Classification and sequencing of hepatitis D virus from a large cohort of chronically infected individuals paired with co-infecting hepatitis B virus sequencing: a genomic characterisation study

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Summary

Background The most severe form of viral hepatitis is caused by co-infection of hepatitis D virus (HDV) and hepatitis B virus (HBV). Phylogenetic analyses classify HBV and HDV into eight major genotypes: HBV GTA to GTH and HDV GT1 to GT8. Paired HBV and HDV sequencing data from participants with chronic hepatitis delta are scarce. We aimed to sequence and genotype HDV and HBV from a large cohort of participants from clinical studies and diverse countries of origin.

Methods 407 participants with chronic hepatitis D from 24 countries were characterised (124 participants from MYR301 clinical trial, 93 from MYR204, 114 from MYR202, and an additional 76 participants from diverse geographical locations). HBV and HDV from participants were analysed using sequencing, enzyme immunoassay, or both to determine HBV and HDV genotypes. BLAST analysis and phylogenetics were used to determine HBV and HDV genotypes with reference sequence libraries. Bulevirtide treatment response (measured by HDV RNA decline and normalisation of alanine aminotransferase) was compared by genotype for MYR trial participants.

Findings HDV sequencing assays were successful for 386 (95%) of 407 participants and HBV sequencing or serology-based HBV genotyping assays were successful for genotyping 395 (97%) participants. For individual genotypes, HBV GTD (336 [83%] participants) and HDV GT1 (364 [89%]) were the most prevalent. For paired HBV–HDV genotypes, HBV–HDV D/1 was most common (320 [79%] of 407) followed by A/1 (30 [7%]). Phylogenetic analyses of HDV full-genome sequences showed distinct clusters of sequences within HDV GT1, and four novel provisional HDV GT1 subgenotypes, HDV GT1fp to HDVGT1ip, were identified. For 218 MYR clinical trial participants, bulevirtide treatment response was similar across HDV GT1 subgenotypes (both established and newly identified).

Interpretation Novel HDV subgenotypes identified in this study indicate a greater genetic diversity of HDV GT1 than previously recognised. This knowledge will be important for developing better diagnostics, and in understanding HDV genotype-specific biology and response to treatment. More extensive HDV sequencing from under-sampled regions, such as Africa, is needed to determine the true breadth of HDV sequence and genotype diversity.

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For **WHO data on HBV** see https://www.who.int/news-room/fact-sheets/detail/hepatitis-b

Introduction

WHO estimates that almost 300 million individuals are living with hepatitis B virus (HBV), making it one of the most globally prevalent chronic viral infections. Chronic HBV can result in complications such as cirrhosis and hepatocellular carcinoma.[1,2] Co-infection with hepatitis D virus (HDV), a small RNA virus that requires HBV envelope proteins for hepatocyte entry and viral propagation, can result in more severe disease outcomes than HBV mono-infection.[3] A 2020 meta-analysis by Stockdale and colleagues[4] estimates that among HBsAg-positive individuals, 4·5% are also HDV positive, which would equate to approximately 12 million people worldwide. The highest prevalence of HDV is in Mongolia, and the largest absolute number of HDV-positive individuals is in China.[5]

The HBV genome is a circular, partially double-stranded DNA molecule of around 3·2 kb.[6] Nucleotide sequence diversity groups HBV into eight confirmed genotypes (GTA to GTH).[7] HBV genotypes have distinct global geographical distribution, with HBV GTD distributed globally but most commonly found in Europe, the Middle East, and the Indian subcontinent.[7] HBV GTA circulates in Africa, Europe, and the Americas, GTB and GTC in Asia, and GTF and GTH in South and Central America.[7]

The HDV RNA genome is around 1·7 kb long and encodes two forms of hepatitis D antigen (HDAg): small HDAg and large HDAg.[8,9] Phylogenetic analyses of nucleotide

Research in context

Evidence before this study

We conducted a PubMed and GenBank search for all publicly available hepatitis D virus (HDV) sequences. The PubMed search was from database inception to March 3, 2025. Keywords included "Hepatitis D", "molecular epidemiology", "chronic Hepatitis D", "Hepatitis Delta Antigen", "Large Hepatitis Delta Antigen", "HBV/HDV coinfection", "Hepatitis D prevalence", and "global genotype distribution", in various combinations. GenBank was searched by organism taxonomic identifier for HDV, then filtered by nucleotide completeness in NCBI Virus. Within both searches, there was a paucity of publications related to HDV genotype distribution. Previous studies were limited to particular geographical regions and HDV genotypes were described only in the introduction and discussion sections. Previous literature also contained little information related to the hepatitis B virus (HBV) genotypes in individuals with HBV–HDV co-infection.

Added value of this study

This study provides the largest number of full-genome HDV sequences (n=386) to date, and these new sequences have confirmed the five HDV GT1 subgenotypes (HDV GT1a to GT1e) reported previously. Of note, new HDV GT1 subgenotypes have also been identified, suggesting greater genetic diversity for HDV GT1 than previously thought. This study also provides 377 paired HBV–HDV genotypes from clinical samples.

Implications of all the available evidence

This study represents the largest dataset of full-genome HDV sequences and the second largest number of HBV–HDV genotype determinations in clinical samples to date. With future HDV therapeutic options becoming available, understanding the genotype distribution will be essential to ensure treatment efficacy across different populations. Additionally, these findings suggest the need for pan-genotypic HDV RNA quantification assays. Finally, this evidence highlights the importance of testing all individuals with HBV for HDV infection and further research will enable a better understanding of the distribution of HDV genotypes.

sequence diversity characterise HDV into eight major genotypes numbered one to eight (HDV GT1 to HDV GT8).[10] HDV GT1 is globally the most prevalent and geographically widespread.[4] The other seven HDV genotypes are associated with distinct geographical regions, with HDV GT2 and HDV GT4 mainly confined to east Asia, HDV GT3 to Latin America, and HDV GT5 to GT8 to Africa.[4] Previous reports have indicated that the majority of HDV genotypes have at least two subgenotypes.[11] For HDV GT1, Karimzadeh and colleagues[12] identified five subgenotypes: HDV GT1a and GT1b, which are more prevalent in sub-Saharan Africa; HDV GT1c, found in central Asian countries such as China, Viet Nam, Japan, and Taiwan; HDV GT1d, more commonly observed in Europe and Asia; and HDV GT1e in European countries Spain, Italy, Germany, and Romania.

HDV is highly sequence divergent, with sequence variability reaching up to 40% over the full genome,[11] whereas HBV sequence diversity across the full genome reaches approximately 13% variability.[7] Limited genomic data are available for HDV (around 1300 sequences) in the public domain compared with HBV (around 10 000 sequences). Consequently, HBV–HDV paired genotype combination data are also scarce, with only approximately 2000 pairs found in the literature.[13] The majority of these HBV–HDV genotype pairs were recently identified by Gerber and colleagues,[14] with HBV–HDV genotype D/1 as the most prevalent combination. In our study, HBV and HDV samples from 407 participants were sequenced and the paired genotypes of 377 participants were determined. To date, this is the largest HDV full-genome sequencing study. Extensive characterisation of HDV sequence diversity will inform the design of more sensitive HDV diagnostic assays. Importantly, knowledge of HDV viral diversity will be important for understanding HDV genotype-specific biology and response to treatment.

