Hepatitis C virus diversity and treatment outcomes in Benin: a prospective cohort study

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Summary

Background 10 million people are chronically infected with the hepatitis C virus (HCV) in sub-Saharan Africa. The assessment of viral genotypes and treatment response in this region is necessary to achieve the WHO target of worldwide elimination of viral hepatitis by 2030. We aimed to investigate the prevalence of HCV genotypes and outcomes of treatment with direct-acting antiviral agents in Benin, a country with a national HCV seroprevalence of 4%.

Methods This prospective cohort study was conducted at two referral hospitals in Benin. Individuals were eligible for inclusion if they were seropositive for HCV and willing to consent to participation in the study; exclusion criteria were an inability to give consent or incarceration. Viraemia was confirmed by PCR. The primary outcomes were to identify HCV genotypes and measure sustained virological response rates 12 weeks after completion of treatment (SVR12) with a 12-week course of sofosbuvir–velpatasvir or sofosbuvir–ledipasvir, with or without ribavirin. We conducted phylogenetic and resistance analyses after the next-generation sequencing of samples with a cycle threshold (Ct) value of 30 or fewer cycles. The in-vitro efficacy of NS5A inhibitors was tested using a subgenomic replicon assay.

Findings Between June 2, 2019, and Dec 30, 2020, 148 individuals were screened for eligibility, of whom 100 were recruited prospectively to the study. Plasma samples from 79 (79%) of the 100 participants were positive for HCV by PCR. At the time of the study, 52 (66%) of 79 patients had completed treatment, with an SVR12 rate of 94% (49 of 52). 57 (72%) of 79 samples had a Ct value of 30 or fewer cycles and were suitable for whole-genome sequencing, from which we characterised 29 (51%) samples as genotype 1 and 28 (49%) as genotype 2. Three new genotype 1 subtypes (1q, 1r, and 1s) and one new genotype 2 subtype (2xa) were identified. The most commonly detected subtype was 2d (12 [21%] of 57 samples), followed by 1s (eight [14%]), 1r (five [9%]), 1b (four [7%]), 1q (three [5%]), 2xa (three [5%]), and 2b (two [3%]). 20 samples (11 genotype 2 and nine genotype 1) were unassigned new singleton lineages. 53 (93%) of 57 sequenced samples had at least two resistance-associated substitutions within the *NS5A* gene. Subtype 2d was associated with a lower-than-expected SVR12 rate (eight [80%] of ten patients). For one patient, with subtype 2b, treatment was not successful.

Interpretation This study revealed a high SVR rate in Benin among individuals treated for HCV with sofosbuvir–velpatasvir, including those with highly diverse viral genotypes. Further studies of treatment effectiveness in genotypes 2d and 2b are indicated.

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For the French translation of the abstract see Online for appendix 1

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Introduction

The hepatitis C virus (HCV), an RNA virus in the genus *Hepacivirus* of the Flaviviridae family, causes chronic liver infection in 58 million people worldwide and 350 000–400 000 deaths per year.[1] Sub-Saharan Africa accounts for 14% of the global HCV burden (10 million people), and west Africa has the highest seroprevalence in this region. Benin, a west African nation with a population of 12 million, is estimated to have an HCV seroprevalence of 4%.[2]

In 2016, WHO outlined an ambitious plan to eliminate viral hepatitis worldwide by 2030, with the aims of a 90% reduction in HCV incidence and a 65% reduction in mortality.[3] To achieve these aims, 80% of individuals with chronic HCV infection need access to care. However, in 2016, only 20% (14 million) of people infected with HCV were diagnosed.[4] By 2019, 21% (15·2 million people) were diagnosed and 13% (9·4 million people) had been treated.[1] Efforts must therefore increase to meet WHO targets. One of the primary tools in this effort is the use of direct-acting antiviral therapies, which have revolutionised the treatment of chronic HCV infection. Affordable, all-oral, short-duration treatment regimens with a favourable side-effect profile mean that direct-acting antivirals can

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Research in context

Evidence before this study

We searched PubMed for hepatitis C virus (HCV)-related articles and the National Center for Biotechnology Information (NCBI) GenBank via HCV GLUE, using the search terms "HCV" and "Benin", from database inception to Dec 11, 2023, without language restrictions. We found only one complete HCV genome sequence from Benin available in the NCBI GenBank database, despite increasing evidence that HCV sequence diversity is far higher in west Africa than in high-income countries, where the majority of clinical trials have been conducted.

Added value of this study

We investigated the prevalence of HCV genotypes in patients in Benin using next-generation sequencing and the response to treatment with direct-acting antiviral therapies; to our knowledge, this is the first such study in west Africa. We sequenced 57 HCV genomes from Benin, detecting a very high level of genotypic diversity in the country, and report four previously undescribed subtypes (1q, 1r, 1s, and 2xa). A lower-than-expected sustained virological response rate was found in people infected with the most prevalent subtype, 2d.

Implications of all the available evidence

Larger genotyping and treatment outcome studies are needed in real-world settings in sub-Saharan Africa to monitor the response to direct-acting antiviral therapies. Population-based studies could be used to guide optimal pan-genotypic treatment strategies without the need for the genotyping of samples from individual patients. Sofosbuvir–velpatasvir is an acceptable pan-genotypic regimen for use in Benin.

realistically be rolled out in low-resource settings.[5] Pan-genotypic regimens also have the potential to simplify HCV treatment by omitting the cost of genotyping. However, considerable gaps remain in our knowledge of HCV diversity in sub-Saharan Africa owing to a scarcity of HCV genomic data from this region.[6] Before this study, only six partial HCV sequences from Benin had been published in the National Center for Biotechnology Information (NCBI) GenBank. Low levels of testing and insufficient access to treatment means that the efficacy of direct-acting antivirals against HCV subtypes endemic to sub-Saharan Africa is poorly reported.[7] Notably, several studies have shown low sustained virological response rates after treatment with first-generation NS5A inhibitors against HCV subtypes 1l, endemic in west Africa, and 4r, endemic in central and east Africa.[8,9] Such results challenge the validity of some (especially first-generation) pan-genotypic regimens, such as sofosbuvir–daclatasvir, which is currently recommended by WHO.[3] To our knowledge, this is the first study from Benin to report HCV treatment outcomes after direct-acting antiviral therapy and HCV genetic diversity.

Methods

Study design and participants

See Online for appendix 2

For this prospective cohort study, patients who were seropositive for HCV were recruited from two referral hospitals in Benin, the Centre National Hospitalier Universitaire in Cotonou and Centre Hospitalier Departmental de Borgou-Alibori in Parakou, between June 2, 2019, and Dec 30, 2020. Seropositive patients were identified during the Benin national screening campaign (2016–19) led by the Société Béninoise d'Hépato-gastro-entérologie, in which 7000 people aged 0–90 years were tested using the InTec HCV Rapid Test (InTec Products; Xiamen, China). Inclusion criteria were positive serology and willingness to provide consent and exclusion criteria were inability to give consent or incarceration. HCV treatment history was self-reported. Gender was self-reported by study participants in the clinical data survey; options were male or female.

