk HBV carriers, and a minimum of 20 for risk HBV carriers. Meta-analyses were done in the R-3.4.2 statistical software package (Metaprop module), and linear regression analyses were done in GraphPad Prism (version 5). Detailed information regarding materials, methods and related references are provided in the online supplementary file. This study was reported in accordance with the preferred reporting items for systematic reviews and meta-analyses (PRISMA).

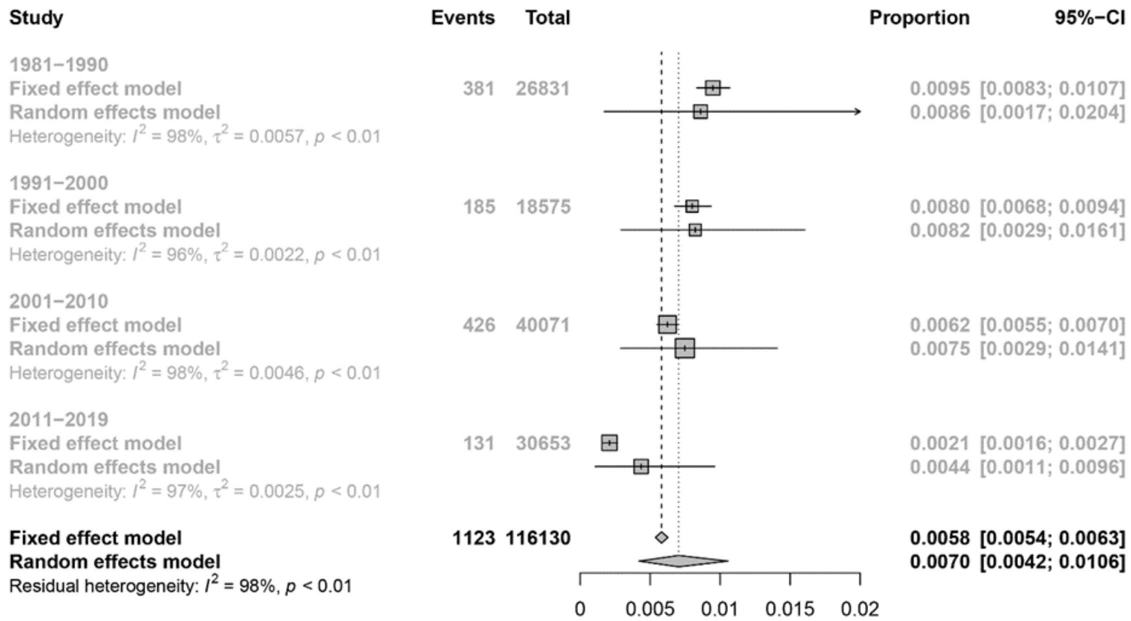
Supplementary Figures



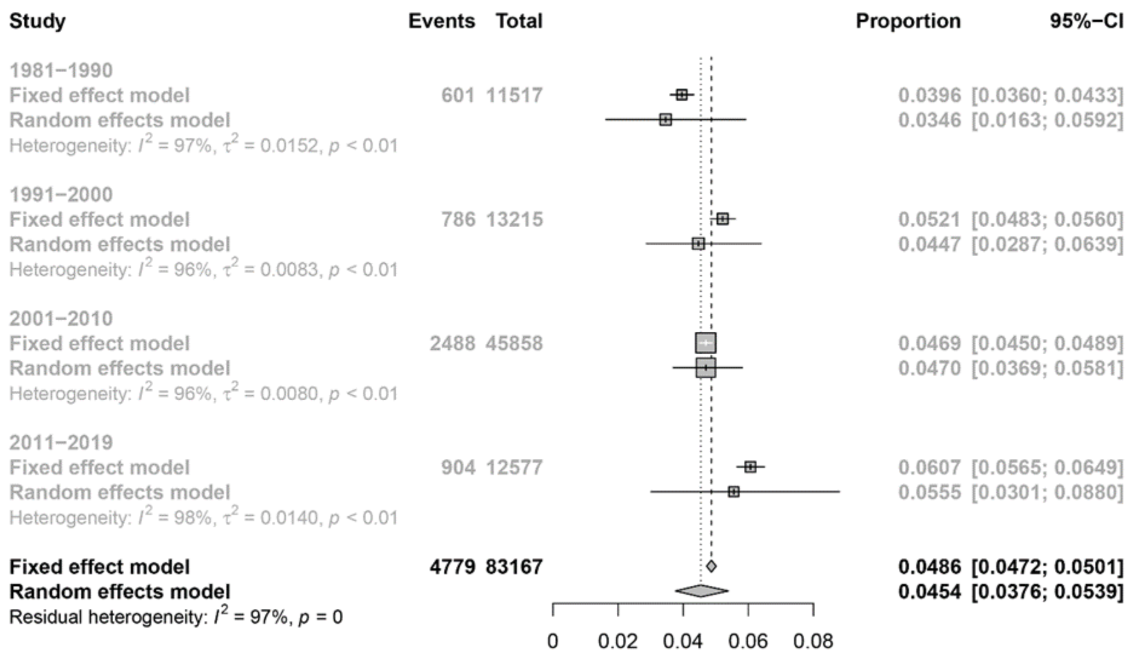
Supplementary Figure 1. Funnel plot of pooled prevalence of HDV infection. (A) General population; **(B)** Non-risk HBV carriers.



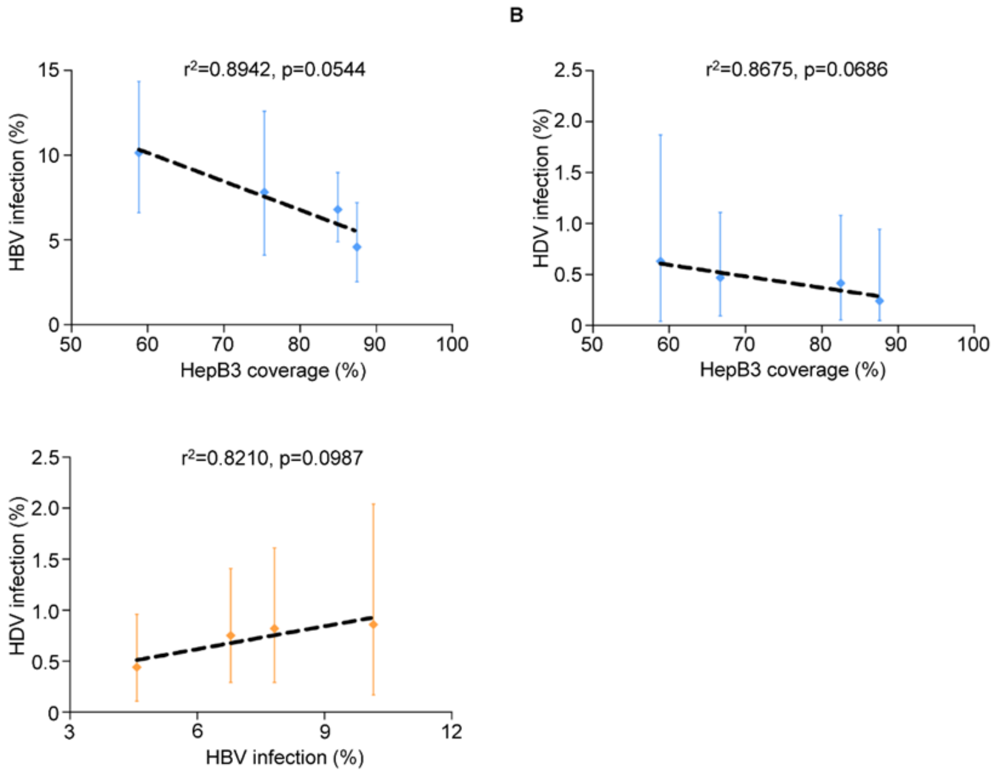
Supplementary Figure 2. Forest plot showing meta-analysis of HBV prevalence among general population. CI, confidence interval.



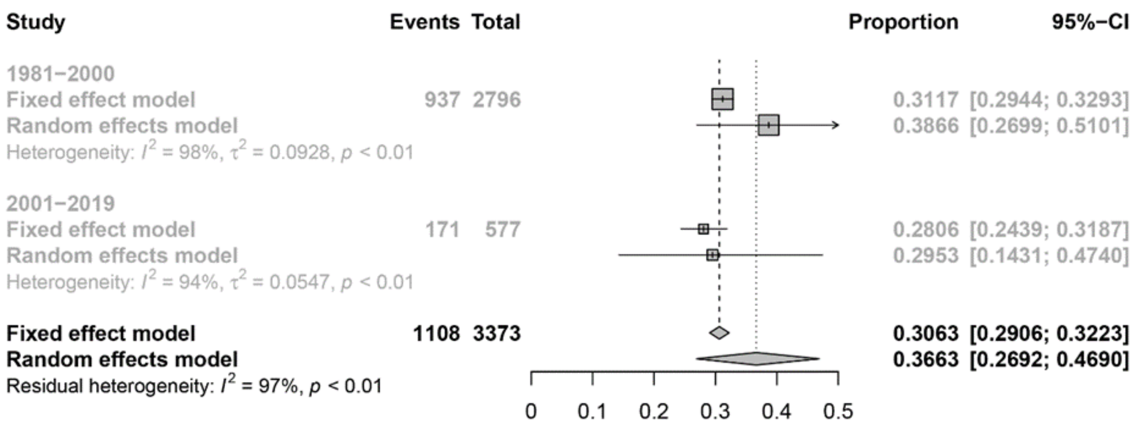
Supplementary Figure 3. Forest plot showing meta-analysis of HDV prevalence among general population. CI, confidence interval.



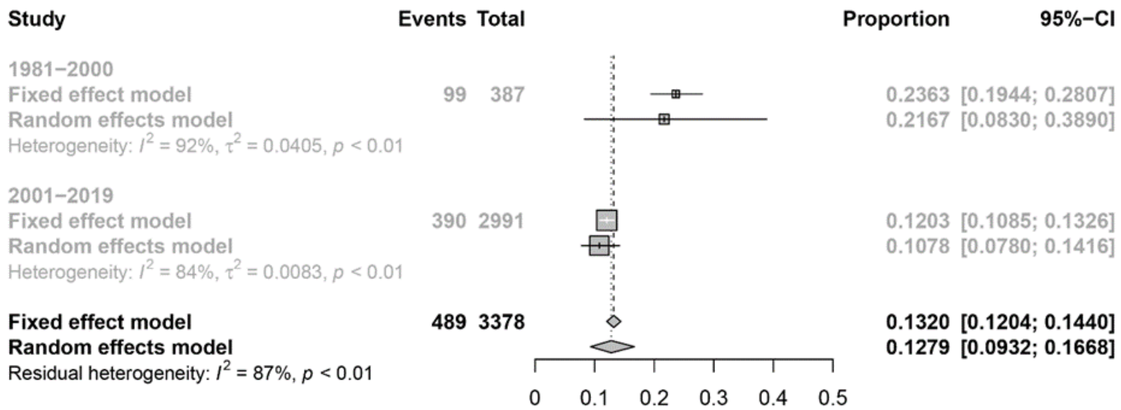
Supplementary Figure 4. Forest plot showing meta-analysis of HDV prevalence among non-risk HBV carriers. CI, confidence interval.



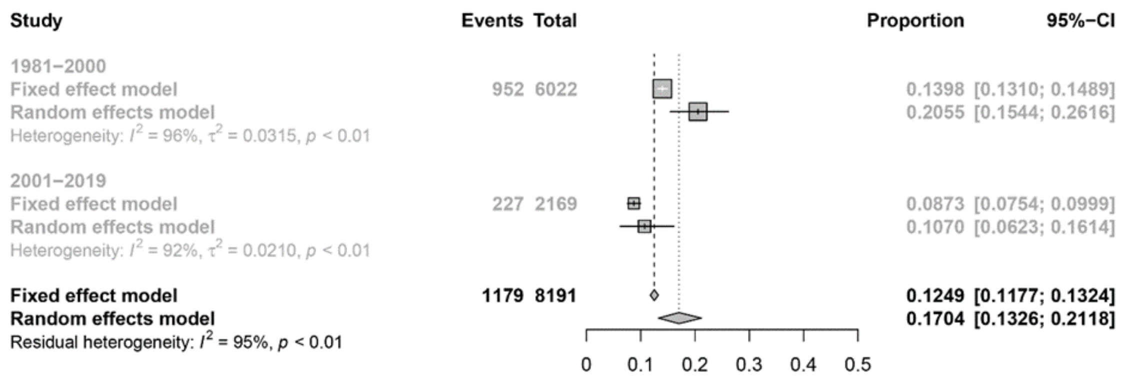
Supplementary Figure 5. Associations between the infections of HBV and HDV and HBV vaccination. (A) HBV infection-HepB3 coverage; **(B)** HDV infection-HepB3 coverage; **(C)** HDV infection-HBV infection. HepB3, three doses of hepatitis B vaccine.



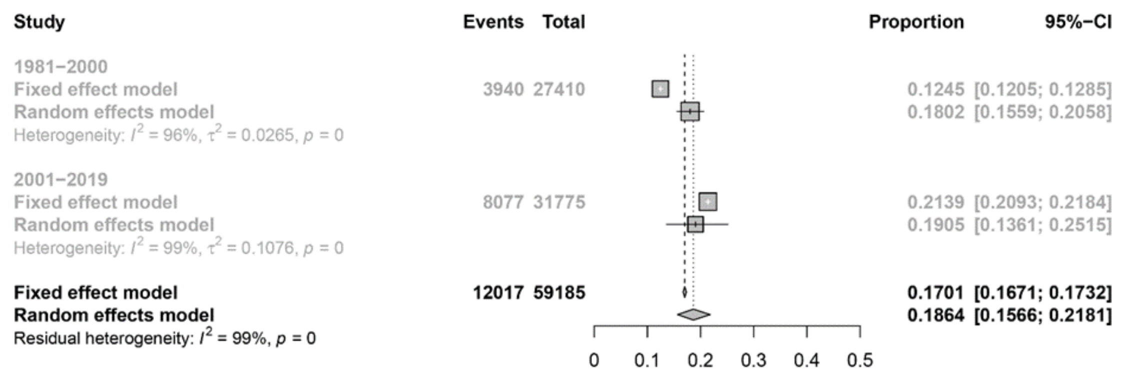
Supplementary Figure 6. Forest plot showing meta-analysis of HDV prevalence among risk HBV carriers related to intravenous drug using. CI, confidence interval.



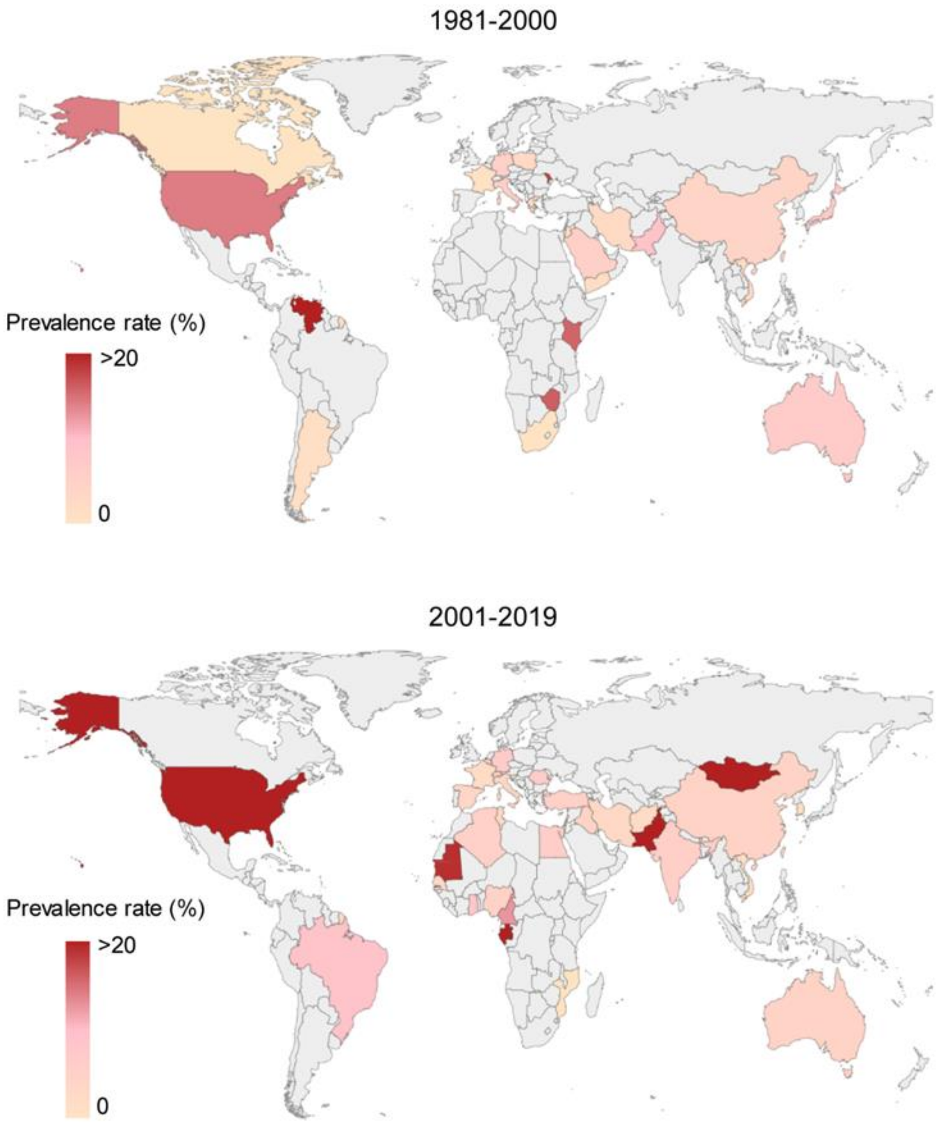
Supplementary Figure 7. Forest plot showing meta-analysis of HDV prevalence among risk HBV carriers related to HIV infection. CI, confidence interval.



Supplementary Figure 8. Forest plot showing meta-analysis of HDV prevalence among risk HBV carriers related to mixed factors. CI, confidence interval.



Supplementary Figure 9. Forest plot showing meta-analysis of HDV prevalence among risk HBV carriers related to symptomatic liver disease. CI, confidence interval.



Supplementary Figure 10. Global hepatitis delta virus prevalence among hepatitis B virus (HBV) carriers (1981-2000, 2001-2019). Blank means no HDV epidemiological data available.

More detailed information are provided in the online Supplementary Data File (https://www.sciencedirect.com/science/article/pii/S2210740121000565?via%3Dihub).

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Chapter 6

The macrolide antibiotic azithromycin potently inhibits hepatitis E virus in cell culture models

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International Journal of Antimicrobial Agents. 2021 Jun 19:106383.

ABSTRACT

Hepatitis E virus (HEV) infection in immunocompromised patients, pregnant women and children requires treatment, but no approved-medication is available. We identified macrolide antibiotic azithromycin as a potent HEV inhibitor. Azithromycin inhibits HEV replication and viral protein expression in multiple cell culture models with genotype 1 and 3 strains. This is largely independent of its induction of interferon-like response. Because of safe and cheap, repurposing azithromycin for treating HEV infection is attractive particularly in resource-limited settings.

KEYWORDS

Azithromycin; Hepatitis E virus; Interferon; Pregnant women

HIGHLIGHTS

- Azithromycin, a FDA pregnancy category B drug, has potent anti-HEV activity.
- The antiviral effect of azithromycin has been confirmed in multiple cell culture models with genotype 1 and 3 HEV.
- Azithromycin activates interferon-like response, but the anti-HEV activity is independent of this innate immune response.
- Repurposing azithromycin for treating HEV infection is attractive particularly in resource-limited regions.