See Online for appendix

Methods

Participants

Overall, 407 participants with HBV–HDV co-infection originating from 24 countries across three continents (Africa, Europe, and Asia) were characterised. Baseline plasma samples were collected from consenting participants with chronic hepatitis D enrolled in Gilead Sciences (Gilead) clinical studies MYR301 (NCT03852719; n=124), MYR204 (NCT03852433; n=93), and MYR202 (NCT03546621; n=114), and from an additional 76 participants with HBV–HDV co-infection originating from geographical locations that potentially harboured rarer, non-GT1 HDV genotypes (hereafter referred to as the diverse cohort; appendix p 1). Baseline clinical characteristics for MYR301,[15] MYR204,[16] and MYR202[17] participants have been previously described and clinical study protocols are provided in the appendix (p 5), along with participant ancestry.

HBV and HDV reference sequence libraries

To add the most diverse set of sequences to the HDV genotyping reference library, any full-genome HDV sequence in the public database having greater than or equal to 5% difference in sequence identity within its genotype was added to the reference library. The final library contained the most diverse set of sequences (n=419) covering all HDV genotypes and major phylogenetic clusters from publicly available data. The HBV reference library

consisted of HBV reference sequences representing all genetic diversity previously identified by Kramvis.[7]

Nucleic acid extraction and cDNA synthesis

HDV RNA was extracted from plasma samples using the chemagic Viral DNA/RNA 300 Kit H96 (PerkinElmer, Shelton, CT, USA) and cDNA synthesis was performed with SuperScript IV (Thermo Fisher Scientific, Waltham, MA, USA) and random hexamers. For HBV, total nucleic acid extraction from plasma samples was performed using the chemagic Viral DNA/RNA 300 Kit H96 or the Qiagen EZ Virus Mini Kit (Qiagen, Hilden, Germany) and the Qiagen EZ1 Advanced XL instrument. HBV cDNA synthesis was performed using the SuperScript IV VILO Master Mix (Thermo Fisher) and the Set 1 outer R primer (appendix pp 10–11).

HDV viral load quantification

HDV RNA levels were measured with the Robogene HDV RNA Quantification Kit 2.0 (Roboscreen, Leipzig, Germany) for MYR301, MYR204, and MYR202 clinical trial participants.[18] For the diverse participant group, HDV RNA viral load was measured using the Eurobioplex HDV kit (Eurobio Scientific, Les Ulis, France).[11] Assay limits are provided in the appendix (p 1). GraphPad Prism version 10.1.2 was used to perform an unpaired two-tailed Student's *t* test when comparing viral loads.

HDV amplification and sequencing

The full HDV genome was amplified by RT-PCR as two overlapping amplicons using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Primers HDV-57 and HDV-08 were used for amplicon 1 and HDV-69 and HDV-54 were used for amplicon 2.[19] PCR cycling parameters are provided in the appendix (pp 1–2). Amplicons were processed and sequenced using the Illumina-MiSeq sequencing platform (Illumina, San Diego, CA, USA). To determine HDV sequence from plasma without HDV-specific amplification, total plasma RNA was sequenced using the Ovation RNA-Seq System V2 kit (NuGEN/Tecan, Männedorf, Switzerland) as described previously[20] and in the appendix (pp 2–3). An overview of the next-generation sequencing analysis pipeline is provided in the appendix (pp 11–12). GenBank accession numbers for sequences are provided in the appendix (p 4).

HBV amplification and sequencing

Synthesised cDNA along with total viral nucleic acids were used in a nested PCR reaction to amplify the full genome or small overlapping regions of large HBsAg (appendix pp 10–11). Standard PCR amplification of HBV from virally suppressed participants is challenging due to low HBV DNA levels. Additionally, individuals with HBV–HDV co-infection exhibit HBV replication suppression.[21] To increase HBV amplification efficiency, an ultrasensitive HBV amplification assay using both HBV DNA and RNA was used.[22] Given that PCR amplifies shorter fragments more efficiently than longer ones, five small (169–482 bp) overlapping fragments across the large HBsAg coding region were targeted for amplification (appendix pp 10–11). The assay also used a 60% reduction in thermal cycler ramping rate from annealing to extension to increase the likelihood of primer binding. PCR cycling parameters are provided in the appendix (pp 1–2). Amplicons were further processed for next-generation sequencing, which included library preparation, multiplexing, and deep sequencing using the Illumina-MiSeq deep sequencing platform. HBV sequences are available upon request made to the corresponding author.

Genotype determinations

For HDV, a BLAST cutoff (percent identity threshold) for confident genotype assignment was determined. All sequences in the reference dataset were evaluated for closest intragenotype match and closest intergenotype match. The threshold for HDV genotype calling was determined to be less than 15% sequence divergence. Participant HDV next-generation sequencing consensus sequences were used to determine genotype based on BLAST analyses against the HDV genotyping reference library. All HDV sequences from the reference library and participant sequences were included to create a cumulative tree using FastTree software, employing the Jukes–Cantor model with 1000 bootstraps, as described in the appendix (pp 3–4). If needed, participant genotypes were further confirmed by identifying the closest phylogenetic cluster of genotype-specific reference sequences in the cumulative tree.

For HBV, consensus sequences from next-generation sequencing were used to determine HBV genotypes based on BLAST analyses against a diverse set of HBV sequences representing all HBV subgenotypes. A BLAST cutoff of 8% was used to confidently assign genotype at the nucleotide level for full-length HBV sequences. Additional cutoffs are provided in the appendix (p 4). For participants that did not have sequencing coverage, a serology-based HBV genotyping assay (Cosmo Bio enzyme immunoassay HBV genotyping kit; Cosmo Bio, Tokyo, Japan) was used as an alternative method for HBV genotype determination. The kit is designed to determine HBV genotype (A–D) by detecting genotype-specific PreS2 epitopes.

HDV recombination

Recombination analysis was performed using Recombination Detection Program 5 (RDP5).[23] Recombination events were identified by each of the seven algorithms (RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, and 3seq) embedded in the RDP5 package. An HDV full-genome sequence was considered a potential recombinant based on the following criteria: (1) the recombination signal was supported by at least three out of seven methods with a p value of less than or equal to 0·05 after Bonferroni correction for multiple comparisons implemented in