Ethical approval was granted by the Comité National d'Ethique pour la Recherche en Santé (number 38; Oct 15, 2019). Written informed consent was obtained from all participants.

Procedures

Treatment

Participants were treated for 12 weeks with a combination of Harvoni (ledipasvir 90 mg and sofosbuvir 400 mg) or Epclusa (velpatasvir 100 mg and sofosbuvir 400 mg) once daily, depending on HCV genotype (sofosbuvir–ledipasvir for genotype 1 and sofosbuvir–velpatasvir for genotype 2). The drugs were donated by Gilead Sciences and sufficient supplies were available to treat the first 52 individuals diagnosed during the study period. As of October, 2022, the remaining patients were awaiting treatment availability on the national scheme. Ribavirin, the dose of which was based on weight (<75 kg, 1000 mg per day; ≥75 kg, 1200 mg per day), was added to the treatment regimen at the discretion of the treating physician if the likelihood of cirrhosis was considered high. The criteria for the diagnosis of cirrhosis were an aspartate aminotransferase-to-platelet ratio index of 2 or higher, a liver stiffness measurement of at least 14 kPa, or clinical evidence of cirrhosis (appendix 2 p 16). Patients for whom HCV RNA was undetectable by PCR at 12 weeks after the completion of treatment were classed as having had a sustained virological response (SVR12). During the 12-week treatment course, patients attended four clinic visits at treatment weeks 1, 4, 8, and 12 at Centre National Hospitalier Universitaire (Cotonou) or Centre Hospitalier Departmental de Borgou-Alibori (Parakou). We measured adherence on the basis of clinic attendance and provision of empty medicine containers at each visit.

PCR testing

HCV was quantified in plasma samples at baseline and 12 weeks after the end of treatment using the Cobas

TaqMan assay (Roche Diagnostics; Rotkreuz, Switzerland) at the laboratory of the Programme National de Lute contre le SIDA (PNLS-Benin) or the Alinity HCV viral load assay (Abbott Molecular Diagnostics; Maidenhead, UK) at the West of Scotland Specialist Virology Centre, Glasgow, UK. Before sequencing, to ensure viability after sample transfer, an in-house, real-time PCR assay was also conducted using SuperScript III reverse transcriptase and Platinum Taq DNA Polymerase (Invitrogen; Waltham, MA, USA) for PCR, as follows: denaturation at 50°C for 15 min, annealing at 95°C for 2 min, and elongation at 95°C for 8 s (40 cycles). The forward primer was JFH1-16–5′-TCTGCGGAACC GGTGAGTAC-3′, the reverse primer was JFH1-17–5′-GCACTCGCAAGCACCCTAT-3′, and the probe (Assay ID C_153270924_10) was 6-FAM-AAAGGCCTTGTGGTACTG-MGB (Thermo Fisher; Waltham, MA, USA).

Next-generation sequencing

All samples with a cycle threshold (Ct) value of 30 cycles or fewer underwent next-generation sequencing as previously described.[10] RNA was eluted in nuclease-free water (11 μL) and reverse-transcribed with Superscript III (Invitrogen; Waltham, MA, USA) using random hexamers. An NEB Second Strand Synthesis kit (New England Biolabs; Ipswich, MA, USA) and a KAPA Library Preparation Kit (KAPA Biosystems; Wilmington, MA, USA) were used for library preparation. Samples were indexed using NEBNext Multiplex Oligos for Illumina (New England Biolabs; Ipswich, MA, USA). Qubit (Thermo Fisher; Waltham, MA, USA) and TapeStation (Agilent; Santa Clara, CA, USA) were then used to quantify and check DNA quality. Libraries were pooled at equimolar concentrations. Target enrichment was carried out using custom NimbleGen SeqCap EZ system RNA probes (Roche, Rotkreuz, Switzerland).[10] Samples were sequenced on the Illumina MiSeq platform using a v3 MiSeq Reagent Kit (Illumina; San Diego, CA, USA).

Bioinformatic analysis

Whole genomes were assembled using an in-house de-novo assembly pipeline. Short and low-quality reads (length <75 bases; Phred score <30) were removed using TrimGalore. Filtered reads were subsampled and enriched in silico for HCV, then assembled using SPAdes.[11] K-mer sizes were selected iteratively to generate contig lengths of more than 8500 bases and then mapped using Tanoti. Samples with low depth across the genome underwent reference-based assembly following a k-mer base approach to identify the best HCV reference. Near-whole genomes (>90% coverage) were selected and aligned using MAFFT, version 7.313.[12] Maximum likelihood phylogenetic trees were constructed using RaxML[13] with a general time-reversible nucleotide substitution model and 1000 bootstraps. The genetic distance between sequences was calculated using MEGAX,[14] with a bootstrap method for variance estimation. Resistance-associated substitutions were analysed with HCV GLUE software.[15] New subtypes were classified according to International Committee on Taxonomy of Viruses (ICTV) guidance and confirmed by the ICTV Flavivirus working group lead.[16]

Sub-genomic replicon assays

A sub-genomic replicon (SGR; pJFH-1), encoding the *Gaussia* luciferase gene and HCV non-structural proteins NS3, NS4A, NS4B, NS5A, and NS5B,[17] was modified by replacing the *NS5A* gene (Codex DNA; San Diego, CA, USA) using clinical sample sequences in a JFH-1 genotype 2a background. Two SGR constructs containing the resistance-associated NS5A polymorphisms Phe28Ser plus Leu31Ile (resistance-associated substitution [RAS] control 1) and Pro29Ser plus Lys30Gly (RAS control 2) were synthesised and tested for replication, as described previously.[18] In brief, the constructs were linearised with FastDigest Xba1 (New England Biolabs, Ipswich, MA, USA), purified (Monarch DNA gel extraction kit; New England Biolabs, MA, USA), used as a template for RNA transcription (T7 RiboMAX Express Large Scale RNA Production System; Promega, Madison, WI, USA), and purified again (RNeasy Mini kit; Qiagen, Hilden, Germany). Cells were electroporated (270 V, 950 mF capacitance, infinite resistance) in 4 mm cuvettes (Thermo Fisher, Waltham, MA, USA) at 2×10^5 Huh-7 cells per reaction and chilled on ice before resuspension in 10 mL 10% fetal bovine serum Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher, Waltham, MA, USA). Cells were seeded into 96-well plates and incubated at 37°C for 4 h. To ensure efficient SGR replication, supernatant was collected at 4, 24, 48, and 72 h for luminescence readings and growth curves plotted in triplicate. Replication efficiency was calculated using luminescence readings as follows: (mutant 72 h/mutant 4 h)/(JFH-1 72 h/JFH-1 4 h). To test the in-vitro efficacy of NS5A inhibitors, infected cells were treated in separate plates with ledipasvir (MedChemExpress; Monmouth Junction, NJ, USA), pibrentasvir (Cayman Chemical; Ann Arbor, MI, USA), or velpatasvir (Cayman Chemical; Ann Arbor, MI, USA) in serial dilutions. Plates were incubated at 37°C for 72 h. 20 μL supernatant was removed for a luciferase assay (Pierce Gaussia Luciferase Flash Assay kit, Thermo Fisher, Waltham, MA, USA). Relative light units (RLUs) were calculated, per well, as the 72-h bioluminescence read divided by the 4 h read. Maximum responses for each construct were set as the mean RLU of untreated wells. RLU values were normalised to the maximal response and expressed as a percentage. The resulting values were used to plot dose–response curves and calculate the drug concentration at which replication was reduced by half (IC_{50}), using non-linear regression (R package drc). Replication capacity was calculated using the luminescence readout as follows: (mutant 72 h/mutant 4 h)/(JFH-1 72 h/JFH-1 4 h).