MAIN TEXT

Hepatitis E virus (HEV), as a small non-enveloped, single-stranded RNA virus, is the most prevalent cause of acute viral hepatitis worldwide. Although HEV infection in healthy individuals are usually asymptomatic, immunocompromised patients infected with genotype 3 or 4 HEV bear high risk of developing chronic hepatitis (1, 2). Pregnant women infected with genotype 1 HEV can develop severe complications such as acute liver failure, miscarriage, preterm delivery, stillbirth and perinatal mortality, resulting in high mortality approaching 15-25% (3). Occurrence of maternal-foetal transmission can seriously affect foetal/neonatal outcomes, including anicteric or icteric hepatitis, hypoglycaemia and neonatal death (3). Since no FDA-approved treatment is available, ribavirin monotherapy has been used as an off-label treatment for some chronic hepatitis E cases (3). However, ribavirin therapy is contraindicative in pregnancy due to teratogenicity (4). Therefore, there is an urgent clinical need for developing new anti-HEV therapies, and repurposing existing medications represents an expedited approach. We recently identified several potential anti-HEV candidates by screening a safe-in-human broad-spectrum antiviral agents library (5). Azithromycin, a commonly used macrolide antibiotic, attracted our great interest because it is classified as a FDA pregnancy category B drug. In this study, we aim to comprehensively evaluate the antiviral activity of azithromycin in genotype 1 and 3 HEV models and explore the potential mechanism-of-action.

To test the potential anti-HEV activity of azithromycin, we first applied a genotype 3 HEV based subgenomic replicon model (p6-Luc), in which HEV replication can be monitored by Gaussia luciferase activity. Azithromycin treatment time- and dose-dependently inhibited viral replication related luciferase activity in the human hepatic Huh7 cells harboring the p6-Luc replicon **(Fig. 1A)**. Treatment with 10 μM or 50 μM azithromycin for 72 hours resulted in 78.66%±3.06 (mean±SEM, n=12, $p<0.0001$) and 88.03%±1.17 (mean±SEM, n=12, $p<0.0001$) inhibition of luciferase activity, respectively. The calculated 50% inhibition and cytotoxicity concentrations (IC50 and CC50) were 4.2 μM and 42.9 μM, respectively, indicating a substantial therapeutic window **(Fig. 1B)**. We further investigated the effect in Huh7 cells harboring a genotype 1 HEV replicon, and significant inhibition of viral replication was also found **(Fig. 1C)**. These results demonstrated that azithromycin can inhibit the replication of both genotype 1 and 3 HEV in cell culture models.

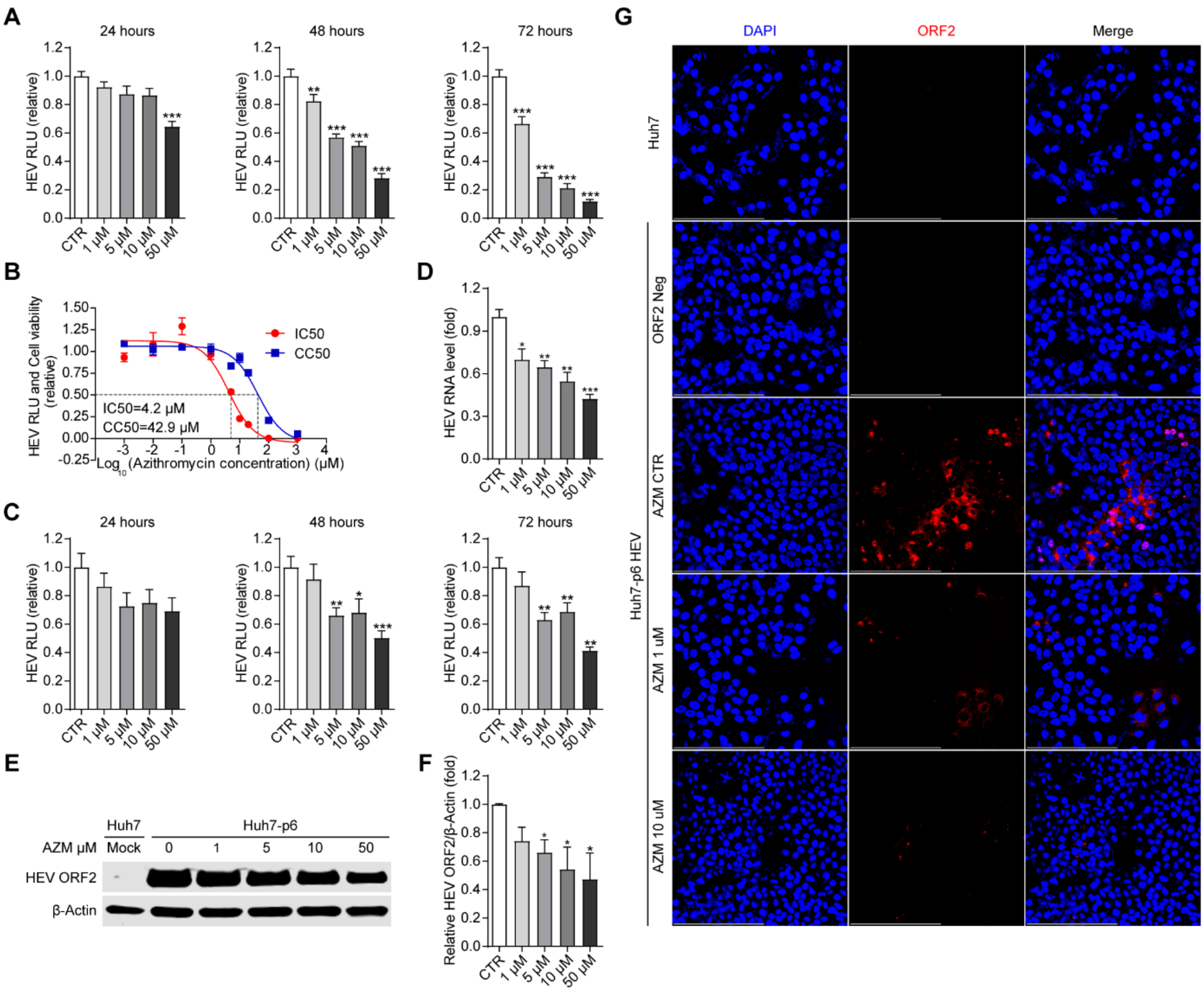

Fig. 1. Anti-HEV activity of azithromycin in Huh7-based cell culture models. (A) The effects of various concentrations of azithromycin (AZM) on HEV in Huh7 cell based genotype 3 replicon model (Huh7-p6-Luc). HEV replication was measured by Gaussia luciferase activity. Data were normalized and compared to the untreated control group (set as 1) (n=12). **(B)** The 50% inhibitory and cytotoxic concentration (IC50 and CC50) of azithromycin calculated by using GraphPad Prism 5 software (n=6-12). **(C)** The effect of azithromycin on genotype 1 HEV replication (n=7). **(D)** Quantification of viral RNA by qRT-PCR in Huh7 cells harboring the p6 HEV infectious model that were treated with azithromycin for 48 hours(n=6-8). **(E and F)** Western blot images and quantification of HEV capsid ORF2 protein level in Huh7-p6 cells treated with azithromycin for 48 hours. The uninfected group (mock) and the infected but untreated group (CTR: AZM=0 μM) serve as negative and positive control, respectively. β-actin serves as an internal reference (n=4). **(G)** Immunofluorescence analysis of viral protein ORF2 (red) in Huh7-p6 cells treated with indicated concentrations of azithromycin for 48 hours. Naïve Huh7 cells incubated with the HEV ORF2 antibody and Huh7-p6 cells incubated with the matched IgG control antibody serve as the negative controls. Untreated Huh7-p6 cells and incubated with the HEV ORF2 antibody serves as the positive control. DAPI (blue) was applied to visualize nuclei. (40 × oil immersion objective). Data are presented as means ± SEM. RLU, relative luciferase unit. (*P < 0.05; **P < 0.01; ***P < 0.001).

To further confirm the inhibitory effect of azithromycin on HEV full-length genome replication and viral protein expression, we used HEV infectious full-length cell model (Huh7-p6). In Huh7-p6 cells, treatment with azithromycin for 48 hours effectively inhibited HEV at viral RNA level determined by RT-qPCR **(Fig. 1D)** and HEV ORF2 protein level measured by western blotting **(Fig. 1E and Fig. 1F)**. The inhibitory effect was finally visualized within host cells by immunofluorescence. Azithromycin treatment effectively inhibited HEV ORF2 expression, and the inhibition is remarkable even at the low concentration (1 μM) and much stronger at higher concentration (10 μM) **(Fig. 1G)**. Notably, both concentrations could not completely inhibit viral protein expression. Altogether, azithromycin dose-dependently inhibited HEV genome replication and viral protein expression.

Although primarily causing hepatitis, HEV infection is also associated with a broad range of extrahepatic manifestations, particularly kidney injury and neurological diseases (6, 7). Thus, we further profiled the anti-HEV activity of azithromycin in a variety of cell models, including hepatic and non-hepatic cell lines harboring the p6-Luc replicon. As expected, azithromycin significantly inhibited HEV replication-related luciferase activity in human kidney 293T-p6-Luc **(Fig. S1A)**, neuronal U87-p6-Luc **(Fig. S1B)** and hepatic PLC-p6-Luc **(Fig. S1C)** models. We determined the IC50 and CC50 concentrations of azithromycin in 293T cells as 9.3 μM and 56.8 μM, respectively. These results indicate that the anti-HEV effect of azithromycin is universal across different cell types.

Emerging evidence suggests that the antiviral activity of azithromycin against particular viruses may be associated with host innate immune response (8, 9). Interferon signaling provides the first-line innate defense against viral infections, and the anti-HEV activity of interferon-alpha (IFN-α) has been demonstrated in cell culture models and indicated in treated chronic HEV patients (10). Binding of IFN-α to its receptor initiates the Janus kinase signal transducer and activator of transcription (JAK-STAT) cascade to recruit the ISGF3 complex, which subsequently binds to IFN-stimulated response element (ISRE) in the nucleus to transcribe interferon-stimulated genes (ISGs) (11). We previously have shown the anti-HEV agents ribavirin and mycophenolic acid (MPA) are capable of activating ISG transcription, although they do not trigger interferon production (12, 13). We found azithromycin treatment dose-dependently activated the transcriptional activity of ISRE-based luciferase reporter, although to a less extent compared to IFN-α stimulation **(Fig. 2A)**. Consistently, azithromycin treatment significantly induced the expression of several ISGs, including MDA5, OAS3, MX1, ISG15 and STAT2, by 1-6 fold in Huh7 cells regardless infected with HEV or not **(Fig. 2B)**. However, the effect on the transcription of RIG-I, STAT1 and TLR-2 is mild.

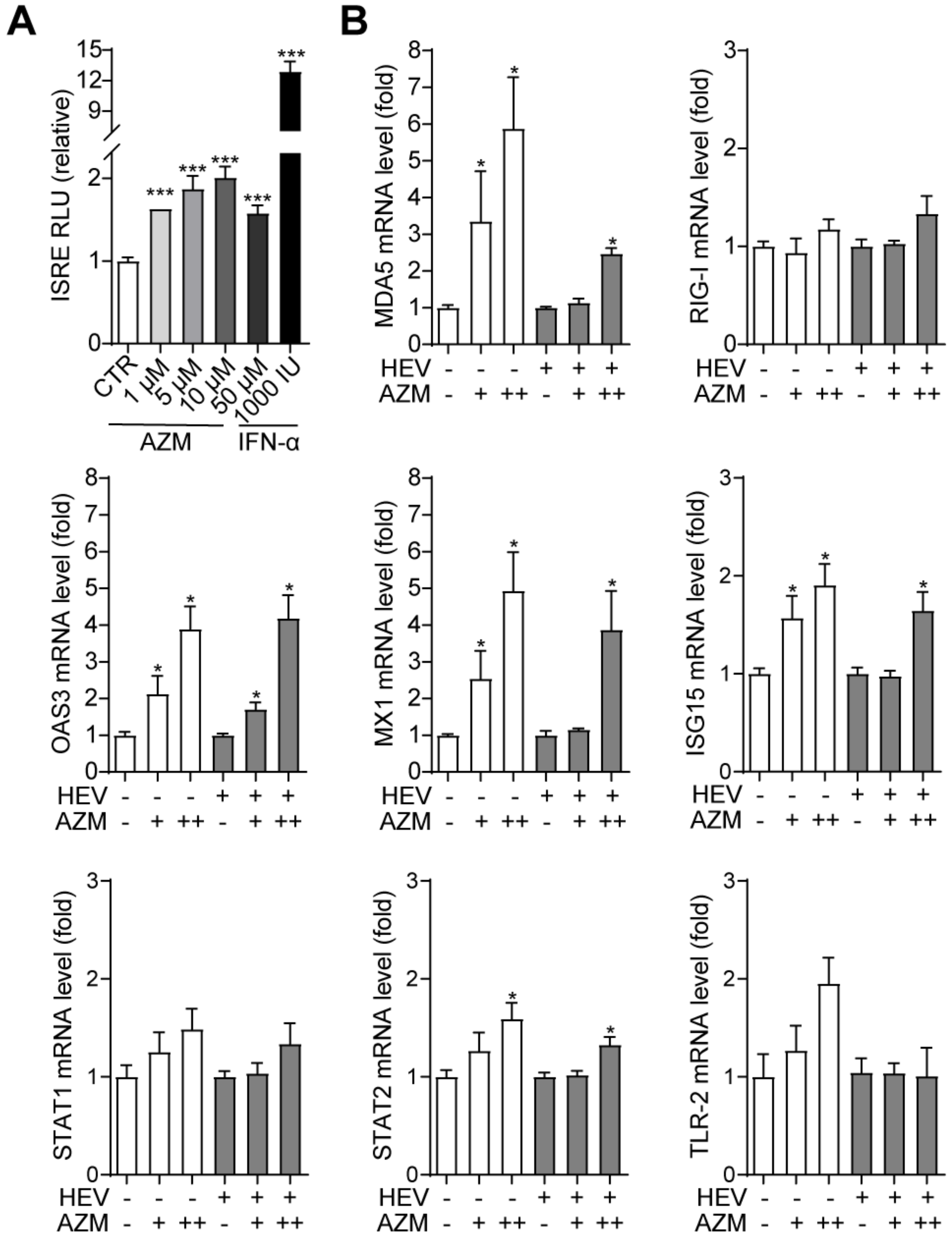

Fig. 2. Azithromycin triggers innate immune response. (A) Analysis of ISRE related firefly luciferase activity in Huh7-ISRE-Luc cells treated with various concentrations of azithromycin (AZM) or IFN-α (1000 IU/Ml, positive control) for 48 hours. Data were normalized and compared to the untreated control group (set as 1) (n=8). **(B)** Analysis of azithromycin induced interferon (IFN)-stimulated genes (ISGs). The mRNA levels of ISGs were measured by RT-qPCR in Huh7 cells treated with azithromycin (10 and 50 μM) for 48 hours with or without HEV infection. Relative gene expression was normalized to the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the 2-ΔΔCt

method. Data were normalized and compared to the untreated control in Huh7 and Huh7-p6 cells respectively (set as 1) (n=4). Data are presented as means ± SEM. MDA5, melanoma differentiation-associated protein 5; RIG-I, retinoic acid-inducible gene I; OAS3, 2'-5'-oligoadenylate synthetase 3; MX1, MX dynamin like GTPase 1; ISG15, interferon-stimulated gene 15; STAT1/2, signal transducer and activator of transcription 1/2; TLR2, Toll-like receptor 2. (*P < 0.05; **P < 0.01; ***P < 0.001).

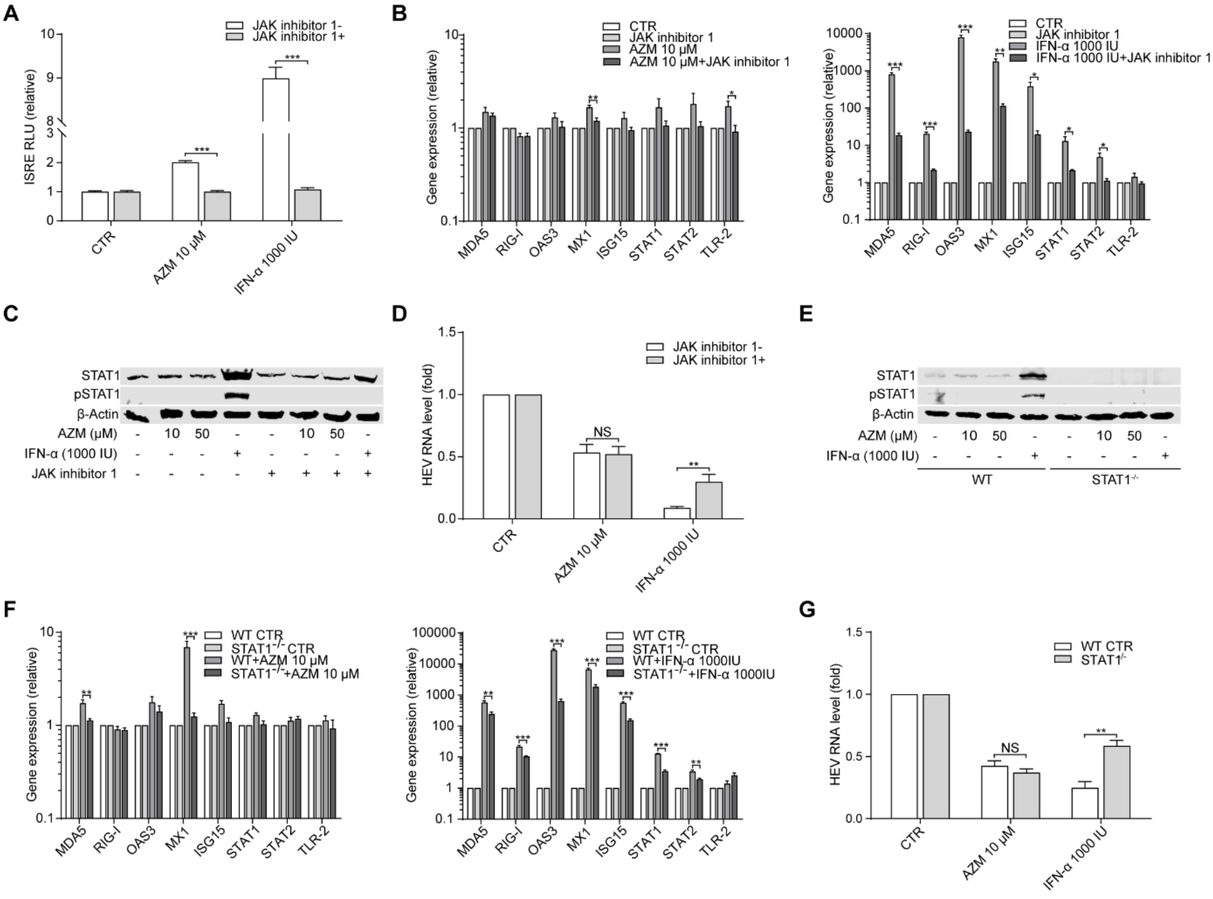

Fig. 3. The effect of azithromycin induced non-canonical interferon-like response on its anti-HEV activity. **(A)** Comparison of ISRE related firefly luciferase activity in Huh7-ISRE-Luc cells after combined treatment of JAK inhibitor 1 (10 μM) with azithromycin (AZM, 10 μM) or IFN-α (1000 IU/Ml, positive control) for 48 hours. Data were normalized to the azithromycin untreated group and JAK inhibitor 1 only group respectively (set as 1) (n=10). **(B)** Comparison of ISGs gene transcription in Huh7 cells after combined treatment for 48 hours. The mRNA levels of indicated ISGs were quantified by qRT-PCR and relative gene expression was normalized to the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the 2-ΔΔCt method (n=4). **(C)** Western blot analysis of total STAT1 or phosphorylated STAT1 (pSTAT1) expression in Huh7 cells after combined treatment for 48 hours. β-actin serves as an internal reference (n=4). **(D)** Comparison of HEV RNA quantification in infectious Huh7-p6 cells after combined treatment for 48 hours. Intracellular relative HEV RNA was measured by qRT-PCR (n=6). **(E)** Western blot analysis of STAT1 and phosphorylated STAT1 (pSTAT1) expression in wild type (WT) and STAT1 knockout ($STAT1^{-/-}$) Huh7 cells after at 48 hours. β-actin serves as an internal reference (n=4). **(F)** Comparison of ISGs gene transcription in WT and $STAT1^{-/-}$ Huh7 cells at 48 hours. Data were normalized to the untreated WT group and $STAT1^{-/-}$ group respectively (set as 1) (n=6). **(G)** Comparison of HEV RNA quantification in WT and $STAT1^{-/-}$ Huh7 cells. Huh7 cells were infected with HEV for 24 hours followed by azithromycin treatment for 48 hours, then intracellular relative HEV RNA was measured by qRT-PCR (n=6). Data are presented as means ± SEM. RLU, relative luciferase unit; NS, not significant; IFN-α, type I interferon α; MDA5, melanoma differentiation-associated protein 5; RIG-

I, retinoic acid-inducible gene I; OAS3, 2'-5'-oligoadenylate synthetase 3; MX1, MX dynamin like GTPase 1; ISG15, interferon-stimulated gene 15; STAT1/2, signal transducer and activator of transcription 1/2; TLR2, Toll-like receptor 2. (*P < 0.05; **P < 0.01; ***P < 0.001).