	Total, n	HDV genotype, n (%)				
		GT1	GT2	GT5	GT6	Undetermined
Europe	357	343 (84%)	6 (1%)	4 (1%)	1 (<1%)	3 (1%)
Russia	267	259 (64%)	6 (1%)	..	..	2 (<1%)
Romania	9	9 (2%)	..	..	..	0
Moldova	5	5 (1%)	..	..	..	..
Sweden	1	1 (<1%)	..	..	..	..
France	17	12 (3%)	..	3 (1%)	1 (<1%)	1 (<1%)
Italy	23	23 (6%)	..	..	..	..
Germany	35	34 (8%)	..	1 (<1%)	..	..
Africa	43	18 (4%)	0	9 (2%)	1 (<1%)	15 (4%)
Benin	2	2 (<1%)	..	..	..	..
Cameroon	11	6 (1%)	..	1 (<1%)	1 (<1%)	3 (1%)
Côte d'Ivoire	8	2 (<1%)	..	4 (1%)	..	2 (<1%)
Egypt	2	2 (<1%)	..	..	..	..
Guinea	3	..	..	2 (<1%)	..	1 (<1%)
Mali	4	1 (<1%)	..	1 (<1%)	..	2 (<1%)
Morocco	1	..	..	..	..	1 (<1%)
Niger	1	..	..	..	..	1 (<1%)
Mauritania	2	..	..	..	..	2 (<1%)
Central African Republic	2	1 (<1%)	..	..	..	1 (<1%)
Chad	1	1 (<1%)	..	..	..	..
Togo	2	1 (<1%)	..	..	..	1 (<1%)
Tunisia	1	1 (<1%)	..	..	..	..
Senegal	3	1 (<1%)	..	1 (<1%)	..	1 (<1%)
Other	7	3 (1%)	1 (<1%)	0	0	3 (1%)
Afghanistan	2	1 (<1%)	..	..	..	1 (<1%)
Lebanon	1	..	..	..	..	1 (<1%)
Türkiye	3	2 (<1%)	..	..	..	1 (<1%)
Unknown	1	..	1 (<1%)	..	..	..
Total	407	364 (89%)	7 (2%)	13 (3%)	2 (<1%)	21 (5%)

Percentages are calculated relative to the total number of participant samples (n=407). HDV=hepatitis D virus.

Table 1: **HDV genotypes by country or region of origin**

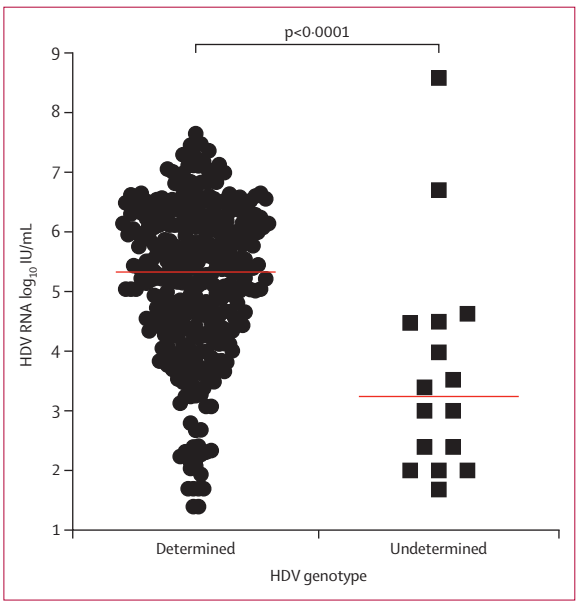

Figure 1: **HDV viral loads for participants with HDV genotype determinations**
The figure shows available HDV RNA levels for participants with successful genotype assignments (n=345) and available HDV viral loads for participants that were not successfully genotyped (n=16). Viral load was not available for some participants (n=46). The red line denotes the median value for each group. HDV=hepatitis D virus.

RDP5; (2) the recombinant segment was at least 200 bp in length. Weak recombination signals denoted by RDP5 output were excluded. A summary of the recombination analysis for each subgenotype sub-cluster is provided in the appendix (p 15). Similarity plots were used to confirm identified recombinants, as described in the appendix (p 4).

Bulevirtide half maximal effective concentration (EC_{50}) determination

The HDV entry inhibitor bulevirtide is a peptide derived from the pre-S1 region of large HBsAg that binds the HDV host entry receptor sodium taurocholate cotransporting polypeptide.[24,25] Bulevirtide EC_{50} values were determined as described previously, where bulevirtide EC_{50} values ranged from a mean of 0·20 nM to 0·73 nM against a panel of sequence-diverse HBV–HDV laboratory strains and clinical isolates.[26] Briefly, primary human hepatocytes were pretreated with bulevirtide, then infected with clinical plasma or an HDV laboratory strain. Immunofluorescence staining against HDAg was performed at day 5 post infection and the percentage of HDAg-positive cells relative to total cell numbers was calculated using HCS Studio Cellomics Scan software (version 6.6.2, Thermo Fisher Scientific, Waltham, MA, USA).

Role of the funding source

The funder was involved in study design, data collection, data analysis, and data interpretation. All authors contributed to the writing of the report. All authors had full access to the data and take responsibility for the integrity of the data and the accuracy of the analysis. The decision to submit for publication was made by the authors.

Results

Serum samples were collected from 407 participants with HBV–HDV co-infection from 24 distinct countries of origin and HDV full-genome amplification and sequencing were performed. HDV full-genome amplicon sequencing was successful for 367 (90%) of 407 participants (appendix p 13). HDV amplification was more successful for the 331 clinical trial samples (MYR301, MYR204, and MYR202) compared with the 76 samples from the diverse cohort (appendix p 13). HDV amplification was successful for 330 (97%) of 331 clinical trial participants and for 37 (49%) of 76 diverse cohort samples (appendix p 13). For a subset of participants from the diverse cohort for whom amplicon sequencing was not successful and who had remaining plasma (n=35), total plasma RNA sequencing using random primers was attempted. From the plasma RNA sequencing, 19 additional participants were successfully sequenced. Overall, HDV sequencing was successful