For more on **TrimGalore** see https://github.com/FelixKrueger/TrimGalore

For more on **Tanoti** see https://github.com/vbsreenu/Tanoti

Outcomes

The primary outcomes were to identify HCV genotypes in all participants with a sufficient viral load for next-generation sequencing and SVR12 after completion of a 12-week treatment course in all treated patients. Secondary

For more on **HCV GLUE** see http://hcv.glue.cvr.ac.uk

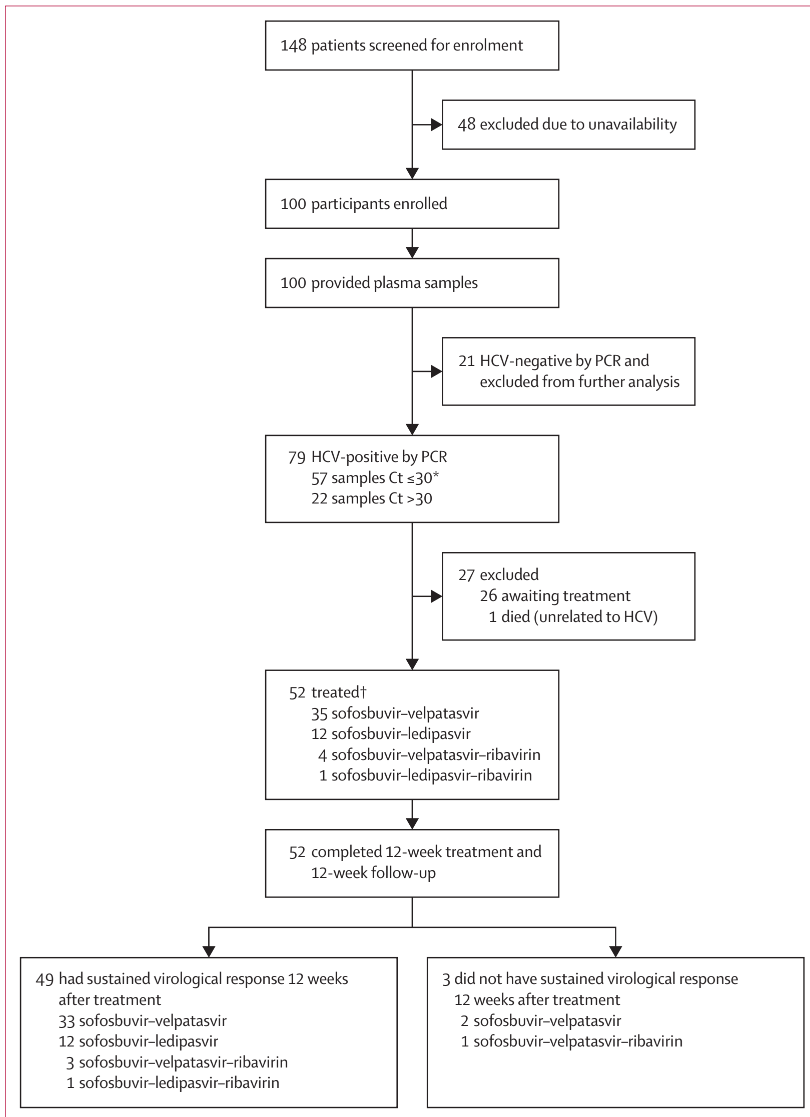


Figure 1: **Trial profile**
Ct=cycle threshold. HCV=hepatitis C virus. *Samples with Ct ≤30 underwent whole-genome sequencing for subsequent analysis; all 57 of these samples were sequenced. †Treatment regimen was chosen on the basis of genotype results.

	Number of participants	No SVR12	SVR12	p value
Overall	52	3 (6%)	49 (94%)	··
Gender				
Male	19 (37%)	2 (11%)	17 (89%)	··
Female	33 (63%)	1 (3%)	32 (97%)	0·62
Age				
<59 years	17 (33%)	1 (6%)	16 (94%)	··
≥59 years	35 (67%)	2 (6%)	33 (94%)	1·00
HCV RNA, log_{10} IU/L				
≤5·99	25 (48%)	2 (8%)	23 (92%)	··
≥6·00	27 (52%)	1 (4%)	32 (96%)	0·95
Treatment group				
Sofosbuvir–ledipasvir–ribavirin	13 (25%)	0	13 (100%)	··
Sofosbuvir–velpatasvir–ribavirin	39 (75%)	3 (8%)	36 (92%)	NA*
Previous treatment				
No	48 (92%)	2 (4%)	46 (96%)	··
Yes	4 (8%)	1 (25%)	3 (75%)	0·55
Cirrhosis				
Yes	11 (21%)	2 (18%)	9 (82%)	
No	22 (42%)	0	22 (100%)	··
Unknown	19 (37%)	1 (5%)	18 (95%)	0·10
Genotype or subtype				
1	16 (31%)	0	16 (100%)	··
1b	4 (8%)	0	4 (100%)	··
2	6 (12%)	0	8 (100%)	··
2b	2 (4%)	1 (50%)	1 (50%)	··
2d	10 (19%)	2 (20%)	8 (80%)	··
Not genotyped	15 (29%)	0	15 (100%)	0·058

Data are n or n (%) unless otherwise indicated. Data on previous treatment were obtained from hospital documentation. p values were calculated by Fisher's exact test. HCV=hepatitis C virus. NA=not applicable. SVR12=sustained virological response at 12 weeks after completion of treatment. *Comparison not applicable as treatment was stratified by genotype as per WHO guidelines.

Table 1: **Treatment outcomes grouped by clinical and virological characteristics**

outcomes were the identification of substitutions associated with resistance to direct-acting antivirals and the association between genotype and treatment outcome.