As expected, blocking the function of Janus kinases by JAK inhibitor 1 largely attenuated IFN-α induced ISRE activation, ISG transcription, STAT1 phosphorylation, and anti-HEV activity **(Fig. 3A-D)**. Surprisingly, although JAK inhibitor 1 affected azithromycin-induced ISRE activation and ISG transcription, it has no effect on the anti-HEV activity of azithromycin **(Fig. 3A, 3B and 3D)**. Of note, azithromycin hardly activates STAT1 phosphorylation, or to a mild level that was undetectable by western blotting assay **(Fig. 3C)**. We next investigated the potential involvement of STAT1 by using STAT1 knockout Huh7 cells **(Fig. 3E)**. Loss of STAT1 significantly attenuated the induction of ISGs and anti-HEV activity of IFN-α **(Fig. 3F and 3G)**. But again, STAT1 knockout had no effect on the anti-HEV activity of azithromycin, although attenuated the induction of ISGs **(Fig. 3E-G)**.

Similar to our observations, previous studies in different viral infection models, including respiratory syncytial viruses, rhinovirus, influenza virus, and ZIKA virus (8, 9, 14), have indicated potential activation of IFN response by azithromycin. However, the overall antiviral activity of azithromycin is not affected by the absence of type I IFN (9), functional inhibition of Janus kinases **(Figs. 3F-D)**, or knockout of IFNAR1 (9) or STAT1 **(Figs. 3E-G)**. Interestingly, ISGs such as pathogen recognition receptors (MDA5 and RIG-I) and the ones (OASs, MXs, and ISG15) functionally related to RNA and protein metabolism were frequently found to be upregulated by azithromycin (8, 9). Overall, azithromycin appears to activate interferon (like) response, but whether this actually contributes to the broad antiviral activity remains largely unclear.

Importantly, as an FDA pregnancy category B drug, azithromycin has been clinically proven to be safe in pregnant women, newborns and young children (15). Furthermore, azithromycin also possesses anti-inflammatory and immunomodulatory properties (16). Severe HEV infections in pregnant women universally accompany with massive inflammatory response (17). Thus, azithromycin could be an idea therapeutic candidate for treating HEV infected pregnant women that can simultaneously inhibit the infection and pathogenic inflammation. Animal study showed that azithromycin can accumulate in the lung and liver, leading to more than 100-fold enrichment in these tissues than the bloodstream (18). Furthermore, pharmacokinetic studies in human showed that it not only accumulates in brain and placenta of the mother but also can be transported to fetal tissue through amniotic fluid and umbilical cord plasma, reaching final concentrations from 2.8 to 21 μM (9, 19). These concentrations are comparable to or even higher than the IC50 of azithromycin demonstrated in our HEV models **(Fig. 1 and Fig. S1)**, indicating that the concentrations of azithromycin in maternal and fetal tissues should be sufficient to inhibit HEV replication. Because azithromycin is cheap and widely available in oral and intravenous formulations. Repurposing for treating HEV infection would be highly accessible for all patients including from resource-limited regions. However, combination of azithromycin with the known anti-HEV agents IFN-α **(Fig. S2A)**, ribavirin **(Fig. S2B)** or MPA **(Fig. S2C)** resulted in antagonistic effects.

In summary, this study has demonstrated azithromycin as a potent inhibitor of HEV replication in cell culture models. Although azithromycin is capable of moderately activating a non-canonical interferon-like response, the anti-HEV activity is largely independent of this innate immune response and the exact mechanism-of-action requires further study. Given the great potential of azithromycin for treating a wide range of HEV patients including pregnant women and children, follow-up in vivo studies are warranted to validate our findings.

MATERIALS AND METHODS

Reagents and antibodies

Azithromycin, human IFN-α, ribavirin and mycophenolic acid were purchased from Sigma-Aldrich. The JAK inhibitor 1 (SC-204021) were obtained from Santa Cruz Biotechnology (Santa Cruz). The diluent used for azithromycin was Dimethyl sulfoxide (DMSO, Sigma) with final concentration below 0.1% for the high concentration treatment, which was confirmed in preliminary experiments not to have any effect on measured outcomes. Ribavirin, MPA and IFN-α were dissolved in 1× Phosphate-Buffered Saline (PBS). The HEV ORF2 antibody (mouse monoclonal, MAB8002) and β-actin antibody (mouse monoclonal, sc-47778) were purchased from EMD Millipore and Santa Cruz Biotechnology (Santa Cruz), respectively. Mouse Control IgG2b antibody was purchased from InvivoGen (mabg2b-ctrlm). Phospho-STAT1 (Tyr701) (58D6, Rabbit mAb, 9167) and STAT1 (Rabbit mAb, 9172) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-rabbit and anti-mouse IRDye-conjugated secondary antibodies (Li-Cor Biosciences, Lincoln, NE, USA) were also used.

Cell culture

Human hepatoma cell line (Huh7) and human glioblastoma cell line (U87) were kindly provided by the Department of Viroscience (Erasmus Medical Center). Human hepatoma cell line PLC/PRF/5 (PLC) and human embryonic kidney epithelial cell line (HEK293T) were originally obtained from ATCC (http://www.atcc.org). These cells were cultured in Dulbecco’s modified Eagle medium (DMEM) contained 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37℃ with 5% CO_2.

Viruses and culture models

Above mentioned cell lines were employed to generate HEV cell as we reported previously (5, 20). For infectious full-length cell model (Huh7-p6), Huh7 cells were electroporated with full-length HEV genomic RNA (genotype 3 Kernow-C1 p6 clone, GenBank accession number JQ679013). To generate luciferase-based replicon models (Huh7-p6-Luc, PLC-p6-Luc, U87-p6-Luc, HEK293T-p6-Luc), a plasmid construct containing subgenomic HEV sequence in which HEV capsid protein ORF2 was replaced by a Gaussia luciferase reporter gene for monitoring viral replication. HEV genotype 1 replicon model (Huh7-GT1-Luc) was based on the Sar 55/S17/luc HEV clone containing a Gaussia luciferase reporter. To produce viral RNA, the Ambion mMESSAGE nMACHINE in vitro RNA transcription kit (Thermo Fisher Scientific Life Sciences) was used. The ISRE reporter system mimicking IFN response (Huh7-ISRE-Luc) and STAT1 knockout cells (Huh7-STAT1$^{-/-}$) we used here were from our previous study, HEV virus preparation and re-infection were same as the reported methods (5, 20).

MTT assay

To perform MTT assay, 10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to the cells seeded in 96-well plate followed by 3 hours’ incubation at 37°C with 5% CO_2. Then the medium was removed and 100 µL DMSO was added to each well. After additional incubation of 15 min, the plate was transferred to the microplate absorbance readers (BIO-RAD) to detect the absorbance of each well at wavelength of 490 nm.

Quantification of HEV replication and gene expression

Viral replication in HEV replicon models was measured by the secreted luciferase activity in the cell culture medium. Luciferase activity was quantified on a LumiStar Optima luminescence counter (BMG Lab Tech) using BioLux® Gaussia Luciferase Flex Assay Kit (New

England Biolabs). For HEV infectious model, intracellular viral RNA was quantified. Total RNA was extracted with Machery-Nucleo Spin RNA II kit (Bioke) and quantified by NanoDrop ND-1000 spectrophotometer (Wilmington). The cDNA was synthesized by cDNA Synthesis Kit (Takara). Applied Biosystems SYBR Green PCR Master Mix (Life Technologies) was used to quantify HEV RNA and mRNA of inflammatory cytokines, IFNs and ISGs. Relative gene expression was normalized to the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the 2-ΔΔCt method. All the primers used in this study were provided in the online Supplementary Table S1.

Western blotting

Total protein lysates were heated at 95°C for 5 min then run on 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) at 90 V for 120 min, subsequently transferred onto polyvinylidene difluoride (PVDF) membrane (0.45 mm, Thermo Fisher Scientific Life Sciences) at 250 mA for 120 min. The membrane was incubated with blocking buffer (Li-Cor Biosciences) for 1 hour at room temperature followed by incubation with primary antibodies mouse anti-HEV ORF2 (1:1000) and anti-β-actin (1:1000) at 4°C overnight. Then the membrane was washed 3 times followed by incubation with anti-mouse (1:5000) or anti-rabbit (1:10000) IRDye-conjugated secondary antibodies (Li-Cor Biosciences) at room temperature for 1 hour. Finally, after washing 3 times, membrane was visualized using an Odyssey infrared imaging system CLx (LICOR Biosciences).

Immunofluorescence

Cells grown on µ-Slide 8 Well (ibidi GmbH, 80826) were washed once with 1× PBS and then fixed for 10 min with 4% (w/v) paraformaldehyde (PFA) at room temperature (RT). After fixation, the cells were washed 3 times with PBS and permeabilized with 0.1% (v/v) Triton X-100 for 10 min, then washed 3 times with PBS. Cells were incubated for 1 hour in blocking solution (5% Normal Donkey Serum, 1% Bovine Serum Albumin, 0.2% TRITON X in 1 × PBS) at RT. Then cells were incubated with primary anti-HEV ORF2 antibody (aa 434-457, clone 1E6, IgG2b) at a 1:200 dilution for 1 hour. The control group was incubated with the matched mouse IgG 2b antibody (InvivoGen; 1:200). The primary antibody mixture was then removed and cells were washed 3 times with PBS. The secondary Anti-mouse-Alexa Fluor® 594-Conjugate antibody (Cell Signaling Technology; 1:5000) was added and the cells were incubated an additional hour at RT. After the incubation the cells were washed 3 times and then cell nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI) for 10 min at RT. Images were taken on a Leica SP4 confocal microscope (lens: 40x), image analysis was performed using ImageJ (NIH).

Statistical Analysis

Statistical analysis was performed using the non-paired, non-parametric test (Mann-Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). All results were presented as mean ± standard errors of the means (SEM). P values <0.05 were considered as statistically significant. Detailed information regarding materials, methods as well as additional results are provided in the Supplementary Data File.

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SUPPLEMENTARY METHODS AND RESULTS

Supplementary Figures

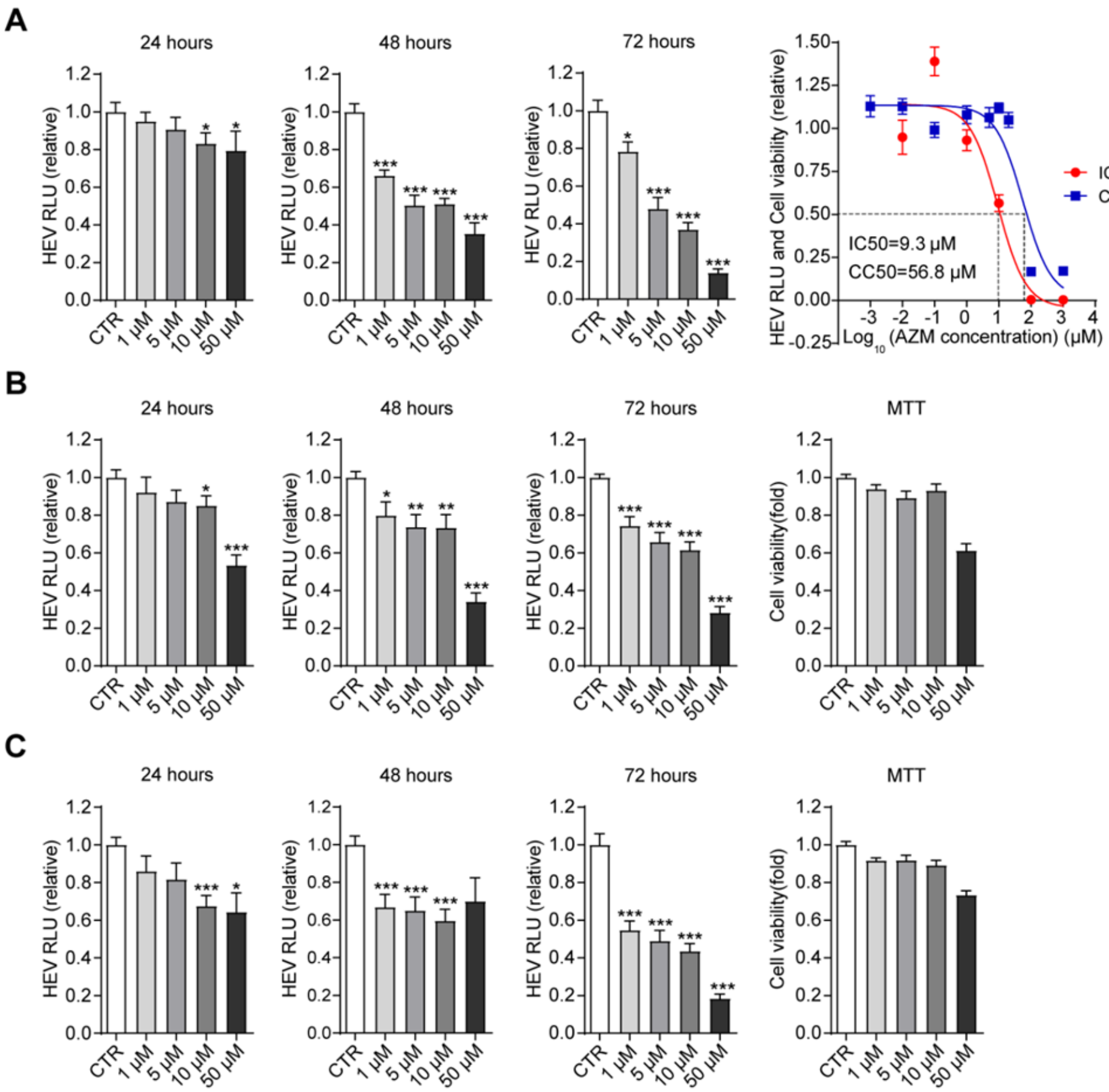

Fig. S1. Anti-HEV activity of azithromycin in diverse cell culture models harboring genotype 3 HEV replicon. (A) The effects of various concentrations of azithromycin (AZM) on HEV replication in kidney 293T cells harboring the p6-Luc replicon. HEV replication was measured by Gaussia luciferase activity (n=12). The 50% inhibitory and cytotoxic concentrations (IC50 and CC50) of azithromycin were calculated using GraphPad Prism 5 software (n=5-6). **(B)** The effect of azithromycin on HEV replication in neuronal U87 (U87-p6-Luc), or **(C)** hepatic PLC/PRF/5 cells (PLC-p6-Luc). The luciferase activity was measured 24, 48 or 72 hours post-treatment. MTT assay was performed at 72 hours post-treatment. Data were normalized and compared to the untreated control group (set as 1) (n=10). Data are presented as means ± SEM. RLU, relative luciferase unit. (*P < 0.05; **P < 0.01; ***P < 0.001).

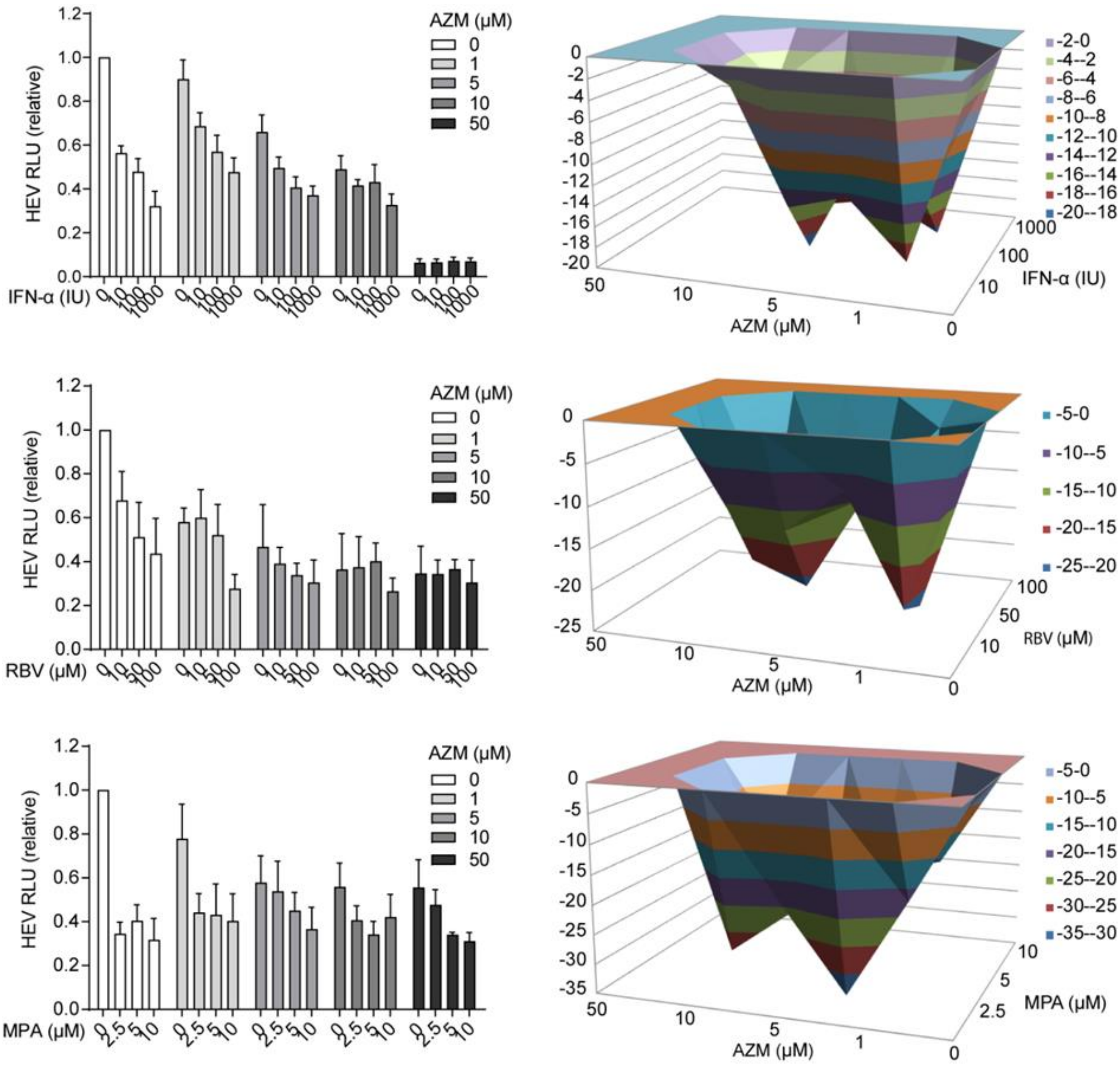

Fig. S2. Combination with azithromycin (AZM) antagonized the anti-HEV effects of human interferon alpha (IFN-α), ribavirin (RBV) and mycophenolic acid (MPA). Azithromycin was combined with IFN-α **(A)**, RBV **(B)**, and MPA **(C)** in Huh7-p6-Luc cell culture model for 48 hours. The inhibitory effects on HEV genotype 3 sub-genome replication was measured by Gaussia luciferase activity. The combinatory effects were analyzed by MacSynergyII model. The three-dimensional surface plot represents the differences (within 95% confidence interval) between actual experimental effects and theoretical additive effects of the combination at various concentrations (n=4). The untreated group serves as control (set as 1). Data are presented as means ± SEM. RLU, relative luciferase unit.