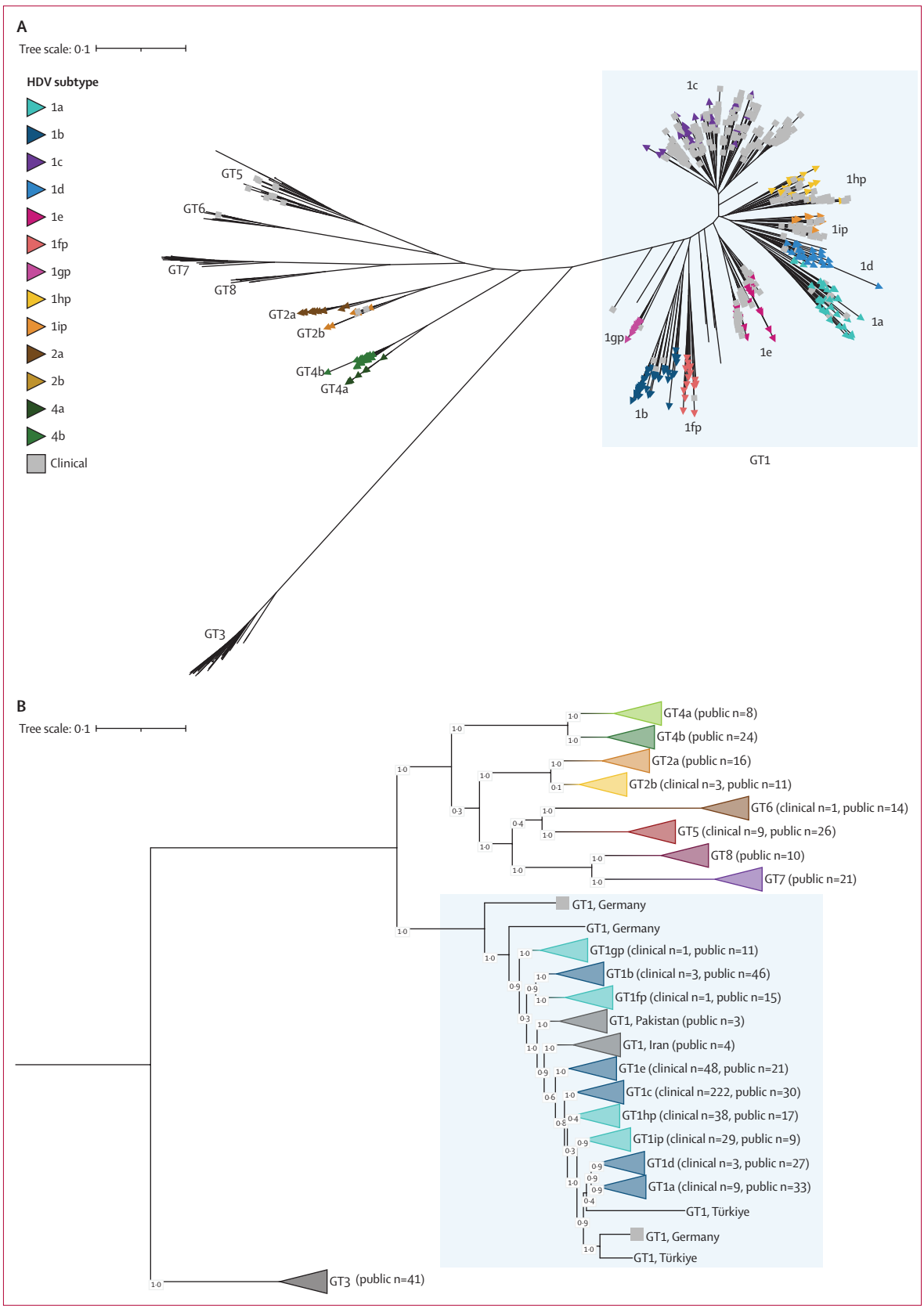

***Figure 2*: Expansion of HDV GT1 subgenotypes through phylogenetic analysis of clinical HDV sequences**
(A) An unrooted phylogenetic tree was generated with 757 HDV full-genome sequences (389 HDV public reference sequences and 368 clinical HDV sequences from this study) using FastTree software employing the Jukes–Cantor model with 1000 bootstraps. Established and provisional HDV subgenotypes are labelled. Branches identified as GT1, GT2, and GT4 HDV subgenotypes are shown with coloured triangles. Newly identified provisional subgenotypes GT1f to GT1i are denoted with a p in the key and on the tree label. (B) A maximum likelihood tree of 759 HDV full-genome sequences (390 HDV public reference sequences and 369 clinical HDV sequences from this study) rooted to HDV GT3 public sequences. The phylogenetic tree was generated with HDV full-genome sequences using FastTree software employing the Jukes–Cantor model with 1000 bootstraps. Branches are collapsed into genotype nodes depicted here as coloured triangles with the number of public and clinical sequences shown in parentheses. Nodes in the light blue box are HDV GT1 and are categorised as follows: HDV GT1 subgenotypes in dark blue indicate previously published subgenotypes HDV GT1a to GT1e,[12] and those in teal indicate the new provisional subgenotypes (GT1fp to GT1ip); nodes in grey are HDV GT1 sequences from Pakistan and Iran that did not cluster to an HDV GT1 subgenotype. Branches ending in a grey square are clinical sequences from this study that did not cluster to an HDV GT1 subgenotype. Also, public sequences that do not cluster to an HDV GT1 subgenotype are shown as GT1 with the country of origin. HDV genotype and subgenotype cluster features and genetic distances are described in the appendix (pp 6, 15). HDV=hepatitis D virus.