Statistical analysis

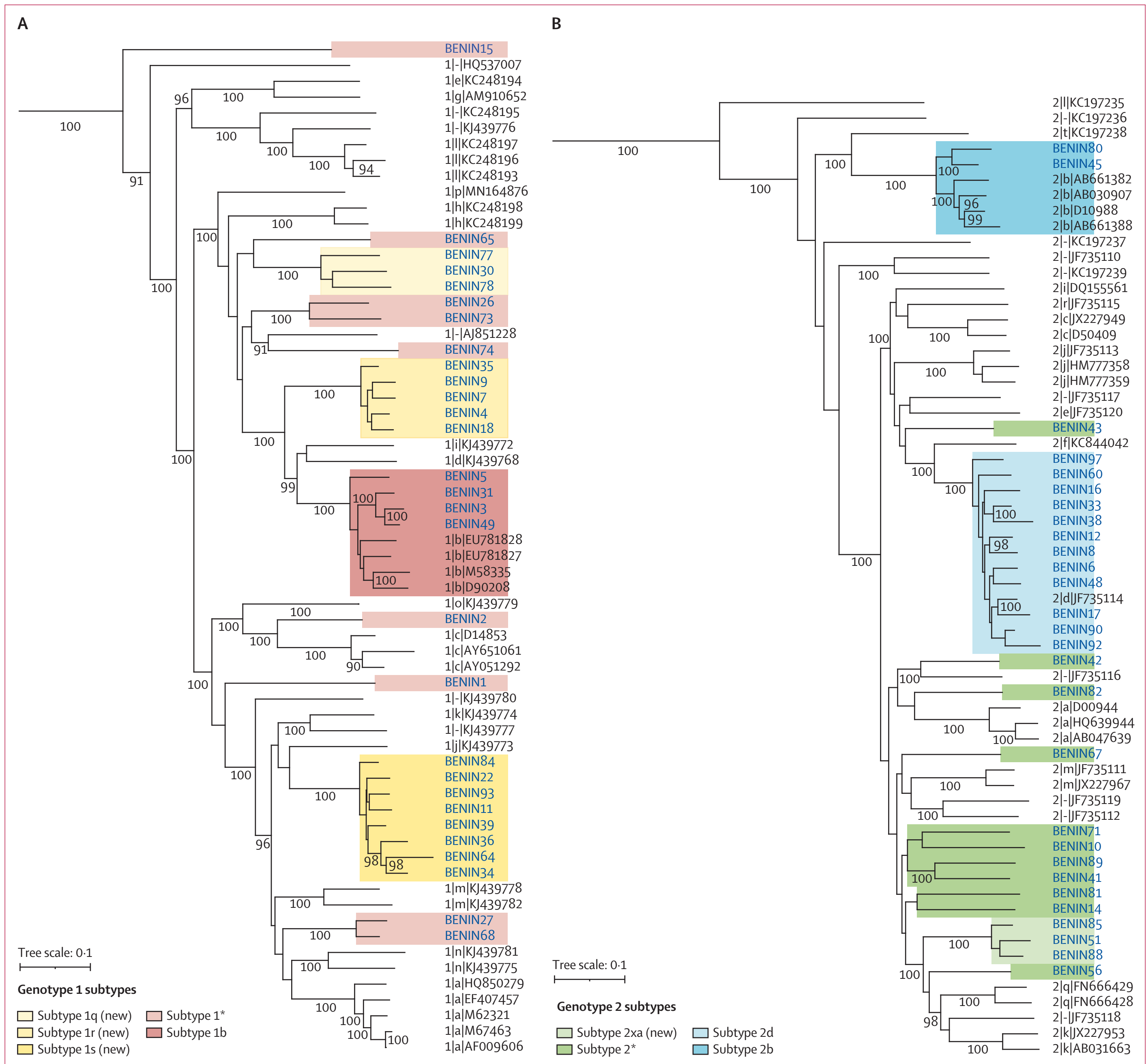
We aimed to recruit 100 participants to the study. We calculated that 36 participants in each genotype group (genotype 1 or genotype 2) would be required to give 80% power of detecting a 20% difference in SVR12 from a baseline of 95%, with a significance level (α) of 0·05. Patient data and SVR12 rates were compared using Fisher's exact test when sample sizes were less than five or non-normally distributed and χ^2 analysis for normally distributed data with a sample size of five or more. Statistical analysis was done using R (version 4.2.3).

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between June 2, 2019, and Dec 30, 2020, we screened 148 patients who were seropositive for HCV, of whom 48 were unavailable for participation. Of the 100 eligible patients we approached, all consented to take part in the study. 79 (79%) of 100 participants tested positive for HCV RNA by PCR; the 21 (21%) who tested negative were excluded from further analysis. 52 (66%) of 79 participants had been treated as of October, 2022 (figure 1). The median age was 65 years (IQR 20, range 27–78). 46 (58%) participants were female and 33 (42%) were male. None of the patients injected drugs and the most likely route of transmission in the majority of cases was iatrogenic. The baseline



(Figure 2 continues on next page)

and clinical characteristics of the 52 treated participants are summarised in table 1.

Samples from 57 (72%) of 79 patients who tested positive for HCV RNA by PCR had a Ct value of 30 cycles or fewer (around 10^4 IU/mL), which is used as a threshold for suitability for whole-genome sequencing, and all 57 (100%) were successfully sequenced. Treatment outcome data were available for 52 individuals, of whom pre-treatment HCV sequence data were available for 37 (71%).

The overall SVR12 rate was 94% (49 of 52 treated patients). 35 (67%) of 52 patients were aged 59 years or older and 17 (33%) were younger than 59 years; 33 (63%) were female and 19 (37%) were male. Four treatment regimens were used: 35 (67%) of 52 patients received sofosbuvir–ledipasvir, of whom 33 (94%) had SVR12. Three (75%) of the four individuals who received sofosbuvir–velpatasvir–ribavirin had SVR12. All 12 (100%) patients with genotype 1 who were treated with sofosbuvir–ledipasvir had SVR12. One