Supplementary Table

Table S1. Primers sequences used to detect HEV, inflammatory cytokines, IFNs and ISGs

Gene		Primer sequence (5'-3')
HEV	Forward	GGTGGTTTCTGGGGTGAC
	Reverse	AGGGGTTGGTTGGATGAA
GAPDH	Forward	GTCTCCTCTGACTTCAACAGCG
	Reverse	ACCACCCTGTTGCTGTAGCCAA
TNF-α	Forward	CTCTTCTGCCTGCTGCACTTTG
	Reverse	ATGGGCTACAGGCTTGTCACTC
IL-1β	Forward	CCACAGACCTTCCAGGAGAATG
	Reverse	GTGCAGTTCAGTGATCGTACAGG
IL-6	Forward	AGACAGCCACTCACCTCTTCAG
	Reverse	TTCTGCCAGTGCCTCTTTGCTG
IL-8	Forward	GTGCAGTTTTGCCAAGGAGT
	Reverse	CTCTGCACCCAGTTTTCCTT
IL-12	Forward	ACCACTCCCAAAACCTGC
	Reverse	CCAGGCAACTCCCATTAG
IFN-α	Forward	TGGGCTGTGATCTGCCTCAAAC
	Reverse	CAGCCTTTTGGAACTGGTTGCC
IFN-β	Forward	CTTGGATTCCTACAAAGAAGCAGC
	Reverse	TCCTCCTTCTGGAACTGCTGCA
IFN-γ	Forward	GAGTGTGGAGACCATCAAGGAAG
	Reverse	TGGTTTGCGTTGGACATTCAAGTC
IFN-λ1 (IL-29)	Forward	GGAAGACAGGAGAGCTGCAACT
	Reverse	AACTGGGAAGGGCTGCCACATT
IFN-λ2 (IL-28A)	Forward	TCGCTTCTGCTGAAGGACTGCA
	Reverse	CCTCCAGAACCTTCAGCGTCAG
MDA5 (IFIH1)	Forward	GCTGAAGTAGGAGTCAAAGCCC
	Reverse	CCACTGTGGTAGCGATAAGCAG
RIG-I (DDX58)	Forward	CACCTCAGTTGCTGATGAAGGC
	Reverse	GTCAGAAGGAAGCACTTGCTACC
OAS3	Forward	CCTGATTCTGCTGGTGAAGCAC

	Reverse	TCCCAGGCAAAGATGGTGAGGA
MX1	Forward	GGCTGTTTACCAGACTCCGACA
	Reverse	CACAAAGCCTGGCAGCTCTCTA
ISG15	Forward	CTCTGAGCATCCTGGTGAGGAA
	Reverse	AAGGTCAGCCAGAACAGGTCGT
STAT1	Forward	ATGGCAGTCTGGCGGCTGAATT
	Reverse	CCAAACCAGGCTGGCACAATTG
STAT2	Forward	CAGGTCACAGAGTTGCTACAGC
	Reverse	CGGTGAACTTGCTGCCAGTCTT
TLR2	Forward	TTTCACTGCTTTCAACTGGTA
	Reverse	TGGAGAGGCTGATGATGAC

Chapter 7

Ivermectin effectively inhibits hepatitis E virus replication requiring the host nuclear transport importin α1

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ABSTRACT

We identified ivermectin, an FDA-approved anti-parasitic drug, effectively inhibited hepatitis E virus (HEV) infection in a range of cell culture models including hepatic and extrahepatic cells with genotype 1 and 3 strains. Long-term treatment showed no clear evidence of drug resistance development. We demonstrated gene silencing of importin-α1, a cellular target of ivermectin and a key member of the host nuclear transport complex, attenuated viral replication and largely abolished the anti-HEV effect of ivermectin.

KEYWORDS

Ivermectin; Hepatitis E virus; Nuclear transport; Drug repurposing

MAIN TEXT

Hepatitis E virus (HEV), as a single-stranded, positive-sense RNA virus, is the most common cause of acute viral hepatitis worldwide (1). Among the classified 8 genotypes, genotype 1, 2, 3, and 4 HEV are well-characterized to infect humans (2). In general, HEV infection is self-limiting with an extremely low mortality rate. In contrast, acute infection with genotype 1 HEV in pregnant women has a high risk of developing severe implications with high death rate of up to 30% (3). Infection with genotype 3 and occasionally genotype 4 HEV in organ transplantation recipients bears a high risk of developing chronic hepatitis E (4-6). Because no FDA-approved medication is available, monotherapy of interferon alpha (IFNα), ribavirin or their combination has been used as an off-label treatment for chronic hepatitis E (7, 8). However, IFNα is generally contraindicated in kidney, pancreas, heart, and lung-transplant recipients because it stimulates the immune system and increases the risk of acute rejection (9). Ribavirin monotherapy is effective in approximately 80% of eligibly treated chronic hepatitis E patients (10). However, treatment failure has been frequently reported, probably attributed to resistance development or poor tolerance (7). Thus, there is a clinical need for developing new antiviral therapies against HEV, and we are interested in repurposing safe-in-human broad-spectrum antiviral drugs for treating hepatitis E (11).

Ivermectin is an approved anti-parasitic medication with an established safety profile since the 1970s and is on the World Health Organization (WHO) list of essential medicines (12, 13). Over the last 30 years, there were more than 2.5 billion doses of ivermectin distributed across the globe, showing an excellent safety profile in the clinic (14). Interestingly, ivermectin has been reported to exert broad antiviral activity against single-stranded RNA viruses and DNA viruses. More interestingly, ivermectin has recently been shown to inhibit SARS-CoV-2 replication, the causative agent of the COVID-19 pandemic (15). In this study, we aim to evaluate the potential of ivermectin in inhibiting HEV replication and possible underlying mechanism-of-action.

To test the potential anti-HEV activity of ivermectin, we first used a subgenomic replicon and a full-length genome HEV infectious model, based on the genotype 3 Kernow-C1 p6 clone **(Fig. S1)**. In the subgenomic model, the open reading frame (ORF) 2 encoding the capsid protein was replaced by a Gaussia luciferase reporter gene for monitoring viral replication. We found that ivermectin treatment significantly inhibited viral replication-related luciferase activity in the human hepatic Huh7 cells harboring the subgenomic replicon in a dose-dependent manner **(Fig. 1A)**. We next tested a series of ivermectin concentrations (0.001-100 μM) in the Huh7 cell model to assess antiviral and cytotoxic effects. The 50% inhibition and cytotoxicity (IC50 and CC50) concentrations of ivermectin were 2.68 μM and 18.83 μM, respectively **(Fig. 1B)**. In the Huh7 infectious p6 cell model, treatment with ivermectin for 48 hours potently inhibited HEV at viral RNA level determined by qRT-PCR **(Fig. 1C and Fig. S2A)** and HEV ORF2 protein level measured by western blotting **(Fig. 1D)**. For instance, treatment with 10 μM of ivermectin for 48 hours resulted in a 71.68% ± 5.78 (mean ± SEM, n=4, $p<0.001$) (Fig. 1C) and 51.67% ± 8.19 (mean ± SEM, n=4, $p<0.01$) **(Fig. 1D)** inhibition of HEV RNA and capsid protein level, respectively. Furthermore, based on calculation of viral RNA copy number **(Fig. S2B)**, treatment with 1 or 5 μM of ivermectin for 48 hours resulted in significant inhibition of secreted viral RNA into supernatant **(Fig. S2C)**, which was consistent with the reduced level of capsid protein in the supernatant **(Fig. S2D)**. The inhibitory effect was further confirmed as shown a dose-dependent decrease of ORF2-encoded capsid protein expression by confocal imaging assay **(Fig. 1E)**. Furthermore, ivermectin also dose-dependently inhibited viral replication in Sar55 clone-based genotype 1 HEV subgenomic replicon **(Fig. 1F)**. Since the p6 genotype 3 clone is capable of producing infectious viral particles, we harvested HEV particles at 48 hours post-treatment of the antiviral drugs, and then performed a re-infection assay in naïve Huh7 cells to further determine the antiviral effects. We included ribavirin as a positive control. We found the amount of produced HEV with infectivity was significantly reduced by ivermectin or ribavirin treatment shown at both viral RNA and protein levels upon re-infection in Huh7 cells **(Fig. S3)**. Notably, HEV is more sensitive to ivermectin than ribavirin. For example, 10 μM ivermectin exerted potent anti-HEV activity, whereas there was hardly an antiviral activity of ribavirin at this concentration **(Fig. S3)**. Drug resistance is one of the main factors that limit the effectiveness of antiviral treatment. Long-term exposure to low dose antiviral drugs is prone to developing resistance (16). To characterize ivermectin in this respect, the HEV p6 cell model was constantly exposed to low dose ivermectin (1 μM). Ivermectin retained its anti-HEV activity even after 21 days, shown at both viral RNA and ORF2-encoded capsid protein levels **(Fig. 1G)**.

Besides hepatitis, HEV infection is associated with a broad range of extrahepatic manifestations, including kidney injury and neurological diseases (17-19). We thus further profiled the antiviral activity of ivermectin in a variety of cell models, including hepatic and non-hepatic cell lines with genotype 3 HEV. Notably, ivermectin significantly inhibited viral replication-related luciferase activity in human kidney 293T **(Fig. 2A)**, hepatic PLC **(Fig. 2B)**, and neuronal U87 **(Fig. 2C)** cells. We determined the IC50 and CC50 concentrations of ivermectin were 2.89 µM and 18.23 µM in 293T cell model, 3.75 µM and 68.78 µM in PLC cell model, and 7.83 µM and 49.51 µM in U87 cell model, respectively.

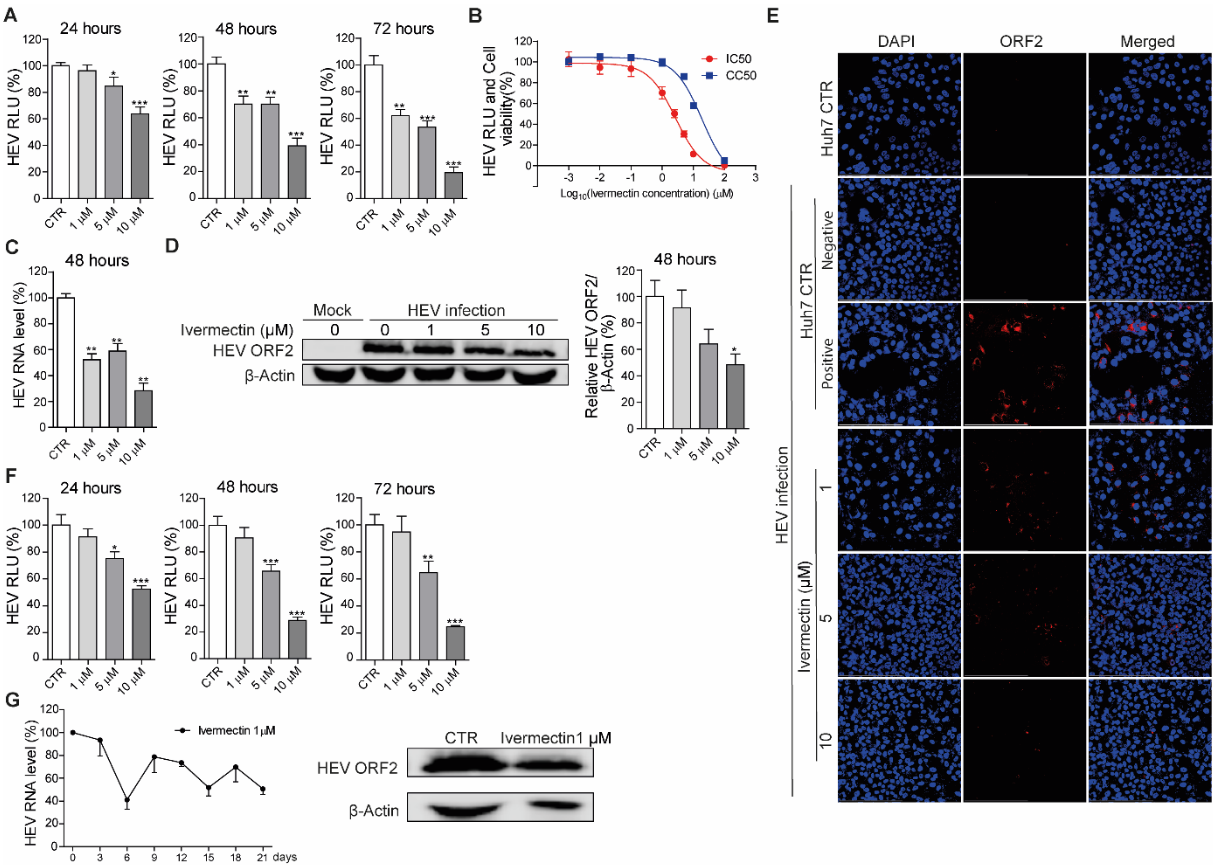

Fig. 1. Anti-HEV activity of ivermectin in Huh7-based cell culture models. (A) The effects of ivermectin treatment for 24, 48 or 72 hours on viral replication related luciferase activity in the genotype 3 subgenomic Huh7-p6-Luc cell model. The untreated group serves as control (CTR) (set as 100%) (n=12). **(B)** The 50% inhibitory concentration (IC50) and 50% cytotoxic concentration (CC50) of ivermectin in Huh7-p6-Luc cell model and Huh7 cell line were calculated using GraphPad Prism 5 software (n=6-12). **(C)** The infectious Huh7-p6 cell model was treated with indicated concentrations of ivermectin for 48 hours. The effects on viral RNA was quantified by qRT-PCR using primers targeting ORF2/ORF3 overlap region (n=4-8). **(D)** Western blot analysis of HEV capsid protein level in Huh7-p6 cells treated with ivermectin for 48 hours. The uninfected group (mock) serves as the negative control, and the infected but untreated group serves as the positive control (set as 100%) (n=4). **(E)** Immunofluorescence analysis of viral ORF2-encoded capsid protein (red) in Huh7 cells treated with indicated concentrations of ivermectin for 48 hours. Huh7 cells incubated with the anti-HEV capsid protein antibody or Huh7-p6 cells incubated with the matched IgG control antibody serves as the negative control. HEV infected Huh7 cells untreated and incubated with the anti-HEV capsid protein antibody serves as the positive control. DAPI (blue) was applied to visualize nuclei. (40 × oil immersion objective). **(F)** Huh7 cell based genotype 1 HEV replicon (Sar55 clone) was treated with ivermectin for 24, 48 or 72 hours and viral replication related luciferase activity was measured (n=10). **(G)** The effects of long-term treatment

with 1 µM ivermectin on HEV RNA in Huh7-p6 cell model were quantified by qRT-PCR. The untreated (CTR) group serve as control (set as 100%) (n=3-4). Western blot analysis of HEV ORF2-encoded capsid protein was performed on 21 days of treatment. RLU: relative luciferase unit. Data are presented as means ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

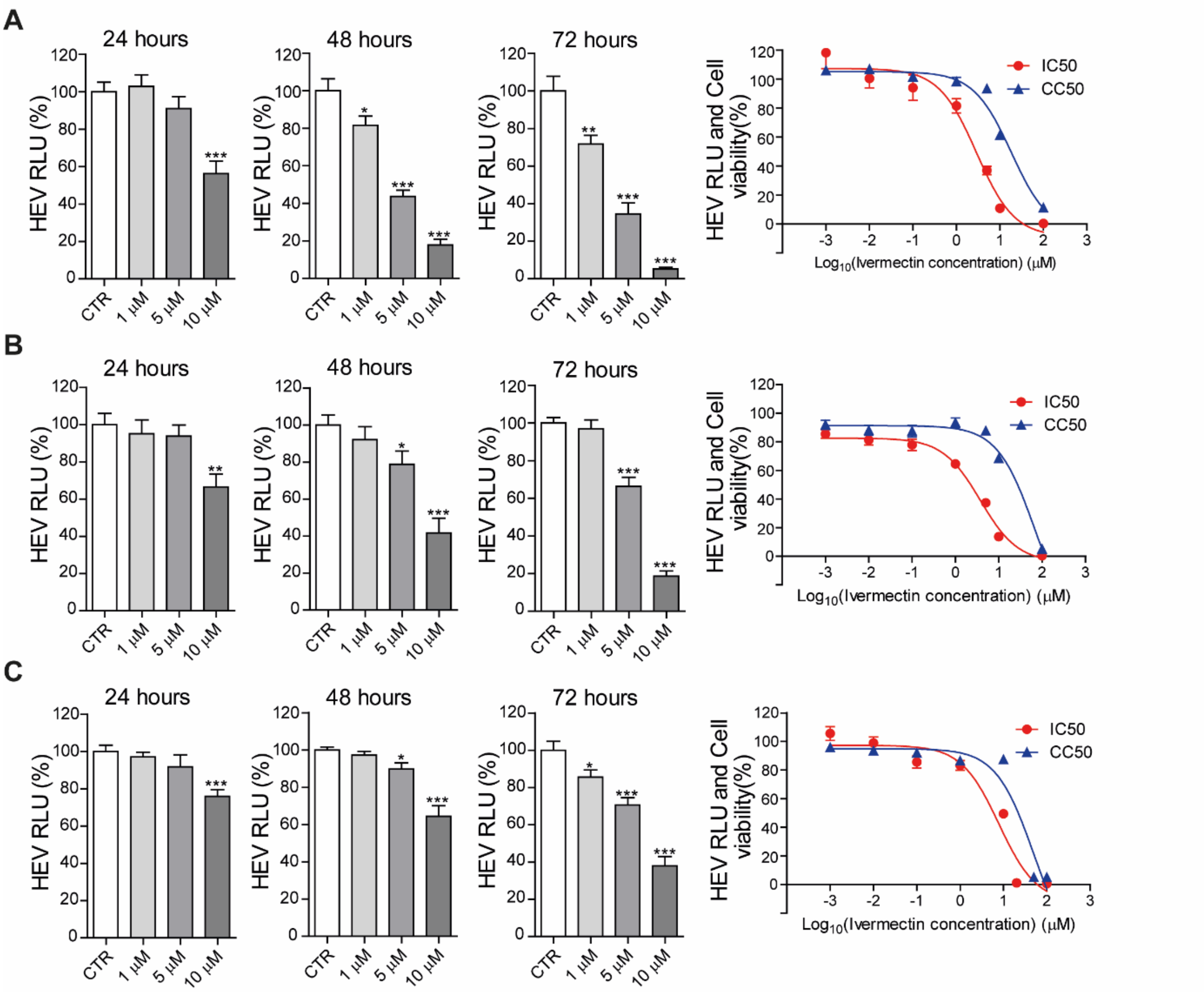

Fig. 2. Anti-HEV activity of ivermectin in different human cell culture models. The effects of ivermectin treatment on viral replication related luciferase activity and the 50% inhibitory concentration (IC50) against HEV replication and 50% cytotoxic concentration (CC50) of ivermectin were calculated using GraphPad Prism 5 software in kidney 293T cells (n=6-12) **(A),** PLC/PRF/5 cells (n=10-12) **(B)**, or neuronal U87 cells (n=10-12) **(C)**. The untreated (CTR) group serve as control (set as 100%). RLU: relative luciferase unit. Data are presented as means ± SEM (*P < 0.05; **P < 0.01; ***P < 0.001).