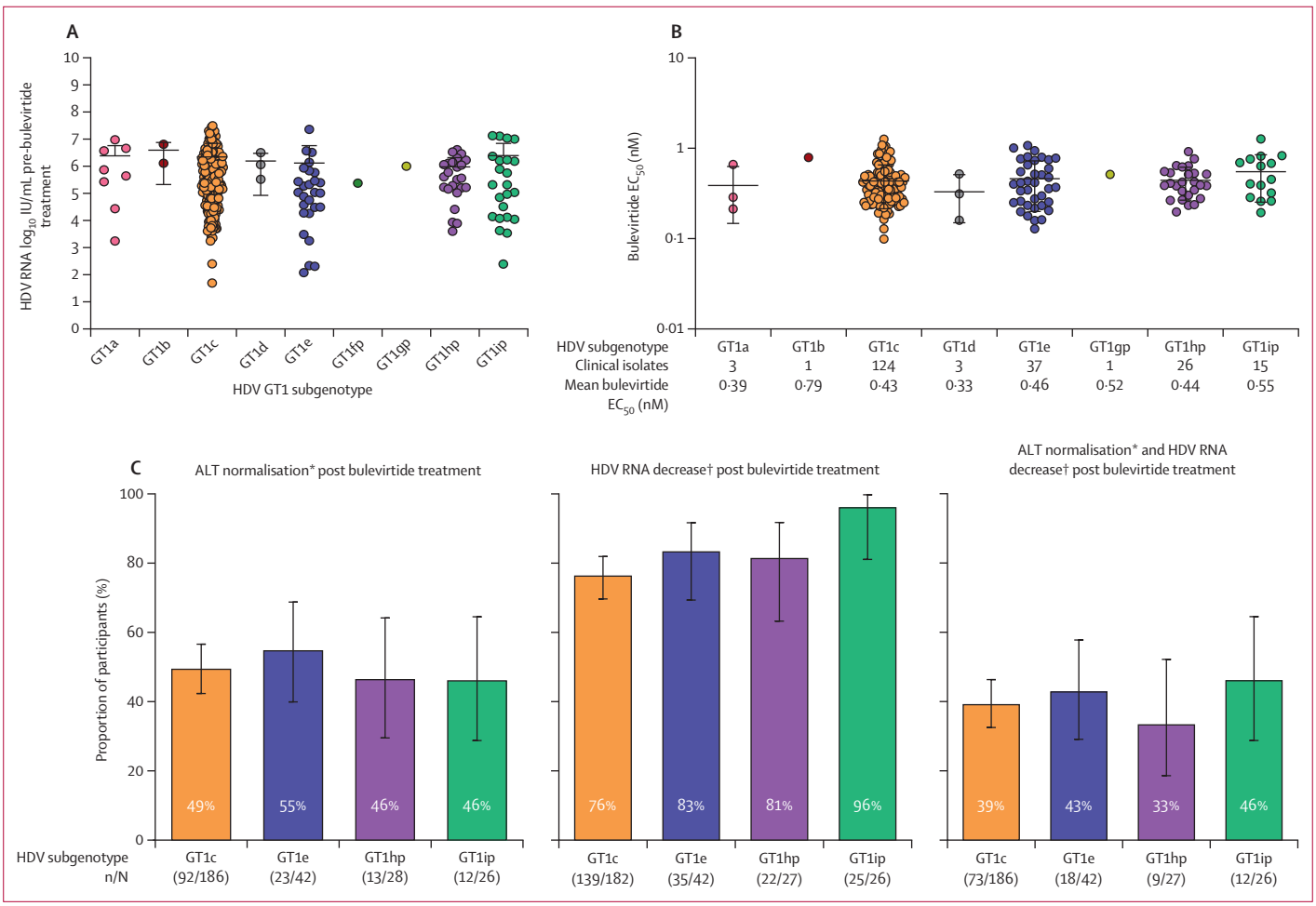

Figure 3: **HDV RNA levels, bulevirtide antiviral activity, and bulevirtide treatment response across genetically distinct HDV GT1 subgenotypes**
(A) HDV RNA levels for participants in this study across HDV GT1 subgenotypes with available data (GT1a n=8, GT1b n=2, GT1c n=179, GT1d n=3, GT1e n=27, GT1fp n=1, GT1gp n=1, GT1hp n=24, GT1ip n=23). Mean HDV RNA level and SD are indicated with a horizontal line and error bars. (B) Bulevirtide antiviral activity for clinical isolates across HDV GT1 subgenotypes. The number of clinical isolates tested and the mean bulevirtide EC_{50} value for each HDV GT1 subgenotype are indicated. Mean bulevirtide EC_{50} value and SD are indicated with a horizontal line and error bars. (C) Bulevirtide treatment response for MYR202, MYR204, and MYR301 participants with HDV GT1 subgenotype designations. The proportions of participants with ALT normalisation, HDV RNA decrease, and combined response (ALT normalisation and HDV RNA decrease) were plotted for MYR202 participants at week 24 of bulevirtide treatment and for MYR204 and MYR301 participants at week 48 of bulevirtide treatment. Participants with full datasets across any bulevirtide-containing treatment regimen were included. Error bars indicate the 95% CI. Clinical study designs are provided in the appendix (p 5). ALT=alanine aminotransferase. EC_{50}=half maximal effective concentration. HDV=hepatitis D virus. *ALT within normal range as assessed according to central laboratory criteria (≤31 U/L for women and ≤41 U/L for men in Russia; ≤34 U/L for women and ≤49 U/L for men in all other locations). †Serum HDV RNA reduction by ≥2 log_{10} IU/mL (or undetectable) from baseline to week 24 of bulevirtide treatment for MYR202 or from baseline to week 48 of bulevirtide treatment for MYR204 and MYR301.

for 386 (95%) of 407 participants using both amplicon and plasma RNA sequencing (appendix p 13).

We investigated potential causes of unsuccessful HDV amplification for 21 participants, who were mostly from the diverse cohort (20 of 21; table 1). HDV RNA viral loads were approximately two orders of magnitude higher (median of approximately 5·3 log_{10} IU/mL) in participants that had successful HDV genotype determination compared with participants with an undetermined genotype (approximately 3·2 log_{10} IU/mL), suggesting that viral load affected successful genotype determination (figure 1). Mismatches at primer binding sites could also cause failure of amplification. For the participants that were genotyped by total plasma RNA sequencing using random primers, the HDV amplification primer binding sites were evaluated to identify mismatches. For the participants that had sequencing coverage at the primer binding sites (n=15), seven had at least one mismatch in at least one primer binding site (appendix p 13).

PCR amplification with an ultrasensitive assay resulted in successful HBV amplification for 281 (69%) of 407 participants (appendix p 13). HBV amplification was successful for 247 (75%) of 331 clinical trial participants and 34 (45%) of 76 participants in the diverse cohort (appendix p 13). To determine HBV genotypes for samples in which the HBV ultrasensitive amplification assay failed, a genotyping

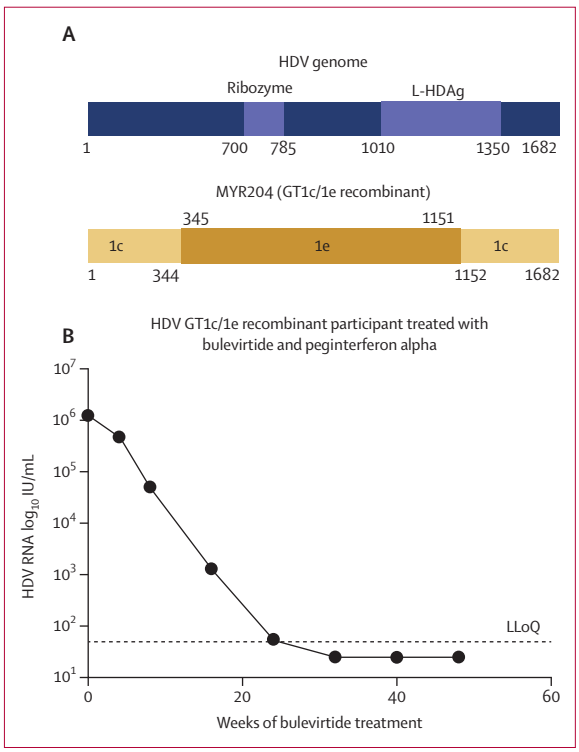

Figure 4: **HDV GT1c/1e recombinant sequence identified in MYR204**
(A) Schematic of the HDV genome with coordinates for the ribozyme and large L-HDAg region, and schematic of potential recombination events in the HDV genome of a MYR204 participant. Coordinates for the ribozyme and L-HDAg region are shown. Nucleotides on the top of the MYR204 participant schematic indicate the starting and ending nucleotides of the GT1e sequence, whereas nucleotides on the bottom indicate the starting and ending positions of the GT1c sequence. A detailed sequence alignment is provided in the appendix (p 7). (B) HDV RNA levels for the GT1c/GT1e MYR204 participant up to 48 weeks of 10 mg bulevirtide treatment with peginterferon alfa. HDV=hepatitis D virus. L-HDAg=large hepatitis D antigen. LLoQ=lower limit of quantification.

	Total, n	HBV genotype, n (%)					
		GTA	GTB	GTC	GTD	GTE	Undetermined
Europe	357	30 (7%)	1 (<1%)	1 (<1%)	315 (77%)	5 (1%)	5 (1·2%)
Russia	267	21 (5%)	..	..	245 (60%)	1 (<1%)	..
Romania	9	3 (1%)	..	..	6 (1%)	..	..
Moldova	5	..	..	..	4 (1%)	..	1 (<1%)
Sweden	1	1 (<1%)	..	..	..	..	..
France	17	3 (1%)	..	1 (0·2%)	9 (2%)	2 (<1%)	2 (<1%)
Italy	23	1 (<1%)	1 (<1%)	..	21 (5%)	..	..
Germany	35	1 (<1%)	..	..	30 (7%)	2 (<1%)	2 (<1%)
Africa	43	10 (2%)	..	..	14 (3%)	12 (3%)	7 (2%)
Benin	2	1 (<1%)	..	..	..	1 (<1%)	..
Cameroon	11	4 (1%)	..	..	3 (1%)	2 (<1%)	2 (<1%)
Côte d'Ivoire	8	..	..	..	4 (1%)	2 (<1%)	2 (<1%)
Egypt	2	1 (<1%)	..	..	1 (<1%)	..	..
Guinea	3	1 (<1%)	..	..	2 (<1%)	..	..
Mali	4	..	..	..	1 (<1%)	1 (<1%)	2 (<1%)
Morocco	1	..	..	..	1 (<1%)	..	..
Niger	1	..	..	..	..	1 (<1%)	..
Mauritania	2	1 (<1%)	..	..	..	1 (<1%)	..
Central African Republic	2	..	..	..	1 (<1%)	1 (<1%)	..
Chad	1	1 (<1%)	..	..	..	..	..
Togo	2	..	..	..	..	2 (<1%)	..
Tunisia	1	..	..	..	1 (<1%)	..	..
Senegal	3	1 (<1%)	..	..	..	1 (<1%)	1 (<1%)
Other	7	..	..	..	7 (2%)	..	..
Afghanistan	2	..	..	..	2 (<1%)	..	..
Lebanon	1	..	..	..	1 (<1%)	..	..
Türkiye	3	..	..	..	3 (1%)	..	..
Unknown	1	..	..	..	1 (<1%)	..	..
Total	407	40 (10%)	1 (<1%)	1 (<1%)	336 (83%)	17 (4%)	12 (3%)

Percentages are calculated relative to the total number of participant samples (n=407). HBV=hepatitis B virus.