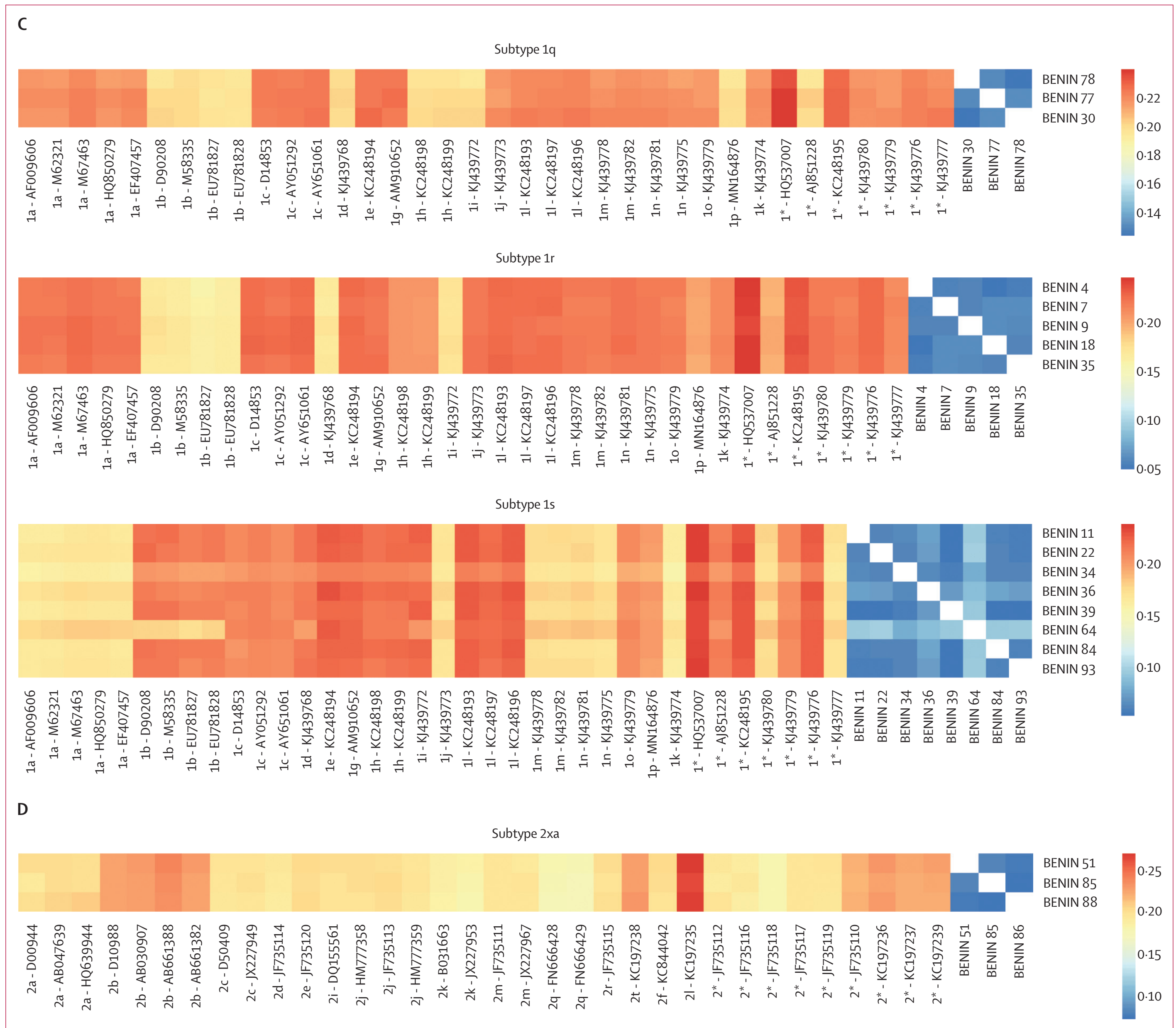


Figure 2: **Genomic diversity of HCV in Benin**
(A) Maximum likelihood phylogenetic tree of HCV genotype 1 sequences from Benin. HCV genotype 1 near-whole-genome reference sequences listed on the ICTV website were downloaded from NCBI GenBank and aligned with Benin HCV genotype 1 sequences. Benin HCV genotype 1 sequences are labelled in blue and coloured by subtype, including the newly assigned subtypes 1q, 1r, and 1s. Subtype 1* sequences are genotype 1 sequences that have not been formally assigned a subtype as fewer than three sequences were identified. Reference sequences are labelled in black. Bootstrap values greater than 90% are shown. (B) Maximum likelihood phylogenetic tree of HCV genotype 2 sequences from Benin. HCV genotype 2 reference sequences listed on the ICTV website were downloaded from GenBank and aligned with Benin HCV genotype 2 sequences. Benin HCV genotype 2 sequences are labelled in blue and coloured by subtype, including the newly assigned subtype 2xa. Subtype 2* sequences are genotype 2 sequences that have not been assigned a subtype. Reference sequences are labelled in black. Bootstrap values greater than 90% are shown. (C) Heat maps of pairwise distance measurements between genotype 1 reference sequences and Benin genotype 1 sequences (subtypes 1q, 1r, and 1s). (D) A heat map of pairwise distance measurements between genotype 2 reference sequences and Benin genotype 2 sequences (subtype 2xa). HCV=hepatitis C virus. ICTV=International Committee on Taxonomy of Viruses. NCBI=National Center for Biotechnology Information.

patient was treated with sofosbuvir–ledipasvir–ribavirin and had SVR12. Of 11 individuals with cirrhosis, nine (82%) had SVR12, and one (50%) of two individuals with cirrhosis who had previously been treated had SVR12.

Of the 37 patients who were treated and genotyped, 22 (59%) were infected with previously unreported HCV subtypes and all 22 had SVR12. In three (8%) of these 37 individuals, treatment was not successful; two were infected with subtype 2d and one with subtype 2b.