Emerging evidence suggests that the antiviral activity of ivermectin against particular viruses is associated with host nuclear transport importin (IMP) α/β, which may be attributed to the inhibition of nuclear import of host and viral proteins (20-22). For example, the antiviral activity of ivermectin against Human immunodeficiency virus 1 (HIV-1) and dengue virus has been attributed to the inhibition of IMP α/β nuclear import (21). A very recent study has demonstrated that ivermectin can dissociate the preformed IMP α/β heterodimer and prevent the complex formation to limit West Nile virus infection (22). Although IMPα and IMPβ are commonly known to form heterodimer complex, they actually represent a large family of proteins encoded by various genes. In human, IMPα includes α1, α3, α4, α5, α6, α7, and α8, which are encoded by KPNA2, KPNA4, KPNA3, KPNA1, KPNA5, KPNA6, and KPNA7 genes, respectively (23). Among these genes, KPNA2 encoding IMPα1 in human and IMPα2 in mice has been implicated in mediating the antiviral activity of ivermectin against West Nile virus infection (22). Thus, we further investigated the role of human KPNA2 gene/IMPα1 protein, in the setting of HEV infection. We used lentiviral shRNA vectors to stably knockdown KPNA2 gene in Huh7 cells, and confirmed the gene silencing efficacy at both mRNA **(Fig. 3A)** and protein **(Fig. 3B)** levels, as compared to control. Two clones with optimal gene knockdown were selected for subsequent functional experimentation. Compared with control, knockdown of KPNA2 by the shKPNA2 clone 2 or 3 led to 73% ± 4.6 (mean ± SEM, n=4, $p < 0.05$) and 64% ± 13 (mean ± SEM, n=4, $p < 0.05$) reduction of intracellular HEV RNA, respectively **(Fig. 3C)**. Thus, KPNA2 knockdown significantly reduced HEV RNA level **(Fig. 3C)**, suggesting that IMPα1 as a host factor supports HEV infection. Importantly, the anti-HEV activity of ivermectin was largely abolished in KPNA2 knockdown cells, demonstrating that IMPα1 is essentially required for the anti-HEV action of ivermectin **(Fig. 3D)**. To assess the specificity of KPNA2/IMPα1, we performed a similar gene knockdown of KPNA1 **(Fig.S4A)**, which encodes IMPα5 in human. In contrast, loss of IMPα5 had no effects on HEV replication **(Fig. S4B)** and did not reverse the anti-HEV activity of ivermectin **(Fig. S4C)**. Thus, we have demonstrated that a component of the nuclear transport machinery, IMPα1, essentially mediates the anti-HEV activity of ivermectin. However, the exact mechanism-of-action of the IMP α/β heterodimer nuclear import complex involved in HEV infection and mediating the anti-HEV activity of ivermectin requires to be further studied.

Combination of multiple antiviral drugs is a common strategy to enhance efficacy and avoid resistance development. Since IFNα, ribavirin and mycophenolic acid (MPA) have been shown anti-HEV activity in cell culture systems (24, 25), a combined treatment of ivermectin with these compounds is thus envisaged. Surprisingly, combination with ivermectin antagonized the anti-HEV effects of IFNα **(Fig. S5A)**, ribavirin **(Fig. S5B)** and MPA **(Fig. S5C)**.

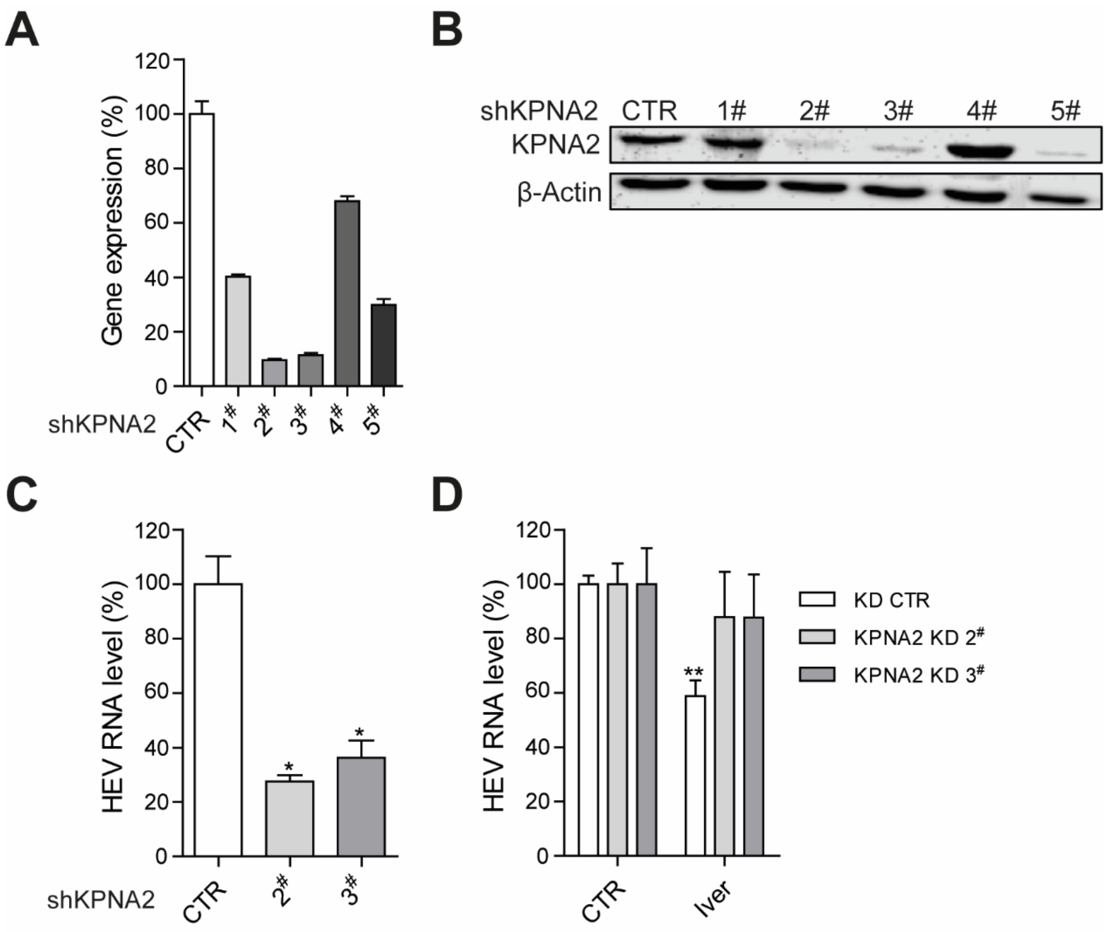

Fig.3. The effects of KPNA2 gene silencing on HEV replication and the anti-HEV activity of ivermectin. **(A)** Knockdown of KPNA2 by lentiviral shRNA vectors. The effects on KPNA2 gene of 5 shKPNA2 clones were quantified by qRT-PCR (n=4). The expression of KPNA2 in control vector transduced cells set as 100%. **(B)** Western blot analysis of KPNA2-encoded IMPα1 protein level. **(C)** The effects of selected KPNA2 knockdown on cellular HEV RNA levels in Huh7 p6 cell model. Viral RNA was quantified by qRT-PCR (n=4). **(D)** KPNA2 knockdown and control Huh7 cells harboring the infectious HEV model were treated with 5 µM ivermectin for 48 hours. The effect on viral RNA was quantified by qRT-PCR (n=6-8). Data are presented as means ± SEM (*P < 0.05; **P < 0.01; ***P < 0.001).

In summary, this study comprehensively demonstrated the anti-HEV activity of ivermectin and the potential mechanism through a host factor IMPα1. Our findings support the further exploration of repurposing ivermectin for treating HEV infection, including for organ transplantation recipients with chronic hepatitis E. Parasitic infections are important complications of organ transplantation that may cause serious morbidity if overlooked. Ivermectin is an effective and commonly used therapy among strongyloides-seropositive organ transplant recipients (26, 27). Although ivermectin use may have been occasionally associated with very mild and self-limiting liver injury, it has been used for treating hyperinfection strongyloidiasis in liver transplant patients with satisfactory outcomes (28, 29).

Thus, ivermectin provides a unique opportunity for treating patients with parasite and HEV co-infections, but also has potential for treating HEV infection in general. Of note, our results are mainly based on the cell culture adapted genotype 3 Kernow-C1 p6 HEV strain that may not fully recapitulate clinical strains. Nevertheless, our findings warrant future studies to further evaluate the efficacy and safety profiles of prolonged ivermectin treatment in treating HEV infection, preferentially in animal models first, before moving into clinical trials.

MATERIALS AND METHODS

Reagents and antibodies

Ivermectin was purchased from Sigma-Aldrich and dissolved in Dimethyl sulfoxide (DMSO, Sigma, Zwijndrecht, the Netherlands) with stock concentration of 100 mM. Human IFNα, ribavirin and mycophenolic acid (MPA) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The anti-HEV capsid protein antibody was purchased from EMD Millipore (mouse monoclonal, MAB8002). Mouse Control IgG2b antibody was purchased from InvivoGen (mabg2b-ctrlm). Anti-KPNA2 antibody (rabbit, #14372) was obtained from Cell Signaling Technology. Anti-β-actin antibody (mouse monoclonal, sc-47778) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human hepatoma Huh7 and PLC/PRF/5 (PLC) cell lines, human embryonic kidney epithelial cell line (293T), human glioblastoma cell line (U87) were kindly provided by the Department of Viroscience (Erasmus Medical Center) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin.

Viruses and cell culture models

Genotype 3 HEV models are based on a plasmid construct containing the full-length HEV genome (genotype 3 Kernow-C1 p6 clone, GenBank accession number JQ679013) or a construct containing subgenomic HEV sequence in which ORF2 was replaced by a Gaussia luciferase reporter gene (p6-Luc). Viral RNA was produced by using the Ambion mMESSAGE nMACHINE in vitro RNA transcription kit. Cells were electroporated with p6 full-length HEV RNA or p6-Luc subgenomic RNA to generate infectious or luciferase-based replicon models, respectively. Similarly, genotype 1 replicon model is based on the Sar 55/S17/luc (GenBank accession number AF444002) HEV clone containing a Gaussia luciferase reporter (Shukla P et al., Journal of virology 86(10), 5697-707 (2012)).

MTT assay

10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to the cells seeded in 96-well plate and cells were maintained at 37℃ with 5% CO2 for 3 hours. Medium was removed and 100 μL of DMSO was added to each well. The absorbance of each well was read on the microplate absorbance readers (BIO-RAD) at wavelength of 490 nm.

Quantification of viral replication

Viral replication in HEV replication models was monitored by the activity of secreted Gaussia luciferase measured by QUANTI-Luc™ Gold (InvivoGen). Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG Lab Tech, Offenburg, Germany). For HEV infectious model, viral RNA was quantified. RNA was isolated using a Machery-Nucleo Spin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (Takara Bio Inc, USA). The HEV RNA level was quantified using a SYBR Green–based real-time PCR assay (Applied Biosystems SYBR Green PCR Master Mix, Life Technologies, CA, USA) according to the manufacturer's instructions. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as housekeeping gene to normalize gene expression using the 2-ΔΔCt method. The primer sequences were as follows: HEV ORF2/ORF3 overlap region sense, 5'-GGTGGTTTCTGGGGTGAC-3'; HEV ORF2/ORF3 overlap region anti sense, 5'-AGGGGTTGGTTGGATGAA -3'; HEV RdRp region sense, 5'-TGAGGAGTCAGTGCTTGCTG-3'; HEV RdRp region antisense, 5'-ATGCCGCACTCCTCCATAAC-3'; GAPDH sense, 5' -GTCTCCTCTGACTTCAACAGCG-3'; GAPDH anti sense, 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Western Blot

Proteins in cell lysates were heated at 95°C for 5 min, followed by loading onto a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), separated at 90 V for 120 min, and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (pore size: 0.45 mm; Thermo Fisher Scientific Life Sciences) for 120 min with an electric current of 250 mA. Subsequently, the membrane was blocked with blocking buffer (Li-Cor Biosciences) for 1 hour at room temperature. Membrane was followed by incubation with primary antibodies mouse anti-HEV ORF2 (1:1000) and anti-β-actin (1:1000) overnight at 4 °C. The membrane was washed 3 times followed by incubation for 1 hour with anti-mouse IRDye-conjugated secondary antibodies (1:5000; Li-Cor Biosciences) at room temperature. After washing 3 times, protein bands were detected with Odyssey 3.0 Infrared Imaging System.

Virus production and re-infection assay

Huh7 cells harboring the infectious genotype 3 HEV were seeded into a multi - well plates HEV particles were harvested by repeated freezing and thawing 3 times, and filtered by 0.45 μm filters. Naïve Huh7 cells were seeded into a multi-well plate and culture medium was discarded when cell confluence was approximately 80%, followed by twice 1×PBS washing. Harvested viruses were added and incubated at 37˚C with 5% CO2 for 24 hours for re-infection, followed by 3 times washing with 1× PBS to remove unattached viruses. Then cells were incubated with culture medium for another 48 hours. The infectivity of produced HEV particles were analyzed by qRT-PCR and Western blotting, respectively.

Long-term treatment assay

For the long-term treatment assay of infectious model (Huh7-p6), the cells were seeded into a 48-well plate with 2×10^4 cells per well. The cells of the control (CTR) or ivermectin treatment groups were passaged and seeded with the same number of cells every 3 days, and cells incubated with normal or contained ivermectin (1 μM) medium were maintained throughout the entire incubation period.

Immunofluorescence

Cells grown on μ-Slide 8 Well (ibidi GmbH, 80826) were fixed with 4% (w/v) paraformaldehyde (PFA) for 10 min at room temperature. After three washes with PBS buffer, cells were permeabilized with 0.1% (v/v) Triton X-100 for 10 min and washed three times with PBS. Block for 1h at Room Temperature with blocking solution (5% Normal Donkey Serum, 1% Bovine Serum Albumin, 0.2% TRITON X in 1 × PBS). Cells were then incubated with primary anti-HEV capsid protein (1:200) antibody (aa 434-457, clone 1E6, IgG2b) or matched mouse IgG 2b antibody (1:200) (InvivoGen) at 4°C overnight and washed three times with PBS, followed by incubation with Anti-mouse-Alexa Fluor® 594-Conjugate antibody (Cell Signaling Technology)

(1:5000) for 1 hour at room temperature. Nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Images were detected with confocal electroscope (lens: 40×).

Gene knockdown by lentiviral vectors

Lentiviral pLKO knockdown vectors (Sigma-Aldrich) expressing shRNAs targeting KPNA1 or KPNA2 and their appropriate controls were obtained from the Erasmus Biomics Center and were produced in HEK 293T cells. These shRNA sequences are listed in table S1. Stable gene knockdown cells were generated after lentiviral vector transduction and selection in medium containing puromycin (3 µg/ml; Sigma). Lentiviral particles were harvested by repeated freezing and thawing 3 times, and filtered by 0.45 µm filters. Naïve Huh7 cells were seeded into a muti-well plate and culture medium was discarded when cell confluence was approximately 80%, followed by twice 1×PBS washing. Harvested viruses were added and incubated at 37˚C with 5% CO_2 for 48 hours. Then stable gene knockdown cells were selected and expanded by adding puromycin (3 µg/ml; Sigma). The KPNA1 or KPNA2 RNA level was quantified using a SYBR Green–based real-time PCR assay according to the manufacturer's instructions. GAPDH was used as housekeeping gene to normalize gene expression using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: KPNA1 sense 5'-TTCCAAAAGCCCA GAGCAACAGC-3'; KPNA1 anti sense, 5'-CCACTACTCCTGGTGTGCTGAT-3'; KPNA2 sense, 5'-CTGTTGGCTCTCCTTGCAGTTC-3'; KPNA2 anti sense, 5'-GCAGGATTCTTGTTGCGGCAAAG-3'.

Statistical Analysis

Statistical analysis was performed using the non-paired, non-parametric test (Mann-Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). All results were presented as mean ± standard errors of the means (SEM). P values <0.05 were considered as statistically significant. Detailed information regarding materials, methods as well as additional results are provided in the Supplementary Data File.

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SUPPLEMENTARY METHODS AND RESULTS

Supplementary Figures

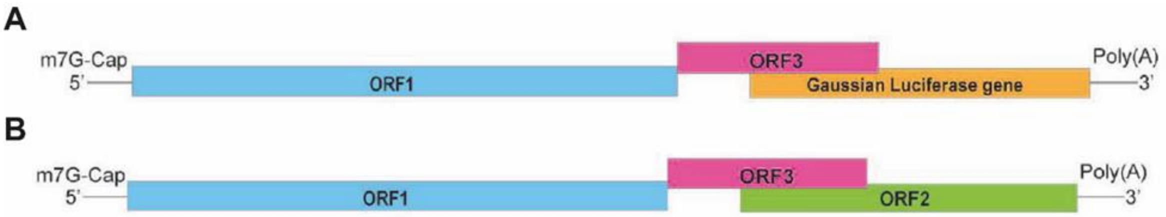

Fig. S1. Diagram of HEV genome. (A) HEV subgenomic replicon model (HEV-GT1-Luc or HEV-GT3-Luc), the open reading frame (ORF) 2 encoding the capsid protein was replaced by a Gaussia luciferase reporter gene for monitoring viral replication. **(B)** Full-length HEV genome (HEV-GT3-p6) for generating the infectious model.