Table 2: **HBV genotypes by country of origin**

enzyme immunoassay was performed. The enzyme immunoassay successfully determined 108 additional genotypes and the HBV genotypes for an additional six participants were determined from the plasma RNA sequencing that was performed for a subset of participants (appendix p 13). In total, using the HBV ultrasensitive amplification assay, genotyping enzyme immunoassay, and plasma RNA sequencing, 395 (97%) of 407 HBV participant genotypes were determined (appendix p 13).

HDV genotype determination from participant next-generation sequencing consensus sequences was based on BLAST analyses against an HDV reference sequence library using a 15% sequence identity cutoff, or phylogenetic clustering. To identify reference HDV sequences required to genotype participants using BLAST, the sequence diversity of publicly available HDV sequences was analysed. Within the public dataset, up to 22% intra-genotype divergence within HDV GT1 was observed and HDV GT2 to HDV GT8 had up to 12% divergence, although the number of sequences available for non-HDV GT1 genotypes was low (appendix p 14).

BLAST analyses identified HDV GT1 as the most prevalent HDV genotype (364 [89%] of 407 participants), followed by HDV GT5 (13 [3%]), HDV GT2 (seven [2%]), and HDV GT6 (two [<1%]; table 1). For the 19 participants that were genotyped using plasma RNA sequencing, five were HDV GT1a, four were GT1d, three were GT1e, two were GT1, two were GT5, one was GT6, and one was GT1b (appendix p 13). HDV GT1 participant sequences were observed in 19 of 24 countries, HDV GT5 in seven countries, HDV GT2 in Russia, and HDV GT6 in France and Cameroon (table 1). Most HDV GT5 participants (nine of 13) originated from Africa (table 1).

Phylogenetic analysis of full-genome HDV sequences from the reference sequence library, containing 389 full-genome sequences across all HDV genotypes, identified distinct clusters of HDV GT1 sequences (HDV GT1a to GT1e) with high bootstrap values (>75%), confirming the previous findings by Karimzadeh and colleagues.[12] Similarly, HDV full-genome sequences from our clinical dataset (n=368) were subjected to phylogenetic analysis using the

	Total, n	HBV–HDV genotype combinations, n (%)											
		A/1	A/2	A/5	A/6	B/1	C/1	D/1	D/2	D/5	E/1	E/5	Undetermined
Europe	357	25 (6%)	4 (1%)	..	1 (<1%)	1 (<1%)	1 (<1%)	310 (76%)	2 (<1%)	..	3 (1%)	2 (<1%)	8 (2%)
Russia	267	17 (4%)	4 (1%)	..	..	..	..	241 (59%)	2 (<1%)	..	1 (<1%)	..	2 (<1%)
Romania	9	3 (1%)	..	..	..	..	..	6 (1%)	..	..	..	..	..
Moldova	5	..	..	..	..	..	..	4 (1%)	..	..	..	..	1 (<1%)
Sweden	1	1 (<1%)	..	..	..	..	..	..	..	..	..	..	..
France	17	2 (<1%)	..	..	1 (<1%)	..	1 (<1%)	8 (2%)	..	..	1 (<1%)	1 (<1%)	3 (1%)
Italy	23	1 (<1%)	..	..	..	1 (0·2%)	..	21 (5%)	..	..	..	..	..
Germany	35	1 (<1%)	..	..	..	..	..	30 (7%)	..	..	1 (<1%)	1 (<1%)	2 (<1%)
Africa	43	5 (1%)	..	2 (<1%)	1 (<1%)	..	..	7 (2%)	..	3 (1%)	4 (1%)	2 (<1%)	19 (5%)
Benin	2	1 (<1%)	..	..	..	..	..	..	..	..	1 (<1%)	..	..
Cameroon	11	1 (<1%)	..	1 (<1%)	1 (<1%)	..	..	3 (1%)	..	..	1 (<1%)	..	4 (1%)
Côte d'Ivoire	8	..	..	..	..	..	..	1 (<1%)	..	2 (<1%)	..	1 (<1%)	4 (1%)
Egypt	2	1 (<1%)	..	..	..	..	..	1 (<1%)	..	..	..	..	..
Guinea	3	..	..	1 (<1%)	..	..	..	..	..	1 (<1%)	..	..	1 (<1%)
Mali	4	..	..	..	..	..	..	1 (<1%)	..	..	..	..	3 (1%)
Morocco	1	..	..	..	..	..	..	..	..	..	..	..	1 (<1%)
Niger	1	..	..	..	..	..	..	..	..	..	..	..	1 (<1%)
Mauritania	2	..	..	..	..	..	..	..	..	..	..	..	2 (<1%)
Central African Republic	2	..	..	..	..	..	..	..	..	..	1 (<1%)	..	1 (<1%)
Chad	1	1 (<1%)	..	..	..	..	..	..	..	..	..	..	..
Togo	2	..	..	..	..	..	..	..	..	..	1 (<1%)	..	1 (<1%)
Tunisia	1	..	..	..	..	..	..	1 (<1%)	..	..	..	..	..
Senegal	3	1 (<1%)	..	..	..	..	..	..	..	..	..	1 (<1%)	1 (<1%)
Other	7	..	..	..	..	..	..	3 (1%)	1 (<1%)	..	..	..	3 (1%)
Afghanistan	2	..	..	..	..	..	..	1 (<1%)	..	..	..	..	1 (<1%)
Lebanon	1	..	..	..	..	..	..	..	..	..	..	..	1 (<1%)
Türkiye	3	..	..	..	..	..	..	2 (<1%)	..	..	..	..	1 (<1%)
Unknown	1	..	..	..	..	..	..	..	1 (<1%)	..	..	..	..
Total	407	30 (7%)	4 (1%)	2 (<1%)	2 (<1%)	1 (<1%)	1 (<1%)	320 (79%)	3 (1%)	3 (<1%)	7 (2%)	4 (1%)	30 (7%)

Percentages are calculated relative to the total number of participant samples (n=407). HBV=hepatitis B virus. HDV=hepatitis D virus.