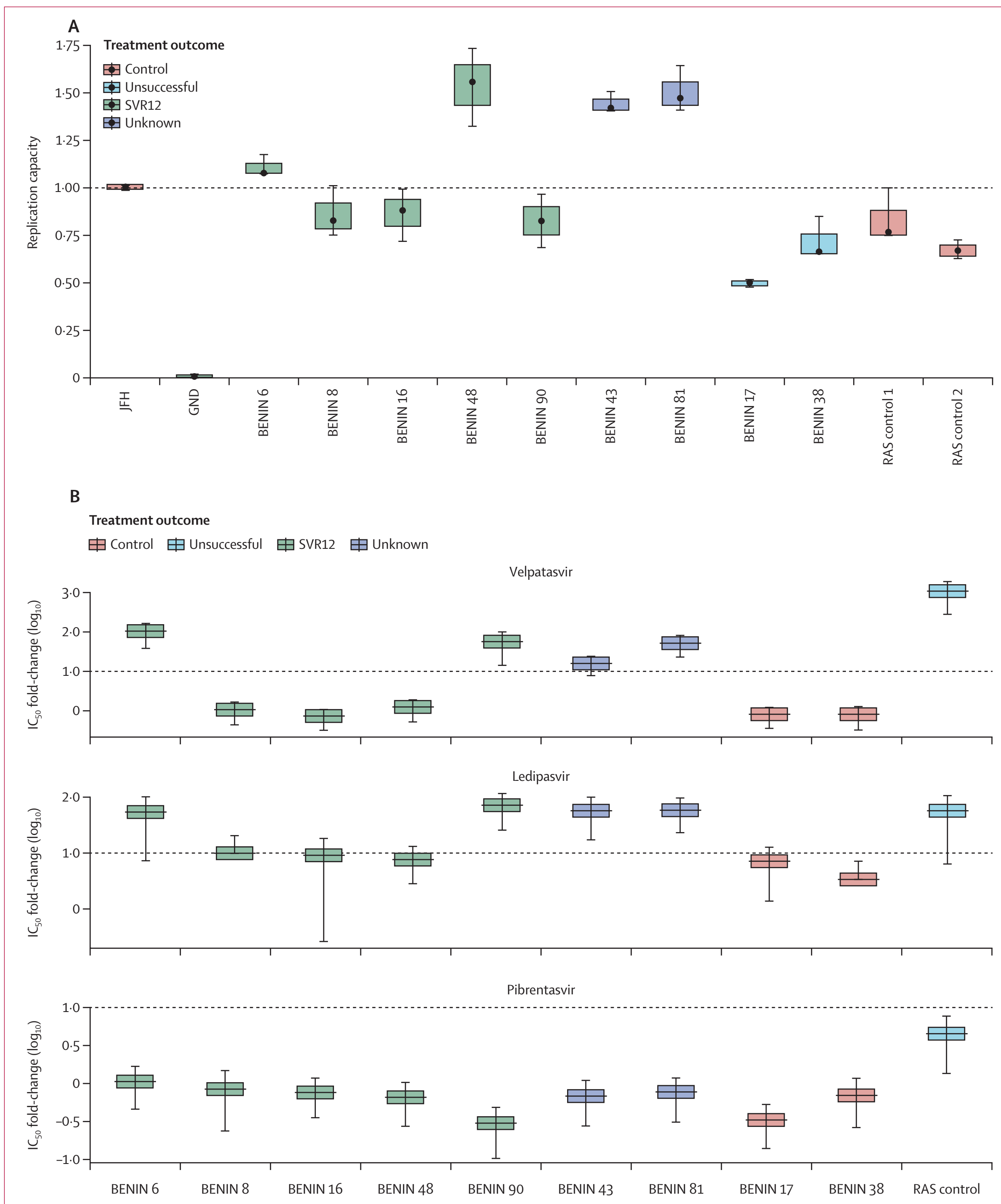


Figure 3: **In-vitro assays of replication and response to treatment**

(A) Replication capacities of nine genotype 2 Benin SGR NS5A constructs, coloured by treatment outcome. GND is a non-replicating SGR construct. RAS control 1 contained the NS5A polymorphisms Phe28Ser and Leu31Ile and RAS control 2 contained NS5A polymorphisms Pro29Ser and Lys30Gly. (B) Fold-changes in IC_{50} (expressed on a log_{10} scale) for the NS5A inhibitors ledipasvir, velpatasvir, and pibrentasvir in each Benin SGR construct compared with JFH-1. Constructs are coloured by treatment outcome. RAS control 1, containing the NS5A polymorphisms Phe28Ser and Leu31Ile, was used for challenge with pibrentasvir and RAS control 2, containing NS5A polymorphisms Pro29Ser and Lys30Gly, was used for challenge with ledipasvir and velpatasvir. HCV=hepatitis C virus. IC_{50}=drug concentration at which viral replication is reduced by half. JFH=construct representing wild-type subtype 2a HCV. RAS=resistance-associated substitution. SGR=subgenomic replicon. SVR12=sustained virological response at 12 weeks after completion of treatment.

	No SVR12 (n=3)	SVR12 (n=15)	p value
Position 24			
Lys/Gln24Lys	0	1 (7%)	1·00
Ser/Thr24Ser	2 (67%)	12 (80%)	1·00
Position 28			
Phe/Leu/Met28Phe	2 (67%)	14 (93%)	0·31
Phe/Leu/Met28Leu	1 (33%)	0	0·17
Phe/Leu/Met28Met	0	1 (7%)	1·00
Position 30			
Gln/Arg30Gln	0	1 (7%)	1·00
Position 31			
Leu/Met31Met	2 (67%)	8 (53%)	1·00
Position 37			
Phe/Leu/Val37Ile	0	1 (7%)	1·00

Data are n (%) unless otherwise indicated. No SVR12 was defined as a positive PCR test for HCV at 12 weeks after completion of treatment. p values were calculated by Fisher's exact test. HCV=hepatitis C virus. SVR12=sustained virological response 12 weeks after completion of treatment.

Table 2: **Presence of known resistance-associated substitutions within the NS5A region in genotype 2 samples by treatment outcome**

Of the 57 HCV samples sequenced in the study, 29 (51%) were genotype 1 and 28 (49%) were genotype 2. The majority of genotype 1 infections (25 [86%] of 29) and 12 (43%) of the 28 genotype 2 infections (figure 2) were with previously unreported subtypes. NCBI GenBank accession numbers for these sequences are listed in appendix 2 (pp 17–18). For the classification of a new subtype, an uncorrected pairwise genetic distance of at least 15% to the nearest subtype is required, represented by three unique sequences.[16] Using these criteria, we identified three new subtypes of genotype 1 and one new subtype of genotype 2 (figures 2, 3; appendix 2 pp 1, 3). Samples Benin30, Benin77, and Benin78 formed the new subtype 1q. Benin4, Benin7, Benin9, Benin18, and Benin35 were newly classified as subtype 1r and Benin11, Benin22, Benin34, Benin36, Benin39, Benin64, Benin84, and Benin93 as subtype 1s. Three sequences within genotype 2 (Benin51, Benin85, and Benin88) were designated as subtype 2xa. Within-genotype sequences had a pairwise distance of less than 9%.

53 (93%) of 57 sequenced samples contained at least two resistance-associated substitutions. Such substitutions were identified in 45 (79%) of 57 samples at position 28, 34 (60%) at position 24, 22 (39%) at position 31, and seven (12%) at position 93 of the NS5A protein (table 2, appendix 2 pp 20–23). No unique mutations were present in the three samples associated with unsuccessful treatment; these samples harboured Ser24Ser, Phe/Leu28Phe, and Leu/Met31Met, which were also present in the majority of individuals who had SVR12. No resistance-associated substitutions were identified in the NS5B protein.

We selected nine genotype 2 HCV sequences to assess replication capacity, including subtype 2d and previously unassigned subtypes. All subtype 2d sequences were from individuals who had been treated with sofosbuvir–velpatasvir, including the two individuals for whom treatment was not successful. The sequences representing unassigned genotype 2 subtypes were from individuals who had not yet been treated. A subtype 2b replicon, which was derived from the NS5A sequence in the patient with viral subtype 2b in whom treatment was not successful, was generated but did not replicate. Details of the constructs and replication capacities are shown in figure 3a and appendix 2 (p 24). Three constructs (Benin48, Benin43, and Benin81) were at least 1·5 times more efficient at replicating than the JFH-1 HCV strain. Benin17 and Benin38, both viruses from individuals for whom treatment was not successful, were 25–50% less efficient at replicating than the JFH-1 strain.