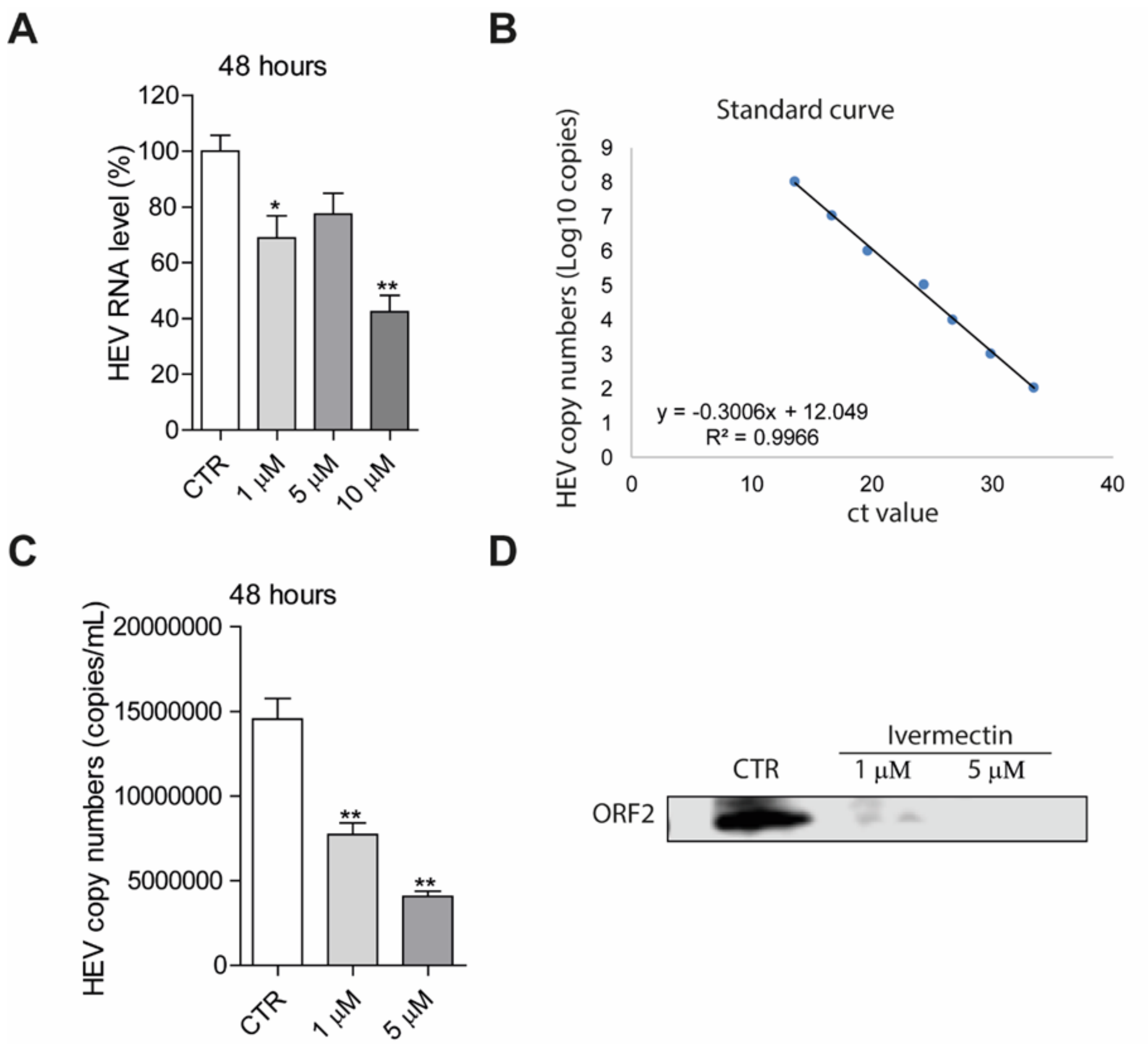

Fig. S2. Anti-HEV activity of ivermectin. (A) The infectious Huh7-p6 cell model was treated with indicated concentrations of ivermectin for 48 hours. The effects on viral RNA was quantified by qRT-

PCR with primers targeting the RdRp region. The untreated group serves as control (CTR) (set as 100%) (n = 4 - 8). **(B)** qRT-PCR determined standard curve for calculating genome copy number. HEV plasmid based standard curve is generated by plotting the log copy number versus the cycle threshold (CT) value. **(C)** qRT-PCR analysis of HEV copy number in Huh7-p6 cells supernatant treated with ivermectin for 48 hours. The untreated group serves as CTR (n = 5 - 6). **(D)** Western blot analysis of HEV capsid protein level in Huh7-p6 cells supernatant. The untreated group serves as CTR.

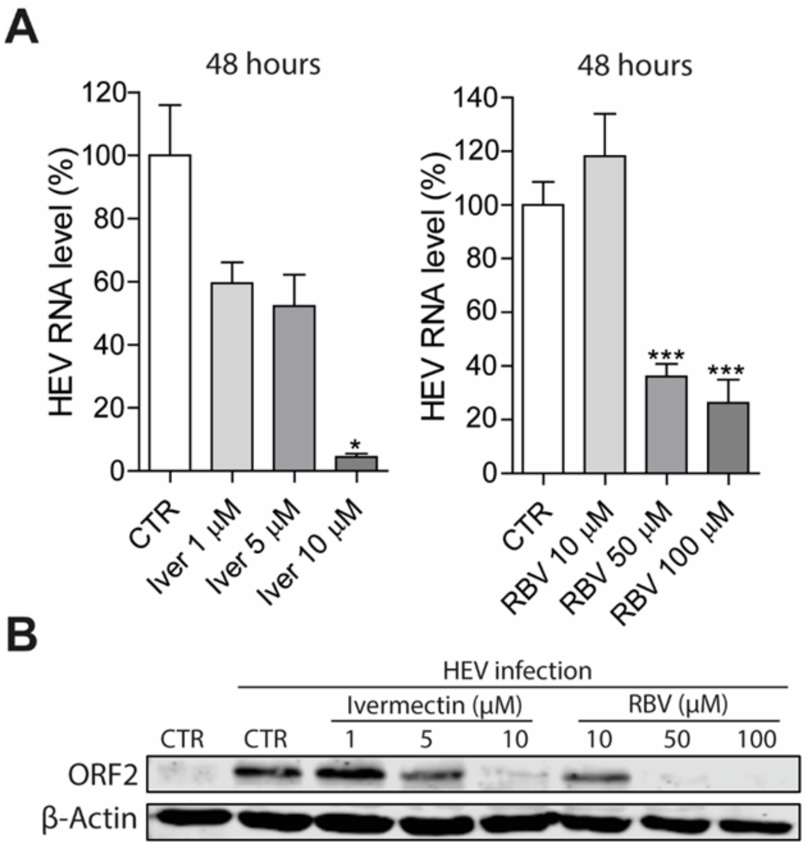

Fig. S3. The effects of ivermectin treatment on the infectivity of produced HEV particles. Naïve Huh7 cells were inoculated with HEV particles produced from HEV infected Huh7 cells 48 hours post-treatment with different concentrations of ivermectin or ribavirin (untreated set as control group). After the inoculation for 48 hours, the Huh7 cells were subjected to qRT-PCR analysis of HEV RNA (n = 4 - 8) **(A)**, Western blotting analysis of HEV ORF2 protein **(B)**. Data are presented as means ± SEM (*P < 0.05; **P < 0.01; ***P < 0.001).

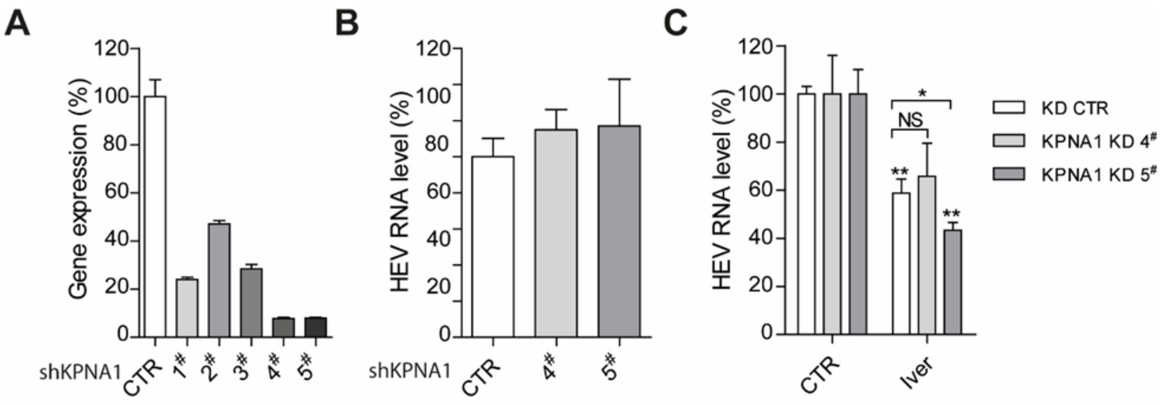

Fig. S4. Gene silencing of KPNA1 by lentiviral RNAi did not affect HEV replication and abolish the anti-HEV activity of ivermectin. (A) Knockdown of KPNA1 by lentiviral shRNA vectors. The effects on KPNA1 gene of 5 shKPNA2 clones were quantified by qRT-PCR (n = 4). The expression of KPNA2 in control vector transduced cells set as 100%. **(B)** The effects of KPNA1 knockdown on cellular HEV RNA in Huh7 cells harboring infectious p6 clone. Viral RNA was quantified by qRT-PCR (n = 4). **(C)** KPNA1 knockdown and control Huh7 cell lines harboring the infectious HEV model were treated with 5 µM ivermectin for 48 hours. The effects on viral RNA was quantified by qRT-PCR (n = 4 - 8). Data are presented as means ± SEM (*P < 0.05; **P < 0.01; ***P < 0.001).

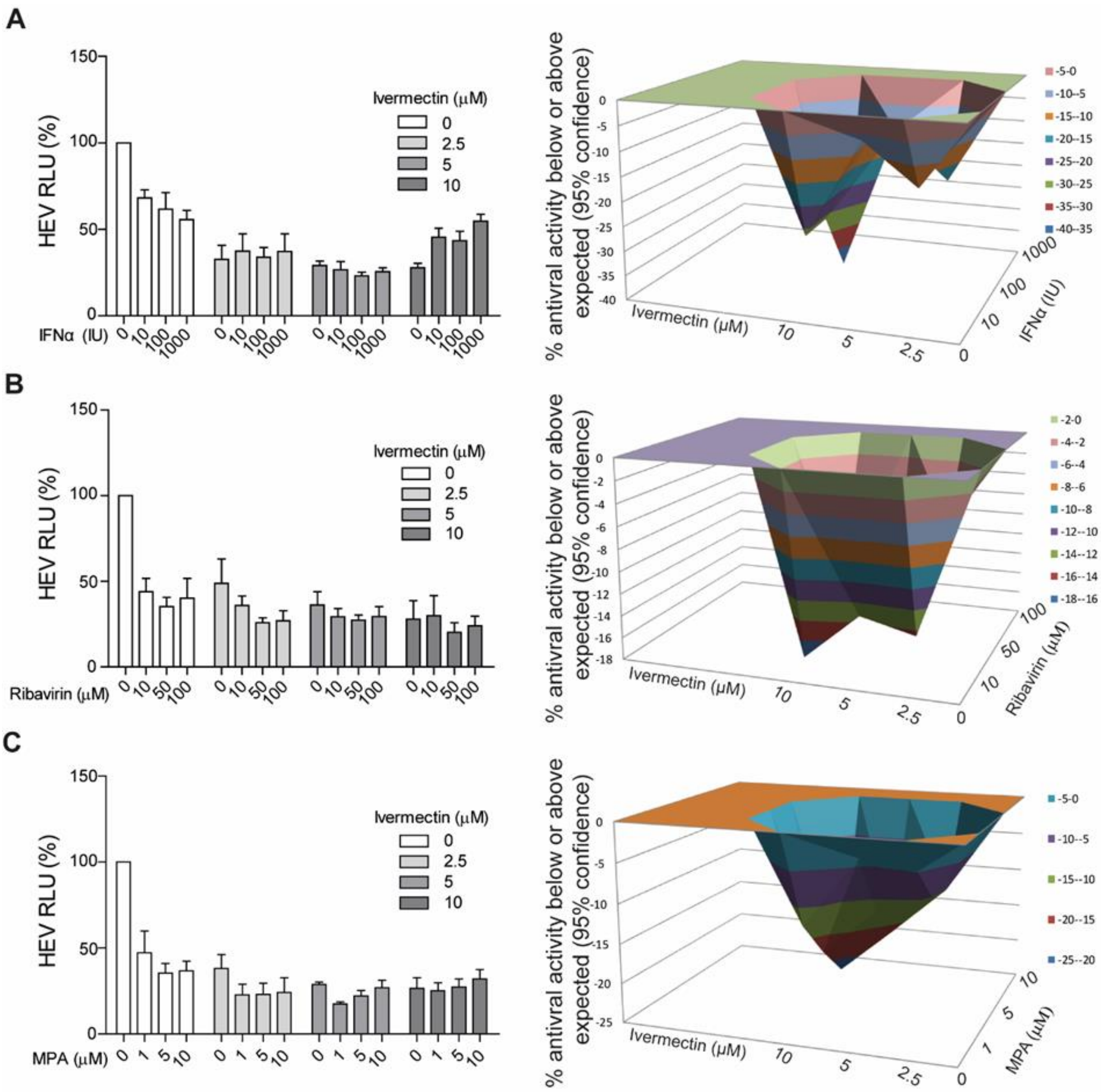

Fig. S5. The combination of ivermectin with IFNα, ribavirin or MPA results in an antagonistic effect against HEV. The antiviral effects of ivermectin in combination with interferon alpha (IFNα) **(A)**, ribavirin **(B)** or mycophenolic acid (MPA) **(C)** were analyzed by the MacSynergyII model. The three-dimensional surface plot represents the differences (within 95% confidence interval) between actual experimental effects and theoretical additive effects of the combination at various concentrations (n = 4 - 5).

Supplementary table

Supplementary table 1. shRNA sequences.

ID	Symbol	TargetTaxonId	TargetSeq	GeneDesc	OligoSeq
1#	KPNA1	Human	GCAGTTATTCAA GCGGAGAAA	Karyopherin α1 (importin α5)	CCGGGCAGTTATTCAAGCGGAGAAAC TCGAGTTTCTCCGCTTGAATAACTGCT TTTTG
2#	KPNA1	Human	CCCTCCTATTGA TGAAGTTAT	Karyopherin α1 (importin α5)	CCGGCCCTCCTATTGATGAAGTTATCT CGAGATAACTTCATCAATAGGAGGGT TTTTG
3#	KPNA1	Human	GCCCTCTCATAT CTATCAGAT	Karyopherin α1 (importin α5)	CCGGGCCCTCTCATATCTATCAGATCT CGAGATCTGATAGATATGAGAGGGCT TTTTG
4#	KPNA1	Human	GCCTTTGATCTT ATTGAGCAT	Karyopherin α1 (importin α5)	CCGGGCCTTTGATCTTATTGAGCATCT CGAGATGCTCAATAAGATCAAAGGCT TTTTG
5#	KPNA1	Human	CTGCTGAATTTC GGACAAGAA	Karyopherin α1 (importin α5)	CCGGCTGCTGAATTTCGGACAAGAAC TCGAGTTCTTGTCCGAAATTCAGCAGT TTTTG
1#	KPNA2	Human	CGAATTGGCAT GGTGGTGAAA	karyopherin α2 (RAG cohort 1, importin α1)	CCGGCGAATTGGCATGGTGGTGAAAC TCGAGTTTCACCACCATGCCAATTCGT TTTTG
2#	KPNA2	Human	CCTGGACACTTT CTAATCTTT	karyopherin α2 (RAG cohort 1, importin α1)	CCGGCCTGGACACTTTCTAATCTTTCT CGAGAAAGATTAGAAAGTGTCCAGGT TTTTG
3#	KPNA2	Human	GCTGGTTTGATT CCGAAATTT	karyopherin α2 (RAG cohort 1, importin α1)	CCGGGCTGGTTTGATTCCGAAATTTCT CGAGAAATTTCGGAATCAAACCAGCT TTTTG
4#	KPNA2	Human	GATGACATTGTC AAAGGCATA	karyopherin α2 (RAG cohort 1, importin α1)	CCGGGATGACATTGTCAAAGGCATAC TCGAGTATGCCTTTGACAATGTCATCT TTTTG
5#	KPNA2	Human	CTACCTCTGAAG GCTACACTT	karyopherin α2 (RAG cohort 1, importin α1)	CCGGCTACCTCTGAAGGCTACACTTCT CGAGAAGTGTAGCCTTCAGAGGTAGT TTTTG

Chapter 8

Summary and Discussion

Viral hepatitis is a major global public health threat and the World Health Organization (WHO) has taken up the mondial elimination of viral hepatitis by 2030 as one of its missions (1). Great advances have been made in the prevention and treatment of HBV and HCV, which collectively contribute the most to the disease burden and thus have become the only targets with respect to the quest to eliminate viral hepatitis (1, 2). However, efforts aimed at achieving the goal of global elimination of viral hepatitis should also involve HDV and HEV. This is because, firstly, the co-infection of HDV with HBV provokes the most severe form of acute and chronic viral hepatitis, and thus represents a major contributor to viral hepatitis-associated cirrhosis, HCC and mortality (3, 4); secondly, HEV, as the most common cause of acute viral hepatitis worldwide, is responsible for most cases of acute hepatitis annually, and is associated with particularly high mortality in specific risk populations (5-7). Establishing the global epidemiology for HDV infection and developing novel and specific therapy for HEV infection are the most urgent issues for these two viruses, hence I chose these subjects as the focuses of this thesis. In this chapter, I will summarize and discuss the main findings obtained in this thesis and propose suggestions for future research.

Firstly, in **Chapter 2**, a standard system and criteria for HDV molecular classification was established. HDV can evolve rapidly as a RNA virus, resulting in high genetic diversity. Heterogeneity of strains can also result from natural recombination in HDV patients infected with multiple strains (8-10). Both mutation and recombination are potent driving forces in the evolution of the HDV. Meanwhile, this phenomena complicates HDV molecular classification in phylogenetic and genetic analyses, in turn leading authors to report inconsistent results in this respect (11, 12). In fact, unlike human immunodeficiency virus (HIV), where recombinants are common and can spread widely as circulating recombinant forms (CRFs) (13), HDV recombinants are rare and do not spread widely, for reasons as yet unknown. Prompted by these and other considerations, I have strived to establish a standardized classification system for HDV, that takes into account previous work. To achieve this, as described in **Chapter 2**, I have identified potential recombinants and excluded these to generate a framework for HDV classification. With respect to the sequences used for phylogenetic and genetic analyses, I had to convert all sequences deposited in public databases to the gene coding form (i.e. sequence of coding strand) that is analogous to that used for reporting messenger RNA (mRNA). Because the HDV genome is a circular negative single-stranded RNA (ssRNA), I also standardized all HDV strain sequences to an antigenomic RNA form with a concordant start site (from 1 to

1678) before the further analyses. After removing the recombinant sequences, I then constructed phylogenetic trees for HDV standardized full-length sequences. In this updated system, HDV strains clearly clustered into eight genotypes, further grouped into 18 subtypes, which presented distinct global distribution with respect to regional presence. Based on the new system and comparisons of nucleotide similarity and genetic distance between strains, I further summarized and reformulated criteria for identifying novel HDV genotypes or subtypes, and I finally also proposed reference genomes for each subtype.

In summary, in **Chapter 2**, I identified the potential recombinants, standardized strain sequences, updated the classification system (including genotype and nomenclature) and criteria, proposed standard reference genomes for HDV subtypes and mapped the global genotypic distribution. Since genotype plays a crucial role in the biological research and clinical treatment of HDV, any molecular classification discrepancy could substantially hinder integrating findings from different academic disciplines, which would in turn slow down progress with respect to understanding this virus and the resulting disease. Hence, in addition to providing updated system, standard classification criteria and reference genomes, I also gave (hopefully) useful guidance for future standardization in the reporting of novel HDV strains helping ensuring high quality of sequences to be submitted to online public database. Although the proposed system will likely evolve as future epidemiological and genomic data will accumulate (14), using standardized classification and criteria should largely avoid potential inconsistency in future research and clinical treatment. I hope my work will foster raising the awareness of the problem and reaching a general consensus in the field.