Table 3: **HBV and HDV genotype combinations by country of origin**

Jukes–Cantor model with 1000 bootstraps, which not only confirmed the existence of these preliminary subgenotypes (HDV GT1a to GT1e) but also revealed novel, previously unidentified HDV GT1 subgenotypes, classified here as provisional (p) subgenotypes HDV GT1fp to GT1ip, with distinct features (figure 2, appendix pp 6, 15). Of the 368 full-genome HDV clinical sequences characterised here, 355 sequences were HDV GT1, with the majority as HDV GT1c (n=221), followed by GT1e (n=48), GT1hp (n=38), GT1ip (n=29), GT1a (n=9), GT1b (n=3), GT1d (n=3), GT1fp (n=1), and GT1gp (n=1; figure 2). Two clinical HDV full-genome sequences from Germany did not cluster with established or provisional HDV GT1 subgenotypes (figure 2). The new provisional subgenotype, GT1hp, emerged from 38 clinical sequences in this study, although with lower bootstrap support (figure 2, appendix p 15). Although genetically distinct, HDV viral loads were similar for participants across HDV GT1 subgenotypes (figure 3A). In total, we provisionally designated four new HDV GT1 subgenotypes (GT1fp to GT1ip). The ongoing collection of HDV full-genome sequences will enable refinement of these provisional classifications, potentially leading to their confirmation or reclassification.

To determine the antiviral activity of bulevirtide against HDV GT1 subgenotypes, clinical isolates were treated with bulevirtide in a primary human hepatocyte infection assay. The mean bulevirtide EC_{50} values were as follows for the established HDV GT1 subgenotypes: 0·39 nM for GT1a (n=3), 0·79 nM for GT1b (n=1), 0·43 nM for GT1c (n=124), 0·33 nM for GT1d (n=3), and 0·46 nM for GT1e (n=37; figure 3B). For the newly identified provisional subgenotypes, the mean bulevirtide EC_{50} values were as follows: 0·52 nM for GT1gp (n=1), 0·44 nM for GT1hp (n=26), and 0·55 nM for GT1ip (n=15; figure 3B). The GT1fp clinical isolate was unavailable for antiviral activity testing. This GT1fp participant was enrolled in the tenofovir arm of MYR202 and did not receive bulevirtide treatment; therefore, bulevirtide treatment efficacy could not be assessed. Overall, bulevirtide had similar antiviral activity against clinical isolates from established and newly identified provisional HDV GT1 subgenotypes.

The potential effect of HDV GT1 subgenotype on bulevirtide treatment response was also explored. MYR301, MYR204, and MYR202 clinical trial participants receiving bulevirtide (2 mg, 5 mg, or 10 mg) alone or in combination with tenofovir disoproxil fumarate or peginterferon alfa were included in this analysis. Virological response, defined as HDV RNA decrease greater than or equal to 2 $\log_{10}$ IU/mL or undetectable from baseline, was reached by 139 (76%) of 182 GT1c participants, 35 (83%) of 42 GT1e participants, 22 (81%) of 27 GT1hp participants, and 25 (96%) of 26 GT1ip participants (figure 3C). ALT normalisation was reached by 46–55% of participants across GT1c, GT1e, GT1hp, and GT1ip subgenotypes, and a combined response of both ALT normalisation and HDV RNA decrease was reached by 33–46% of participants across GT1c, GT1e, GT1hp, and GT1ip subgenotypes (figure 3C). Overall, bulevirtide treatment response was similar across HDV GT1 subgenotypes.

Phylogenetic analyses have shown evidence of HDV recombination both within each individual HDV genotype (intra) as well as between two different HDV genotypes (inter).[27,28] To identify potential HDV recombinant sequences in this study, a recombination analysis was performed using RDP5, as described previously.[23,27] Similarity plot curves were then used to reveal regional variation in sequence similarity, supporting subgenotype classification and enabling detection of potential recombination events or divergence. Here, one clinical HDV GT1c sequence from a MYR204 participant was identified as a potential GT1c and GT1e recombinant sequence (figure 4A). The GT1c sequence similarity spanned nucleotide regions one to 344 and 1152 to 1682 whereas the GT1e sequence similarity was located from nucleotide 355 to 1151 (figure 4A, appendix pp 7–8). The clinical isolate from this participant was sensitive to bulevirtide activity with a mean bulevirtide EC_{50} value of 0·69 nM. This participant experienced virological suppression on bulevirtide with an HDV RNA decrease of greater than or equal to 2 $\log_{10}$ IU/mL, ultimately reaching undetectable levels (figure 4B).

HBV consensus sequences from next-generation sequencing were used to determine HBV genotypes based on BLAST analyses against an HBV reference sequence library. If PCR amplification was unsuccessful, HBV genotype was determined using a commercial genotyping enzyme immunoassay. HBV GTD was the most prevalent HBV genotype (336 [83%] of 407 participants), followed by GTA (40 [10%]), GTE (17 [4%]), GTC (one [<1%]), and GTB (one [<1%]; table 2). HBV GTD sequences were observed in participants from 17 of 24 countries, GTA in 13 countries, GTE in 12 countries, GTC in France, and GTB in Italy (table 2). Most HBV GTA participants (30 of 40) originated from Europe, whereas most HBV GTE participants (12 of 17) originated from Africa (table 2). HBV genotypes were undetermined for 12 participants, of whom seven were of African origin (two from Cameroon, two from Côte d'Ivoire, two from Mali, and one from Senegal; table 2).

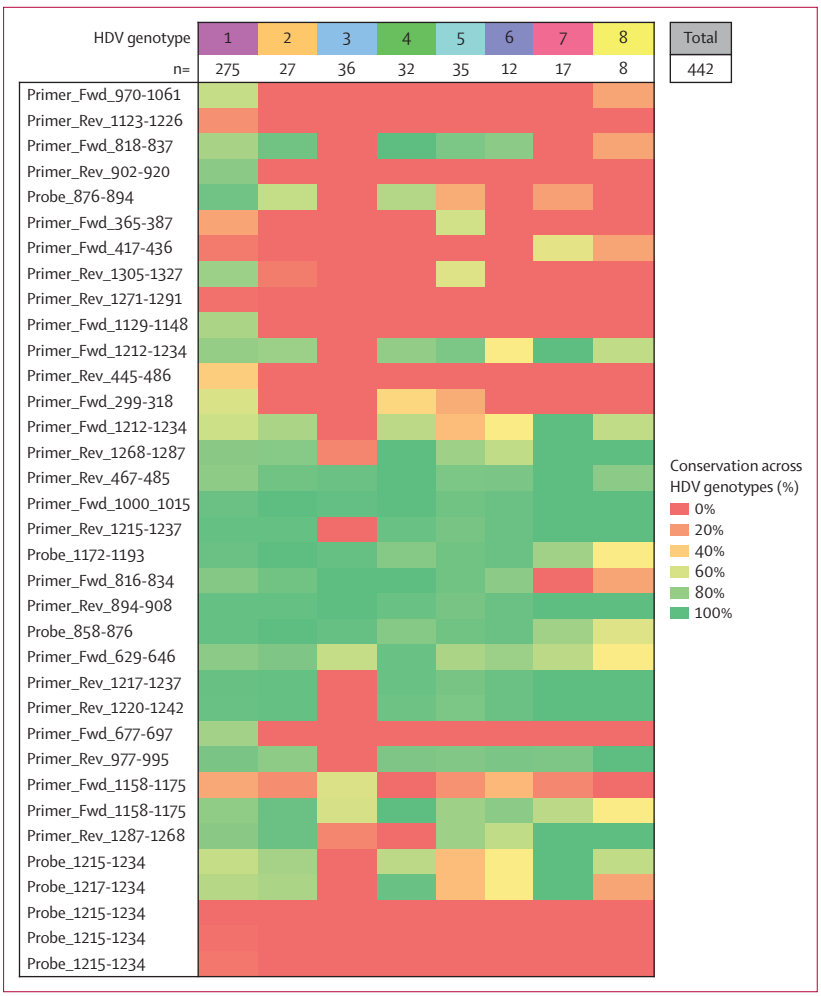

Figure 5: **HDV primers and probe conservation across HDV genotypes**
Each row indicates a unique HDV primer or probe. Matches were determined by calculating the proportion of HDV sequences in our database that completely match a given primer or probe sequence. The binding region on the HDV genome sequence is indicated after the primer or probe name (patent number US20160032411A1[19,29,30]). HDV=hepatitis D virus.