We next measured the in-vitro efficacy of ledipasvir, velpatasvir, and pibrentasvir against each genotype 2 construct and calculated the change in IC_{50} relative to JFH-1 (figure 3b). IC_{50} values for ledipasvir were higher in all genotype 2 Benin viruses than in JFH-1; this drug is not recommended for the treatment of genotype 2 HCV by WHO.[3] For velpatasvir, IC_{50} values were not higher in any of the genotype 2 viruses than in RAS control 1. Although Benin6 was successfully treated, the IC_{50} (2·71 nM) of velpatasvir in this sample was 90 times higher than in JFH-1 (0·03 nM). IC_{50} values of velpatasvir were around 15 times higher in Benin43 (0·42 nM) and around 50 times higher in Benin81 (1·45 nM) than in JFH-1, but the individuals from whom these samples were obtained have not yet been treated. Pibrentasvir showed excellent activity against all Benin virus constructs. Dose–response curves for all constructs are shown in appendix 2 (pp 5–8).

Discussion

This study is the first in Benin (and in west Africa) to report on HCV genetic diversity and treatment outcomes using direct-acting antivirals. Before this study, only six HCV genome sequences were available from Benin, and only one of these was full length.[8,19] Among the 57 full genomes generated in this study, we detected a high diversity within HCV genotypes 1 and 2. Genotype 2 is the most frequently reported HCV genotype in west African countries, followed by genotype 1.[20] Although all genotype 2 subtypes are found in west Africa or in west African communities in Europe,[21] there is a dearth of HCV genomic data from this region. In this study, we identified several new HCV subtypes (1q, 1r, 1s, and 2xa) and multiple unassigned genotype 1 and 2 subtypes. The most common subtype detected in Benin was 2d, followed by 1s and 1r. These endemic subtypes have not been studied in large clinical trials and are highly divergent from the less variable founder subtypes found in high-income countries.

The median age of patients at diagnosis was 65 years, in keeping with several other studies in the African region.[22–24] This advanced age at diagnosis probably reflects changes in risk factors for iatrogenic transmission, but could also reflect the higher recruitment of symptomatic rather than asymptomatic participants in this hospital setting. As in many other countries in sub-Saharan Africa, HCV acquisition was not associated with people who inject drugs. Despite the older median age of patients in our study, the

lower limit of the age range (27 years) indicates that younger patients are still being infected and transmission risk has not yet been eliminated in Benin.

Treatment and outcome data were available for 52 individuals and the SVR12 rate in this real-world study of the Benin national treatment programme was 94%, despite a high diversity of detected HCV sequences. Unfortunately, state-sponsored treatment is not available to all patients in Benin and has to be paid for at a current cost of around 525 000 west African CFA francs (around US$870; approximately an average yearly salary) as of March, 2024. For those able to access treatment, we found slightly higher SVR12 rates than those of patients of west African origin treated in the UK for diverse genotype 1 subtypes,[9] although we did not detect any HCV subtype 1l infections—a subtype detected in other west African countries—in our study. In a UK study,[9] three individuals with subtype 1l infection did not have SVR12 (two were from Nigeria and one was from Cameroon). In this study, the SVR12 rate among genotype 1 infections was 100%, despite the majority of individuals being infected with a previously unassigned genotype 1 subtype (1s was the most prevalent, followed by 1r). Three individuals in whom treatment was not successful were infected with either genotype 2b or 2d, of whom two had cirrhosis and one had unknown liver disease status. A real-world study conducted in the USA reported similar SVR12 rates in patients with genotype 2 infections treated with sofosbuvir–velpatasvir, at around 94%.[25] In our study, only two patients with genotype 2b were treated and so SVR12 rates in this group are not generalisable. Higher SVR12 rates for genotype 2b have also been previously reported in New Zealand,[26] although NS5A mutations were detected in only six (32%) of 19 patients—a number far lower than in this study in Benin.

93% of genomes sequenced had least two resistance-associated substitutions in the NS5A protein, including Phe/Leu28Phe, Leu/Met28Met, Ser/Thr24Ser and Leu/Met31Met.[3,5] Although baseline resistance-associated substitutions in NS5A were present in a high proportion of treated individuals, the majority had SVR12, highlighting the utility of direct-acting antivirals in reaching SVR in this real-world setting. However, subtype 2d had an SVR12 rate of 80% (eight of ten patients), which is lower than expected in the era of direct-acting antivirals.[25] This subtype was the most commonly detected in Benin and further studies are indicated to substantiate this lower-than-expected SVR12 rate. The resistance-associated substitutions in NS5A in the two patients with genotype 2d in whom treatment was not successful (Benin17 and Benin38) were a serine at position 24 and a phenylalanine at position 28. These mutations are intrinsic to this subtype and could increase the risk of unsuccessful treatment. Two patients with subtype 2d had an additional resistance-associated substitution: methionine at position 31 (Benin6 and Benin60). We did not observe a histidine at position 93 in NS5A, a commonly observed resistance-associated substitution among patients with genotype 2 infections in whom treatment is unsuccessful, although in this study we were not able to sample patients after unsuccessful treatment.

Using SGRs modified to reflect the *NS5A* gene of clinical isolates, we showed that velpatasvir inhibited a range of genotype 2 viruses, including those derived from samples associated with unsuccessful treatment. One genotype 2d SGR (from sample Benin6)—harbouring Ser24Ser, Phe/Leu28Phe, and Leu31Met mutations in NS5A—was associated with SVR12, but the IC_{50} of velpatasvir was 90 times higher than in JFH-1. This strain might be harder to treat in individuals with cirrhosis. The replication capacities of some resistant SGRs were approximately half those of the wild type, suggesting that these treatment-resistant viral strains might be less fit. However, the Benin6 SGR showed a slight increase in replication capacity, indicating that resistance in this subtype (2d) is not limited by fitness cost. We also showed that ledipasvir had reduced efficacy across a range of genotype 2 constructs. Because WHO do not recommend ledipasvir for the treatment of HCV genotype 2 infection, this finding does not affect treatment options in Benin.[3] Pibrentasvir had highly potent in-vitro activity against all Benin virus constructs tested. Although pibrentasvir is not readily available in sub-Saharan Africa, a case can be made for investing in this drug in regions with diverse HCV lineages, where a drug with truly pan-genotypic activity might be the most suitable treatment option.

Our study has limitations. Recruitment at hospital sites increases the chances of recruiting older individuals presenting with symptoms rather than younger patients with asymptomatic infection. Additionally, as the genetic diversity of HCV in Benin is high, studying the response of different subtypes to treatment was limited by small numbers, and samples were not available to assess the evolution of resistance-associated substitutions in patients in whom treatment was not successful.

We report a high real-world SVR rate following the treatment of HCV infection in Benin, in keeping with trials in high-income countries despite the high genetic diversity of the virus in west Africa. Treatment was unsuccessful in some patients with genotype 2b and 2d infection, indicating the need for larger studies of SVR in these subtypes. We describe several new HCV subtypes from an undersampled region that harbours high HCV diversity and is probably close to the evolutionary origin of HCV genotypes 1 and 2. In Benin, sofosbuvir–velpatasvir is an effective treatment (and is recommended by WHO) for patients with HCV that has not been genotyped and sofosbuvir–ledipasvir is effective in patients with genotype 1 infection.