Secondly, in **Chapter 3, 4 and 5**, I established a reliable global epidemiology for HDV infection by literature review, meta-analysis and data synthesis, and I also provided a fair historical overview on this topic. Despite being a defective satellite virus of HBV that leads to devastating liver disease, HDV has received inadequate public awareness and effective treatment is generally lacking (15, 16). Since reliable epidemiological estimates are the cornerstone of any public health responses, in **Chapter 3** I aimed to address the urgent issue of HDV global prevalence. In order to provide a fair and realistic estimate, except applying strict inclusion/exclusion criteria and quality assessment, as well as standard meta-analysis procedure and random-effects modeling, I also employed two methodological approaches to calculate HDV global prevalence. Firstly, as a hepatotropic pathogen, HDV infection was occasionally screened in the general population, irrespective of the HBV infection status of the individuals involved (17, 18). Secondly, as a satellite virus that requires HBV surface antigens (HBsAgs) to propagate, HDV infection was predominantly tested for in HBsAg-positive carriers (17, 18). Although the epidemiological data on HDV infection from general population screening were more scarce compared to data derived from testing of HBsAg-positive carriers, I was able to pursue two alternative approaches for calculating HDV global prevalence. Most importantly, this then allowed me to verify the pooled results from HBsAg-positive carriers with the pooled results from general population. I thus feel the data provided to be very robust.

With the above in mind, in **Chapter 3**, I categorized the extracted data of HDV infection from literature review into two major groups: the general population and HBsAg-positive carriers, which were further composed by various subgroups respectively. For the general population, to avoid overestimation that might be caused by the extreme high infection rate among risk populations (e.g., intravenous drug users, commercial sex workers, HIV patients, and patients with liver diseases), I only included representative populations (composed of 72% general subjects and 28% blood donors) followed by minor adjustments, as methodologically necessary. The pooled prevalence of HDV infection in this reflection of the general population was 0.8%, corresponding to 60 million infections if extrapolated to global population at large. This prevalence is relatively lower than the previous estimate of 0.98% (17, 19), which was considered by other researchers as an overestimation (20, 21). This supports the notion that the results I obtained are credible and even precise. However, for HBsAg positive carriers, for which more data are available and with the availability of data of the average infection rate for HDV among different subgroups in such carriers (general carriers/blood donors versus specific risk populations), the actual fraction of HDV-infected individuals can also be reliably estimated. From my work a realistic burden provoked by HDV among HBsAg-positive carriers was calculated to be 13%, corresponding to 48 million infections. Again, I want to emphasize that this estimate is likely more precise and credible than the previously estimated prevalence of 14.57%.

Although my work in this was well-received by the community and perceived to be timely and important with respect to the ongoing debate on HDV global epidemiology, it may also have practical clinical implications for HDV testing (22). I have to admit the fact that, just as I elaborated in the introductory **Chapter 1**, that estimating HDV global burden has been a true challenge, efforts being hampered by many factors including the availability of reliable data, regional/populational heterogeneities, and alternative methodology and data processing underlying published data (20-22). Further problems are the result of inadequate assumptions by investigators as how to interpret results. For example, in a recent meta-analysis, limited data on the presence of HDV signals in low prevalent populations (think of healthy carriers and blood donors) was used to extrapolate to HDV burden on a global scale (23, 24). Consequently, the HDV prevalence of 4.5% among all HBV carriers, was translated into a prevalence of only 0.16% of the global population, corresponding to 12 million people worldwide. Even though it was described at the time as “sounds much more reasonable and would be in line with previous rough estimates” by the expert who initially proposed HDV global prevalence to be 15 to 20 million (25, 26), the underlying assumption is clearly questionable as HDV infected individuals are likely to be underrepresented in asymptomatic carriers (24). Indeed, this earlier meta-analysis was clearly discordant with an regional estimation also conducted by the same research team, that proposed 7 million HDV infections in sub-Saharan Africa alone (18). The Asia endemic region is the major reservoir of HBV infection and the sub-Saharan Africa result is thus not in line with the global results from the same team (27-29). Hence, in **Chapter 4**, to better estimate and understand HDV global burden, I revisited this estimation. Based on the summary of all of the previous relevant calculations, I proposed that the global prevalence of HDV infection was approximately 50 million with a prevalence of 0.7% among the general population and 13% among HBV carriers. To further understand HDV global burden, however, a comprehensive and historical overview on the dynamics of HDV prevalence over the past decades would also be required. Moreover, the impact of HBV vaccination on HDV global prevalence during this period also needed to be assessed. Therefore, in **Chapter 5**, I aimed to address these issues. Based on subgroup analyses, I found HDV prevalence declined by 50% (from 0.86% to 0.44%) among the general population over the past four decades, but increased by 60% (from 3.46% to 5.55%) among non-risk HBV carriers. Further results from risk HBV carriers showed that the infection rates of HDV declined in IDUs, HIV patients, and mixed carriers, but increased in HBV patients with liver disease. Of note, as I indicated in **Chapter 4**, HBV patients living with liver disease rather than those general carriers used earlier, were more likely to be the major reservoir of HDV (24, 28). As a consequence, HDV global burden has been sustained and remained substantial over the past period, with minor changes from the initial 45 million to the present 53 million, even in the presence of substantial vaccination efforts. To explain this unexpected result, my further calculation suggested that HBV vaccination actually has prevented approximately 12 million new cases of HDV infection by now. By contrast, the growth of world population had yielded about 10-20 million HDV cases during the same period, which clearly plays an important role in the maintenance or augment of HDV global burden. These findings show that, even though HBV vaccination is effective prevention for HDV infection, the current situation with respect to HDV infection is still critical, calling for enhanced awareness, screening and interventions. I hope my findings will prove useful and contribute to HDV elimination.

Despite chronic hepatitis D (CHD) is considered the most severe form of chronic viral hepatitis linked to liver cirrhosis and HCC, a data synthesis of the available global evidence remains lacking. Thus, also in **Chapter 3**, I comprehensively profiled the disease mode, infection pattern, clinical progression and outcome of HDV by data synthesis. Synthesizing the global evidence indicated that HDV infection significantly increased the risk of developing severer liver diseases including cirrhosis and HCC in HBV patients and is associated with extremely fast progression of disease. As a result, compared with HBV mono-infection, double infection with HDV resulted in more symptomatic outcomes. For example, more than 30% of HBV/HDV double-infected patients were cirrhotic, whereas this was just 14% for HBV mono-infected patients. This is important because it means that HDV patients are more likely to have liver problems and visit hepatology clinics in real life. This, again, further implies that HDV patients attending hepatology clinics are probably the major contributors of HDV burden and thus cannot be neglected in burden calculation (24, 28). Meanwhile, it also highlights the importance and significance of HDV screening and testing among general HBV carriers, especially in Asia and Africa, which are the regions for the associated liver cirrhosis and HCC and which have to cope with poor sanitation and medical infrastructure (18, 28, 29).

In summary, in **Chapter 3, 4 and 5**, I have strived to make an accurate estimate of HDV infectious disease. Based on abundant and comprehensive evidence, I uncovered that despite HBV vaccination had indeed prevented more people from HDV infection, HDV global burden remained substantial at present. While the misunderstanding of the real epidemiology of HDV global infection might obscure the problem, we should not forget the lesson that the initial underestimation had possibly resulted in the long-term negligence and severe consequence in the past history (15, 22). Most importantly, these estimates involve thousands of millions of lives, but not just numbers (29). Hence, given the significant risk and considerable contribution of HDV to liver cirrhosis and HCC, HBV-related hepatitis elimination would be impossible to achieve without significant progress on the HDV front.

Next, in **Chapter 6 and 7**, I turned my focus to the antiviral therapy of another type of viral hepatitis, hepatitis E, caused by HEV infection. Despite HEV being a self-limiting hepatotropic virus, HEV induces many acute episodes of hepatitis each year. These episodes have a high chance for serious consequences of acute or chronic hepatitis emerging, especially in high-risk populations (featuring pregnant women and immunocompromised patients) (3, 5-7, 30). Owing to the limitations of general antiviral medications (which often are not suitable for pregnant women), developing specific therapeutics for treationg HEV infection in such high-risk populations is a major issue (5, 6, 31). Fortunately, powerful tools (HEV infectious clones and replicons) and practical strategy (repurposing safe-in-human broad-spectrum antiviral agents) have been established for facilitating anti-HEV therapeutic development (32-35). Hence, in these two Chapters, I further explored the potential of repurposing azithromycin and ivermectin, two potential anti-HEV candidates prospectively selected by drug screening, for treating HEV infection.

Azithromycin, a FDA pregnancy category B drug, is primarily used as an antibacterial agent in the clinic (36, 37). But in recent years, this macrolide antibiotic has been reported to inhibit a variety of viruses including respiratory syncytial virus, rhinovirus, influenza virus, ZIKA virus, and it also showed potential antiviral activity in our HEV drug screening (34, 38-42). Therefore, in **Chapter 6**, I firstly comprehensively evaluated the anti-HEV activity of azithromycin. I found it could effectively inhibit HEV RNA replication and viral protein expression in multiple cell culture models for both genotype 1 and 3 strains, and this anti-HEV activity was also confirmed in extrahepatic cells. In addition, I further established that although azithromycin could activate an interferon-like response including the induction of several interferon-stimulated genes (ISGs), but the anti-HEV activity was independent of this innate immune response. Despite the exact mechanism-of-action requires further study, I have convincingly demonstrated azithromycin as a potent HEV inhibitor. This is significant because azithromycin has been clinically proven to be safe in pregnant women, newborns and young children and it is cheap and widely available in oral and intravenous formulations (37, 43-48). Thus, repurposing azithromycin for treating HEV infection would be highly accessible for general patients and pregnant women, particularly in resource-limited regions. Except antiviral activity, azithromycin also possesses anti-inflammatory and immunomodulatory properties

(38, 39, 49, 50). Since severe HEV infection in pregnant women is usually associated with a massive inflammatory response (51), azithromycin should prove an attractive therapeutic candidate drug for treating HEV infected pregnant women that can simultaneously inhibit the infection and pathogenic inflammation and injury in both liver organ and extrahepatic organs. I hope my work will open the doors for follow-up *in vivo* research, preferentially clinical trials, to test the great potential of repurposing azithromycin for treating HEV infection.

Ivermectin, an approved antiparasitic drug with a proven safety profile, is included in the list of WHO essential medicines (52). Ivermectin has been reported to exert broad antiviral activity against single-strain RNA viruses and DNA viruses, and recently it was identified as a potential anti-HEV candidate by our drug screening (34). Therefore, in **Chapter 7**, its anti-HEV activity was comprehensively evaluated. It was found that ivermectin could effectively inhibit HEV RNA replication, viral protein expression and infectious virus production in human liver cells harboring a genotype 3 infectious clone. Then, this anti-HEV activity was further confirmed in hepatic and extrahepatic cell models with genotype 1 and 3 HEV strains. Notably, long-term treatment with low dose ivermectin for 21 days remained the antiviral potency without evidence of drug resistance development. In addition, further gene silencing experimentation found that importin-α1 (53, 54), a key member of the host importin α/β nuclear transport complex, was a host factor that could support HEV infection. Furthermore, it also seems to be the target for ivermectin effects on HEV infection as in importin-deficient cells ivermectin no longer affects HEV replication. These findings support the further exploration of repurposing ivermectin for treating HEV infection. In fact, ivermectin is a commonly used effective therapy for strongyloides-seropositive organ transplant recipients, and it has also been used for treating hyperinfection strongyloidiasis in liver transplant patients, with satisfactory outcomes (55-58). Because HEV-infected immunocompromised patients, including organ transplant recipients and cancer patients, bear a high risk of developing chronic hepatitis (5, 6, 59), it is clear that ivermectin provides a unique opportunity for treating patients with parasite and HEV co-infections, but it also has potential for treating HEV infection in general. I hope this study will lay the groundwork for future *in vivo* studies to further evaluate the efficacy and safety profiles of ivermectin in treating HEV infection.

In summary, in **Chapter 6 and 7**, I have attempted to identify new anti-HEV agents that are suitable for use in HEV high-risk populations through drug repurposing. I have demonstrated that the macrolide antibiotic azithromycin and the antiparasitic ivermectin are potent HEV inhibitors, and I further uncovered their mode of action. These findings not only extend the antiviral spectra of azithromycin and ivermectin to HEV, but also extend our knowledge on their mechanisms if this medication will be applied in the real world. Most importantly, azithromycin and ivermectin provide some clues for the development of HEV specific treatment in pregnant women and organ transplant patients. Thus, in conjunction I hope my studies open new rational avenues for combating HEV-associated disease.

In conclusion, eliminating viral hepatitis is an ongoing major public health challenge. Major advances have been made towards achieving this goal, especially in the prevention and treatment of HBV and HCV infections. However, these efforts will prove not sufficient for the ultimate elimination of viral hepatitis to be achieved by 2030. Additional public health awareness and substantially more resources should also be dedicated to combating HDV and HEV infections. The degree to which the international response is focused on HDV and HEV will be directly related to the quality and results of the hepatitis elimination campaign.

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Chapter 9

Nederlandse Samenvatting

Dutch Summary

Nederlandse samenvatting voor niet-ingewijden.

Virale Hepatitis (leverontsteking) kent vijf verschijningsvormen, *in casu* Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D en Hepatitis E. Honderden miljoenen mensen lijden aan de gevolgen van deze virusziekten. Daarmee is virale hepatitis is wat betreft infectieziekten één der grootste gesels der mensheid. Virale infectie van de lever kan zowel in de acute fase als ook in de chronische fase levensbedreigend zijn, in het laatste geval ook omdat de leverfunctie verloren kan gaan en er leverkanker kan ontstaan. Daarom heeft de WHO (World Health Organisation) besloten dat het elimineren van virale hepatitis een belangrijk en urgent doel is. Het jaar 2030 wordt hierin genoemd als streefdatum. De meeste aandacht gaat in dit opzicht uit naar Hepatitis B en C. Als we echter kijken naar ernstige ziekte en dood, dan is Hepatitis D eigenlijk belangrijker dan Hepatitis B of Hepatitis C. Als we kijken naar het veroorzaken van acute ernstige leverontsteking dan is wereldwijd Hepatitis E het belangrijkst. Dit was mijn overweging om in dit proefschrift mij bezig te houden met juist Hepatitis D en Hepatitis E.

In mijn proefschrift beschrijf ik in **Hoofdstuk 1** mijn analyse van die problemen die nu het meest urgent moeten worden aangepakt. Voor Hepatitis D blijkt dit het gebrek aan inzicht te zijn in de moleculaire taxonomie (taxonomie is de vakwetenschap binnen de biologie, die soorten organismen ordent [classificeert] op grond van hun evolutionaire verwantschap) alsook de onduidelijkheid wat betreft de epidemiologie van het Hepatitis D virus. Voor Hepatitis E ligt het probleem met name in de afwezigheid van medicatie waarmee infectie met het Hepatitis E virus kan worden bestreden. Het waren dan ook deze zaken waarvoor ik met dit proefschrift een bijdrage aan de contemporaine biomedische literatuur heb proberen te leveren.

Een probleem waar onderzoekers bij het ontwerpen van rationele strategieën om Hepatitis D te bestrijden tegen aan liepen was dat de taxonomie van dit virus een ratjetoe was. Verschillende stammen zijn sowieso verschillend, er bestaan vele fusies tussen verschillende stammen en in het verleden zijn er bij het aanbieden van virale sequenties aan publieke databases van dit cirkelvormige virus vaak verschillende startposities gebruikt. Hierdoor was het bijvoorbeeld onmogelijk om een nieuwe variant op de rationele wijze te classificeren of benoemen. In **hoofdstuk 2** van mijn proefschrift doe ik iets aan deze situatie door een HDV standaard genoom voor te stellen en een systematiek over hoe virussen te classificeren. Mijn werk hierover werd gepubliceerd in het vooraanstaande vaktijdschrift *Journal of Viral Hepatitis.*

Vervolgens heb ik geprobeerd iets te doen aan een andere belangrijke vraag voor wat betreft Hepatitis D: hoe groot is het klinisch probleem nu eigenlijk op mondiale schaal? Zowel het aantal mensen dat Hepatitis D had alsook het aantal patiënten dat met Hepatitis D een ernstig klinisch beloop vertoonde van de ziekte, bleef erg onduidelijk. Dit had er mee te maken dat de ziekte in verhouding tot Hepatitis B en Hepatitis C wat stiefmoederlijk werd behandeld en slecht op het netvlies stond bij zowel patiënten, artsen en beleidsmakers. In **hoofdstuk 3** kom ik met antwoorden en die laten zien dat momenteel ongeveer 48 miljoen mensen besmet zijn met Hepatitis D, wat ongeveer het dubbele is van wat vroeger werd aangenomen. Ook laat ik zien dat de ziekte vaak een ernstig beloop kent, terwijl het vaccineren tegen Hepatitis B (het hepatitis D virus heeft het Hepatitis B virus nodig om zich te kunnen vermenigvuldigen) nog niet leidt tot een afname van het aantal Hepatitis D gevallen, althans in absolute zin (relatief ten opzichte van de wereldbevolking is er wel een gunstig effect zichtbaar). Ik wist mijn studie te publiceren in het vooraanstaande vaktijdschrift *The Journal of Infectious Diseases*. In **Hoofdstuk 4** en **Hoofdstuk 5** diep ik deze problematiek nog verder uit, zowel kwalitatief als kwantitatief uit en constateer ik dat met name de toename in de wereldbevolking een dominant effect hier is. Deze uitweidingen wist ik ook goed weg te zetten door een publicatie in het vooraanstaande vaktijdschrift *Journal of Hepatology* en ook het vaktijdschrift *Cliincs in Gastroenterology and Hepatology*. Ik hoop met deze resultaten en vooraanstaande publicaties bewustheid te hebben gecreëerd over het belang van dit wereldgezondheidsprobleem.

Vervolgens heb ik mijn aandacht gevestigd op het Hepatitis E virus. Voor de ziekte veroorzaakt door dit virus is nog geen registreerde medicatie. Er zijn wel middelen die off-label gebruikt kunnen worden, maar deze hebben veel bijwerkingen en ook behandeling van zwangere vrouwen is niet mogelijk. Omdat met name aanstaande moeders een zeer ongunstig ziektebeeld kunnen vertonen na besmetting met bepaalde genotypen van het Hepatitis E virus is dit een groot probleem. Ik besloot daarom celkweekmodellen te gebruiken die het vermogen van het Hepatitis E virus om zich te repliceren goed kunnen meten. Ook maakte ik gebruik van bibliotheken met medicatie die goedgekeurd is voor menselijk gebruik en waar geen patentbescherming meer op zit. Op zoek naar geschikte opties om Hepatitis E mee te behandelen trof twee interessante hits aan. De eerste hit (dit is beschreven in **Hoofdstuk 6**) was Azitromycine, dat de groei van bacteriën (antibioticum) remt. Het wordt gebruikt bij luchtweginfecties, zoals infecties van longen, huidinfecties, geslachtsziekten (gonorroe en chlamydia), Lymeziekte (tekenbeetziekte) en reizigersdiarree. Maar het remt ook de vermenigvuldiging van Hepatitis E en kan dus aantrekkelijk zijn bij het ontwerpen van nieuwe behandeling van deze ziekte, zeker voor de Derde Wereld. Dit resultaat wist ik te publiceren in het vaktijdschrift *International Journal of Antimicrobial Agents*. De tweede hit (zie hiervoor het **hoofdstuk 7**) betrof Ivermectin, een anti-parasitair middel wat ook de aandacht trekt bij de bestrijding van het Coronavirus. Dit laatste aspect heb ik niet onderzocht, maar wat betreft het eerste, ik kon laten zien dat het middel voorkomt dat het genoom van het hepatitis E virus de kern van de gastheercellen binnen dringt. Daardoor kan het virus zich niet vermenigvuldigen. Ook dit middel kan dus een goede optie zijn om verder te onderzoeken wat betreft haar mogelijkheden om Hepatitis E te beschrijden. Deze resultaten heb ik wereldkundig gemaakt middels een publicatie in het vaktijdschrift *Archives of Virology*.