Paired HBV–HDV genotypes were determined for 377 (93%) of 407 participants and were as follows: 320 (85%) of 377 D/1, 30 (8%) A/1, seven (2%) E/1, four (1%) E/5, four (1%) A/2, three (1%) D/2, three (1%) D/5, two (<1%) A/5, two (<1%) A/6, and one (<1%) participant each had B/1 and C/1 combinations (table 3). Participants with D/1 genotypes were observed in 13 of 24 countries, A/1 in 11 countries, E/1 in seven countries, E/5 in four countries, D/5 in Côte d'Ivoire and Guinea, A/6 in France and Cameroon, A/2 and D/2 in Russia, A/5 in Cameroon, B/1 in Italy, and C/1 in France (table 3). The HBV–HDV paired genotype combinations were undetermined for 30 (7%) of 407 participants, most of whom (19 [63%] of 30) were of African origin (table 3).

HDV viral load quantification was performed by two quantitative PCR-based assays, RoboGene and Eurobioplex, using unique primers and probes for 20 participants from the diverse cohort. One participant from Mali

had undetectable HDV RNA in one assay and had a viral load of 8·6 $\log_{10}$ IU/mL in the other assay (data not shown); this participant had HBV GTE and had an undetermined HDV genotype. Given the high sequence diversity of HDV and the lower success rate of our HDV amplification-based sequencing assay in the diverse cohort (appendix p 13), the HDV sequence diversity at published primer and probe sites was investigated. Primer binding across 442 HDV sequences was quantified by calculating the percentage of sequences where at least one mismatch was observed. Across all known HDV genotypes (GT1–GT8), a broad range of sequence diversity that might directly affect the ability of primers to bind, detect, and amplify highly diverse HDV sequences was observed (figure 5). Specifically, we observed that, whereas most primer sets efficiently bind HDV GT1 sequences with few to no mismatches, non-HDV GT1 sequences showed a striking reduction in primer binding, with most primers containing mismatches (figure 5). This highlights a potential detection bias favouring GT1 over other HDV genotypes.

Discussion

In this study, we sought to sequence and genotype HBV and HDV from 407 participants with HBV–HDV co-infection from geographically diverse regions of origin. Overall, the HBV–HDV D/1 genotype combination was most common, followed by A/1. Phylogenetic analyses identified four novel HDV GT1 provisional subgenotypes, GT1fp to GT1ip. Both HDV sequence diversity and HDV viral load are likely to have contributed to the success rate of genotyping of participants in this study. Indeed, the ultrasensitive assay used in this study resulted in successful HBV amplification for 281 (69%) of 407 participants (appendix p 13), which is a marked improvement from previously observed HBV amplification success rates of around 30% for participants with chronic hepatitis D.[14] Determining the breadth of HDV sequence diversity is essential to develop sensitive and accurate HDV detection assays to ensure all individuals that could benefit from treatment are being identified.

The HDV genotype of 21 participants remained undetermined. Participants with an undetermined HDV genotype had significantly lower viral loads than successfully genotyped samples, suggesting that viral load affects HDV amplification success rates. Higher HDV sequencing success rates were observed in participants from Europe compared with those from Africa, suggesting that these sequences could be novel and not covered by the current primer sets. Additionally, potentially reduced amplification efficiency from mutations outside primer regions, variable sample quality affecting RNA integrity, and potential inefficiencies in cDNA synthesis steps could all affect amplification success.

A limitation of this study is the small number of samples from geographical regions outside of Europe. However, some samples from participants of African ancestry were obtained. In this study, 43 participants were from Africa, of whom 11 were from Cameroon and had HDV genotypes as follows: HDV GT1 (n=6), HDV GT5 (n=1), HDV GT6 (n=1), and undetermined (n=3). A 2018 study from Cameroon identified HDV GT1 in 138 participants, HDV GT7 in 61, HDV GT6 in 11, and HDV GT8 in one participant.[31] Although HDV sequencing and genotyping data from Africa are scarce, the available data suggest unique HDV sequence divergence in this understudied region when compared with Europe.

HBV and HDV genotype determination is clinically relevant given reports describing genotype-specific biology and therapeutic efficacy. A poor response to interferon alfa therapy has been reported in participants infected with HBV GTC and GTD compared with GTA and GTB.[32] Similarly, subgenotypes of HDV GT1 have different response rates to interferon alfa.[33] Another example is the increased risk of hepatocellular carcinoma associated with HBV GTA in certain populations.[34] The HDV entry inhibitor bulevirtide is currently the sole approved HDV antiviral treatment in Europe.[35] Amino acids associated with virological resistance to bulevirtide have not been detected in participants during up to 24 weeks of treatment in clinical trials.[36] Here, bulevirtide had potent antiviral activity across genetically distinct HDV GT1 subgenotypes and similar treatment responses across HDV GT1 subgenotypes. Nearly all HDV GT1ip participants had an HDV RNA decrease compared with between 78% and 83% for HDV GT1c, GT1e, and GT1hp. Although statistically significant when compared with the other HDV subgenotypes, more clinical data will be essential to verify this observation.

In conclusion, this is the most comprehensive study to date sequencing full-genome HDV from 386 individuals with HDV infection. The data show greater genetic diversity within HDV GT1 subgenotypes than was previously known. Additionally, as more HDV sequences become available, particularly from understudied global regions, we anticipate efforts in molecular and phylogenetic methods to refine and reclassify HDV subgenotypes.

Contributors

TA and EM conceived the study with input from HM, JF, DM, PL, and DA. SM, SC, AL, and RMar contributed to figures, data analysis, data interpretation, and writing of the report. TA performed data analysis, interpretation, and writing. YL, SX, SN, and RMat performed data analysis and interpretation. SM and SC accessed and verified the data. All authors had full access to all the data in the study and take final responsibility for the decision to submit for publication.

Declaration of interests

All authors affiliated with Gilead Sciences may hold stock or stock options in Gilead Sciences. TA received payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from: AbbVie, Antios, Aligos, Bluejay, Eiger, Roche, Gilead Sciences, GSK, Janssen, and VIR Biotechnology. TA participates on a data safety monitoring board or advisory board for eLilly. TA receives support for attending meetings or travel from AbbVie and Gilead Sciences. PL received payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from: Sbring Bank, MYR, Eiger, Antios, Aligos, Grifols, Altona, ROBOSCREEN, Bristol-Myers Squibb, Roche, Gilead Sciences, GSK,

AbbVie, MSD, Arrowhead, Alnylam, Janssen, and VIR Biotechnology. All other authors declare no competing interests.

Data sharing

HDV sequencing data associated with this publication will be deposited in GenBank upon publication (accession numbers PZ111496 and PZ111813).

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