Contributors

LA, RS, and ECT designed the study. LA and RS collected and analysed the data. ARK, KSA, RK, JS, NK, SEG, and LA recruited the patients. MN, CW, and SR carried out statistical analysis. CD, AF, and LT ran the sequencing. RS and CD ran the replicon assays. EG and RG ran viral load assays. MN, CD, ECT, SV, LA, and RS carried out bioinformatic analysis. RS and LA produced the tables and figures. PFK, SJG, PO, and ECT provided supervision. LA, RS, and ECT accessed and verified all the data in the study and wrote the manuscript. All authors had full access to all the data

in this study, critically reviewed the manuscript, approved the final version, and had final responsibility for the decision to submit for publication.

Declaration of interests

ECT declares funding from the Medical Research Council (MRC) for the MRC Preparedness Platform and the MRC World Class Labs award 2023/24, and her institution has received research funds from Novavax, AstraZeneca, the University of Oxford, and the University of Southampton. She has acted as an external consultant for WHO (HCV and Ebola virus), is the Chair of the BHIVA hepatitis subcommittee, and is a member of UK Health Security Agency technical groups. All other authors declare no competing interests.

Data sharing

GenBank accession numbers (OM525854–OM525910) are provided in appendix 2 (pp 17–18). The custom code developed for data analysis in this study, along with the raw data from replicon experiments, are available upon reasonable request to ECT. Due to strict adherence to data protection regulations and the ethical approvals governing our study, de-anonymised clinical data cannot be made available.

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References

1 WHO. Hepatitis C. July 18, 2023. https://www.who.int/news-room/fact-sheets/detail/hepatitis-c (accessed Oct 30, 2023).
2 Blach S, Zeuzem S, Manns M, et al. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *Lancet Gastroenterol Hepatol* 2017; **2:** 161–76.
3 WHO. Guidelines for the care and treatment of persons diagnosed with chronic hepatitis C virus infection. July 1, 2018. https://www.who.int/publications/i/item/9789241550345 (accessed April 30, 2021).
4 WHO. Global hepatitis report, 2017. April 19, 2017. https://www.who.int/publications/i/item/9789241565455 (accessed April 30, 2021).
5 Pawlotsky J-M, Negro F, Aghemo A, et al. EASL recommendations on treatment of hepatitis C 2018. *J Hepatol* 2018; **69:** 461–511.
6 Shah R, Ahovegbe L, Niebel M, Shepherd J, Thomson EC. Non-epidemic HCV genotypes in low- and middle-income countries and the risk of resistance to current direct-acting antiviral regimens. *J Hepatol* 2021; **75:** 462–73.
7 Sonderup MW, Afihene M, Ally R, et al. Hepatitis C in sub-Saharan Africa: the current status and recommendations for achieving elimination by 2030. *Lancet Gastroenterol Hepatol* 2017; **2:** 910–19.
8 Childs K, Davis C, Cannon M, et al. Suboptimal SVR rates in African patients with atypical genotype 1 subtypes: implications for global elimination of hepatitis C. *J Hepatol* 2019; **71:** 1099–105.
9 Gupta N, Mbituyumuremyi A, Kabahizi J, et al. Treatment of chronic hepatitis C virus infection in Rwanda with ledipasvir-sofosbuvir (SHARED): a single-arm trial. *Lancet Gastroenterol Hepatol* 2019; **4:** 119–26.
10 Thomson E, Ip CL, Badhan A, et al. Comparison of next-generation sequencing technologies for comprehensive assessment of full-length hepatitis C viral genomes. *J Clin Microbiol* 2016; **54:** 2470–84.
11 Bankevich A, Nurk S, Antipov D, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19:** 455–77.
12 Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* 2002; **30:** 3059–66.
13 Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 2006; **22:** 2688–90.
14 Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 2018; **35:** 1547–49.
15 Singer JB, Thomson EC, McLauchlan J, Hughes J, Gifford RJ. GLUE: a flexible software system for virus sequence data. *BMC Bioinformatics* 2018; **19:** 532.
16 Smith DB, Bukh J, Kuiken C, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. *Hepatology* 2014; **59:** 318–27.
17 Bamford CGG, Aranday-Cortes E, Filipe IC, et al. A polymorphic residue that attenuates the antiviral potential of interferon lambda 4 in hominid lineages. *PLoS Pathog* 2018; **14:** e1007307.
18 Shah R, Barclay ST, Peters ES, et al. Characterisation of a hepatitis C virus subtype 2a cluster in Scottish PWID with a suboptimal response to glecaprevir/pibrentasvir treatment. *Viruses* 2022; **14:** 1678.
19 Jeannel D, Fretz C, Traore Y, et al. Evidence for high genetic diversity and long-term endemicity of hepatitis C virus genotypes 1 and 2 in west Africa. *J Med Virol* 1998; **55:** 92–97.
20 Candotti D, Temple J, Sarkodie F, Allain JP. Frequent recovery and broad genotype 2 diversity characterize hepatitis C virus infection in Ghana, west Africa. *J Virol* 2003; **77:** 7914–23.
21 Jordier F, Deligny ML, Barré R, De Micco P, Cantaloube JF. Evidence for two phylogenetic clusters within hepatitis C virus (HCV) genotype 2 inferred from analysis of complete coding sequences of 15 HCV strains. *J Med Virol* 2013; **85:** 1754–64.
22 Iles JC, Abby Harrison GL, Lyons S, et al. Hepatitis C virus infections in the Democratic Republic of Congo exhibit a cohort effect. *Infect Genet Evol* 2013; **19:** 386–94.
23 Davis C, Mgomella GS, da Silva Filipe A, et al. Highly diverse hepatitis C strains detected in sub-Saharan Africa have unknown susceptibility to direct-acting antiviral treatments. *Hepatology* 2019; **69:** 1426–41.
24 Audu RA, Okwuraiwe AP, Ige FA, Adeleye OO, Onyekwere CA, Lesi OA. Hepatitis C viral load and genotypes among Nigerian subjects with chronic infection and implication for patient management: a retrospective review of data. *Pan Afr Med J* 2020; **37:** 335.
25 Belperio PS, Shahoumian TA, Loomis TP, Mole LA, Backus LI. Real-world effectiveness of daclatasvir plus sofosbuvir and velpatasvir/sofosbuvir in hepatitis C genotype 2 and 3. *J Hepatol* 2019; **70:** 15–23.
26 Gane EJ, Hyland RH, Yang Y, et al. Efficacy of ledipasvir plus sofosbuvir for 8 or 12 weeks in patients with hepatitis C virus genotype 2 infection. *Gastroenterology* 2017; **152:** 1366–71.