Een overkoepelende discussie van alle resultaten in dit proefschift en hun plaats in het contemporaine biomedische literatuurlandschap is te vinden in **hoofdstuk 8**. Ik concludeer in dit hoofdstuk dat ik inderdaad vooruitgang heb geboekt in het gevecht der mensheid met virale ontsteking van de lever.

Appendix

Publications

PhD Portfolio

Acknowledgements

Curriculum Vitae

Publication list

1. **Zhijiang Miao**[1], Zhenrong Xie[1], Li Ren, Qiuwei Pan*. Combating hepatitis D: advances and challenges, **Chinese Medical Journal**, 2021 (Invited Review). (IF=2.6; JCR Q3)
2. Ziqin Dian, Yi Sun, Guiqian Zhang, Ya Xu, Xin Fan, Xuemei Yang, Qiuwei Pan, Maikel Peppelenbosch and **Zhijiang Miao***. Rotavirus-related systemic diseases: clinical manifestation, evidence and pathogenesis, **Critical Reviews in Microbiology**, 2021. (IF=7.6; JCR Q1)
3. **Zhijiang Miao**, Ruyi Zhang, Peifa Yu, Yang Li , Qiuwei Pan* and Yunlong Li*. The macrolide antibiotic azithromycin potently inhibits hepatitis E virus in cell culture models, **International Journal of Antimicrobial Agents**, 2021 Jun 19:106383. (IF=5.3; JCR Q1)
4. **Zhijiang Miao** and Qiuwei Pan*. Revisiting the estimation of hepatitis D global prevalence, **Journal of Hepatology**, 2020. (IF=20.6; JCR Q1)
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6. **Zhijiang Miao**, Shaoshi Zhang, Zhongren Ma, Mohamad S. Hakim, Wenshi Wang, Maikel P. Peppelenbosch and Qiuwei Pan*, Recombinant identification, molecular classification and proposed reference genomes for hepatitis delta virus, **Journal of Viral Hepatitis**, 2018. (IF=4.2; JCR Q2)
7. **Zhijiang Miao**, Yunlong Li, Peifa Yu, Bingting Yu, Maikel P. Peppelenbosch and Qiuwei Pan*, The dynamics of hepatitis delta virus prevalence and its potential association with hepatitis B virus vaccination, **Clinics and Research in Hepatology and Gastroenterology**, 2021. (IF=2.9; JCR Q3)
8. **Zhijiang Miao**[1], Li Gao[1], Yindi Song, Ming Yang, Mi Zhang, Jincheng Lou, Yue Zhao, Xicheng Wang, Yue Feng*, Xingqi Dong* and Xueshan Xia*. Prevalence and clinical impact of human pegivirus-1 infection in HIV-1-infected individuals in Yunnan, China. **Viruses**, 2017. (IF=3.5; JCR Q2)
9. **Zhijiang Miao**[1], Zhenrong Xie[1], Jing Miao, Jieyu Ran, Yue Feng* and Xueshan Xia*, Regulated Entry of Hepatitis C Virus into Hepatocytes. **Viruses**, 2017. (IF=3.5; JCR Q2)
10. Yunlong Li, **Zhijiang Miao**, Pengfei Li, Ruyi Zhang , Denis E Kainov , Zhongren Ma , Robert A de Man, Maikel P Peppelenbosch and Qiuwei Pan*, Ivermectin effectively inhibits hepatitis E virus replication, requiring the host nuclear transport protein importin α1. **Archives of Virology**, 2021. (IF=2.6; JCR Q3)
11. Peifa Yu, **Zhijiang Miao**, Yang Li, Maikel P. Peppelenbosch, Qiuwei Pan*. cGAS-STING Effectively Restricts Murine Norovirus Infection But Antagonizes the Antiviral Action of

N-terminus of RIG-I in Mouse Macrophages. **Gut Microbes**, 2021. (IF=10.2; JCR Q1)

12. Ling Wang, Jiaye Liu, **Zhijiang Miao**, Qiuwei Pan* and Wanlu Cao*. Lipid droplets and their interactions with other organelles in liver diseases. **International Journal of Biochemistry & Cell Biology**, 2021. (IF=5.1; JCR Q2/3)
13. Peifa Yu, Yang Li, Yunlong Li, **Zhijiang Miao**, Maikel P. Peppelenbosch and Qiuwei Pan*. Guanylate-binding protein 2 orchestrates innate immune responses against murine norovirus and is antagonized by the viral protein NS7. **Journal of Biological Chemistry**, 2020. (IF=4.2; JCR Q2)
14. Peifa Yu, Yang Li, Yunlong Li, **Zhijiang Miao**, Yining Wang, Maikel P. Peppelenbosch and Qiuwei Pan*. Murine norovirus replicase augments RIG-I-like receptors-mediated antiviral interferon response. **Antiviral Research**, 2020. (IF=4.1; JCR Q1/2)
15. Xumin Ou, Buyun Ma, Ruyi Zhang, **Zhijiang Miao**, Anchun Cheng, Maikel P. Peppelenbosch and Qiuwei Pan*. A simplified qPCR method revealing tRNAome remodeling upon infection by genotype 3 hepatitis E virus. **FEBS letters**, 2020. (IF=2.2; JCR Q3)
16. Yindi Song[1], Yue Feng[1], **Zhijiang Miao**, Binghui Wang, Ming Yang, A-Mei Zhang, Li Liu and Xueshan Xia*. Near-Full- Length Genome Sequences of a Novel Hiv-1 Circulating Recombinant Form, Crf01_Ae/B'/C(Crf78_Cpx), in Yunnan, China. **AIDS research and human retroviruses**, 2016. (IF=1.9; JCR Q4)
17. Yanping Li[1], Jing Miao[1], **Zhijiang Miao**, Yindi Song, Min Wen, Ya Zhang, Shimin Guo, Yuan Zhao, Yue Feng* and Xueshan Xia*. Identification of a Novel HIV Type 1 Circulating Recombinant Form(CRF86_BC)Among Heterosexuals in Yunnan, China. **AIDS research and human retroviruses**, 2016. (IF=1.9; JCR Q4)
18. Li Ren, Binghui Wang, **Zhijiang Miao**, Pan Liu, Shiyi Zhou, Yun Feng, Shuting Yang, Xueshan Xia* and Kunhua Wang*, A correlation analysis of HHV infection and its predictive factors in an HIV-seropositive population in Yunnan, China, **Journal of Medical virology**, 2019. (IF=2.0; JCR Q4).

PhD Portfolio

Name of PhD student	Zhijiang Miao
Department	Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam
PhD Period	October 2017 – October 2021
Promotor	Prof.dr. Maikel P. Peppelenbosch
Copromotor	Dr. Qiuwei Pan

PhD training

Seminars

- 2017-2021, Weekly MDL seminar program in experimental gastroenterology and hepatology (attending); (42 weeks/year; @1.5 h) (ECTS, 9.0).
- 2017-2021, Weekly MDL seminar program in experimental gastroenterology and hepatology (presenting); (preparation time 16 h; 2 times/year) (ECTS, 4.6).
- 2017-2021, Biweekly research group education (attending); (20 times/year; @1.5 h) (ECTS, 4.3).
- 2017-2021, Biweekly research group education (presenting); (preparation time 8 h; 4 times/year) (ECTS, 4.6).

General Courses and Workshops

- 2018, Course in Virology 2018 (ECTS, 1.4).
- 2018, SCORE PhD course: Stem Cells, Organoids & Regenerative Medicine (ECTS, 1.0).
- 2018, English Social Skills Course March-April 2018
- 2019, Basic course on R (ECTS, 1.8).
- 2019, Workshop presenting skills for PhD students and Post Docs (ECTS,1.0).
- 2019, The Introduction in Confocal Course.
- 2020, Microscopic Image Analysis: From Theory to Practice (ECTS, 0.8).
- 2020, Biomedical English Writing Course for MSc and PhD students (ECTS, 2.0).

- 2021, The Course Scientific Integrity.

Teaching Activities

- May-August 2018, Supervising two Junior Med School students' theses; (20 h/week)
- May-August 2019, Supervising two Junior Med School students' theses; (20 h/week)

Conferences

- 2017-2020, Annual Day of the Molecular Medicine, Postgraduate School Molecular Medicine, Rotterdam, the Netherlands.

Serving as Reviewer of Scientific Journals

- Liver International (IF=5.2; JCR Q1); 2019, handled 1 paper.
- AIDS research and human retroviruses (IF=1.9; JCR Q4); 2020-2021, handled 3 papers.

Direct Leaded & Guided Grants (Total:¥ 530,000 (≈ $ 83 000))

- "云南省丁型肝炎病毒分子流行病学及其演化变异规律研究(Molecular Epidemioloigical Study of Hepatitis Delta Virus in Yunnan and Its Evolution)", National Natural Science Foundation of China (NSFC), 2020, **¥ 350,000**;
- "丁型肝炎病毒核酸检测新技术研发团队建设(R&D Workshop for Novel Hepatitis D Virus Nucleic Acid Detection Technology)", Chenggong District, Kunming City, 2020, **¥ 80,000**;
- "HPgV-2 遗传变异规律及基因型分类系统研究 (Study on Genetic Variation and Genotype Classification System of HPGV-2)", Yunnan Province, 2020, **¥ 100,000**.

Awards & Honors

- 2017, China Scholarship Council (CSC) Scholarship (File No. 201708530234)
- 2017, Scholarship of Academic Excellence (Grade 1), KMUST (¥ 8,000).

- 2016, Yunnan Provincial Scholarship, Yunnan (¥ 10,000).
- 2015, Outstanding Freshman Scholarship (Grade 2), KMUST (¥ 5,000).

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To **Dr. Shan Li**, I regret we did not meet earlier, too. In your research, you are cautious, strict and diligent. In your life, you are bright, optimistic and enthusiastic. To me, only god knows how much help, support and care you provided! Any words cannot express my gratitude for you. How many times did we drink together, talk together, and inspire and support one another? Since you are a gentle and polite man, a good husband, you have met a beautiful and virtuous wife, **Ms. Fang Wang**, who can understand you, support you and give you enough freedom! You know how much you pleased me by naming me the "Godfather" of your adorable little **princess Jiayue** (Chenchen); it is a great honor and good fortune for me! Another piece of good news is that your **prince** is on his way, and indisputably I am his "Godfather", too. Good job! In the past, current, and the future, you will always be my big brother. There is so much pleasure and memory, I fell it and fell it good, I remember it and will remember it forever. Wish you all the best of luck and I am confident you will have a bright and prosperous future.

To **Xiangyu**, **Xuejin** and your lovely daughter **Yiyi**, as well as **Qiyu** and **Duanyang-Yanmin** (and your baby **Zhuangzhuang**), a million thanks for bringing me so much fun and excitement. Because of you, my doctoral life escaped being tedious, it also had dinner parties, BBQ, drinking, talking, playing cards. All made my life far colorful and unforgettable in Rotterdam. Good luck!

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To **Dr. Na Zhan**, you are a happy girl, best wishes to you and hope you can graduate soon. Keep in touch and see you around in China. Oh, welcome to Kunming!

To **Dr. Jiejing Shi** and **Dr. Peiqian Liu**, it is such a nice opportunity to meet and be friends with you, keep us in touch and see you around in China.

To **Dr. Li Zhang**, special thanks to you for your encouragement, support and concern. Although it is a pity that you have left Kunming and returned to work in your hometown (Xinxiang), I am happy for you that you got a good job and bought yourself a house. Congratulations! See, you can actually do it very well. So, please be more confident! Hope you live a happy life there and best wishes to you.

To **Dr. Zhenrong Xie** (Head of Biobank, the First Affiliated Hospital of Kunming Medical University), my significant appreciation should go to you. You supervised and taught me a lot during my Bachelor's study, and we even worked together for a while during my Master's study, and finally thanks to your introduction and recommendation I successfully got my doctoral study chance at Erasmus MC. You just fully trust me and support me without ever asking for anything in return. This is also true for my job hunting! I find no words or an other approach for expressing my appreciation to such pure care and affection. But my heart knows the truth. You, as a woman, never really impressed me, but your bravery, assertiveness, fortitude, and ability deserves my respect. I am so pleased to see that you have already made great strides in establishing a human medical sample bank in Kunming. I also hope my return will contribute to this great work as well. You are not alone, you have got my word! Hold on! I am coming!

To two unforgettable women who were very important to my Master's study and abroad study, **Ms. Yingdi Song** and **Dr. Fengmei Zhou**, both of you have undoubtedly believed in me and in my future, and your words remained in my head, always giving me tremendous confidence and strength to do my job better. Best wishes to you and your loved ones.

To **my love lost**, you ever leaving me means that winning the world is pointless and it means less to me than you disappearing from my life. I can only hope you have a happy family, and, yes, I actually finished my PhD study abroad (just as I ever told you).

To **Dr. Li Ren** (Department of Obstetrics and Gynecology, The First People's Hospital of Yunnan Province) and **Specialist Pan Liu** (Department of Urology, Yan'an Hospital of Kunming Chenggong Hospital), really thank you for your assistance, contribution and collaboration. I am so glad and lucky that we have formally allied and continued as a research team in Kunming in 2020. The best part is that we have successfully applied for three grants so far, bringing our overall funding to ¥ 530,000. This money will allow us develop our projects and studies in Kunming. But this is, I believe, only just the beginning and the future will surely bestow even greater things on us.

To **Dr. Ziqin Dian** (Department of Clinical laboratory, The First People's Hospital of Yunnan Province) and **Specialist Xiaoyan Xu** (Department of Pediatrics, The First People's Hospital of Yunnan Province), thank you for your cooperation, which allow me to broaden my research area to Rotaviruses and associated systemic diseases. Hopefully, we will be able to develop our collaboration and start Rotaviruses-related cohort studies in Kunming soon.

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To my good friends who live in China but cared for and supported my Dutch life and doctoral study far too much and far too often, **Jieyu Ran**, **Jing Miao**, **Qi Wang**, **Chunyue Zhang**, **Chongmao Bao** (you just acted as my personal bank, every time when I had economic problem then you proved a reliable solution; and you also responsed all my requests in life), **Hebiao Huang** (and your wife **Lei Zhu**), **Guangrui Gu** (and your wife **Caixu Duan**), **Mingchao Mao**, **Yahong Lv**, **Chunyan Nie**, **Jia long**, **Yuhou Li**, **Ruixiang He**, **Huaxian Yang**, **Lichun Kong**, **Huifen Wei**, **Zengguang Duan**, **Yuncai Chen**, **Zhiyuan Ding**, **Xianling Shan**, **Chunfeng Yin**, **Luxiong Wang**, **Hao Pu**, and there are **too many people to be named here**. You gave me the happy feeling of being missed, which is a fortunate thing. Even a few simple words or short conversations were always enough to warm my heart, bring me peace, and inspire me to face and overcome difficulties. Without your attention, company and inspiration I would not have made it! Wish all of you have a bright future and a happy life. I cannot wait to see you all again.

Finally, my most significant gratitude should go to **my parents**, who not only gave life to me and raised me, but also gave me the opportunity to receive good education. You had great expectations during my youth and now I am so proud I can tell you that I do have not disappointed you, I have made it! Nonetheless, of course it is depressing to think of the company lost as a result of my university education and my studies abroad, but now I shall be in the position to provide you with a good and happy life. My only request and wish for you is that you please stay healthy and enjoy life to the fullest! Also, **my sister**, **Qiuxue Miao** (with your husband, **Yongfeng Ge**, a typical Shandong Good-Man), I am so grateful that you played the parental role for so many times in my life, that you also took on the burden to take care for our parents over the past seven years, and that you prepared so many wonderful meals for me when I was in your home. You have my word that I will repay your dedication by making a contribution to the education of your son, **Hongqi Ge**, my charming nephew, born with intellect. Please stay healthy and have a happy life. In addition, **my aunts** (**Yuexian Miao**, **Shilian Miao** and **Donglian Miao,** as well as **Meihua Lu**), and my cousins **Shangli Ning**-**Huazhong Gao**, **Chunyan Song**-**Zonghu Li**, **Chunrui Song**, **Jing Lu**, **Feng Lu**, **Zhifang Miao**, as well as **all the rest of my relatives**, your words and care have always been so warm and comforting, this was the inspiration to try to be unstoppable brave and strong. Best wishes to you and your family. The good news is that I have decided to work in Kunming, which means I will be closer to you and we will have more family time together. What a wonderful and happy thing!

Curriculum Vitae

Zhijiang Miao (缪志江), was born on July 12, 1990, in Xuanwei, Yunnan province, China. He grew up and finished his primary, secondary, and high school in his hometown.

In 2010, he moved to Kunming to start his Bachelor's study in Biotechnology at Yunnan Normal University. Out of keen interest in biological scientific research, he learned cell culture technology in the Developmental Biology Lab and molecular clone technology at Biological Engineering Center during this period.

In 2014, he initiated his Master's study in Biochemistry and Molecular Biology at the Faculty of Life Science and Technology, Kunming University of Science and Technology. In the Lab of Molecular Virology, under the co-supervision of Prof. Yue Feng and Prof. Xueshan Xia (Head of Lab), he received formal and systematic scientific training in Molecular Epidemiology and Molecular Virology. He graduated in 2017 after successfully completing his research projects on human pegiviruses (HPgVs) and hepatitis C virus (HCV).

Between 2017-2021, he proceeded with PhD training at the Department of Gastroenterology and Hepatology, Erasmus University Medical Center Rotterdam, the Netherlands, thanks to scholarship funding from the China Scholarship Council. Under the supervision of Dr. Qiuwei Pan and Prof. Maikel P. Peppelenbosch, he worked primarily on the molecular epidemiology of hepatitis D virus (HDV) and the antiviral research of hepatitis E virus (HEV), with the positive intention of contributing to the Global Hepatitis Elimination Program.

To better implement his research ideas and improve his academic leadership skills, he organized and built a research team in Kunming in 2020, the crews enrolled comprising doctors and clinicians from The First People's Hospital of Yunnan Province (Departments of Clinical laboratory/Obstetrics and Gynecology/Pediatrics) and Yan'an Hospital of Kunming Chenggong Hospital (Department of Urology). Under his leadership and guidance, together with several team members, successful applications for national, provincial and regional grants were performed, receiving in total more as ¥ 530,000 (≈ $ 83 000).

In conclusion, as a self-motivated and open-minded young scholar, he has shown strong practical ability and good team skills, and he has fostered a broad spectrum of specialties, including liver diseases caused by HCV, HDV, HEV, HPgV-1 and HPgV-2, as well as gastroenteritis induced by enteric Rotavirus infection.

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