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Hepatitis C Virus Diversity in Sub-Saharan Africa and implications for treatment with Direct Acting Antivirals

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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March 2024

Abstract

Hepatitis C virus (HCV) is a leading cause of chronic liver disease worldwide with an estimated 58 million suffering with chronic HCV infection and approximately 1.5 million new infections occurring each year. The World Health Organization (WHO) has set ambitious goals to eliminate viral hepatitis by 2030. A key strategy behind these goals is the discovery of Direct Acting Antivirals (DAAs), which have revolutionised the treatment of HCV infection. However most clinical trials and real world DAA treatment programs have been conducted in high income countries where the predominant circulating -genotypes are epidemic lineages. However, in Sub-Saharan Africa endemic lineages are more common and less well characterised. There is also growing evidence that certain sub-genotypes are more challenging to treat, for example sub-genotypes 1l and 4r. The aims of this thesis were to explore the diversity of HCV using datasets from Uganda and Benin. Furthermore, the prevalence of HCV and associated liver disease from people who inject drugs (PWID) in Coastal Kenya was assessed and HCV genetic data was used to explore PWID networks and estimate the origin of HCV in this community. We found highly divergent HCV genomes in Uganda and Benin including the discovery of multiple new subtypes (1q, 1r, 1s and 2xa in Benin and 4xa in Uganda) as well as numerous subtypes that have previously been uncharacterised. Interestingly, we found only epidemic lineages 1a and 4a in Kenyan PWID. Molecular clock analysis suggests the introduction of HCV 1a from Europe into Kenyan PWID, as recently as 2001, whereas HCV 4a is likely to have been introduced around 1980, from Egypt. Network analysis using HCV genetic data suggests that Kenyan PWID networks are very closely linked, with repeated transmissions between individuals likely. The prevalence of chronic HCV infection in Kenyan PWID is high at 36%, of which 62% were viraemic. Among the 34% of PWID that had HCV-HIV coinfection, 76% were HCV viraemic. The prevalence of baseline NS5A RASs in diverse HCV sub-genotypes from Uganda and Benin is high compared to HCV 1a and 4a seen in Kenyan PWID. However, i*n vitro* experiments using a sub-genomic replicon system suggest that genotype 2 from Benin is largely susceptible to DAAs. Data from this thesis supports the WHO's ambitious goals to achieve an 80% reduction in incident HCV infections worldwide by 2030 using appropriate DAA regimens.

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Publications

1.1 Publications arising from contributions made to HCV studies

The manuscripts of all the publications listed in this section can be found in the appendices.

R. Shah, L. Ahovegbe, M. Niebel, J. Shepherd, and E. C. Thomson, "Non-epidemic HCV genotypes in low- and middle-income countries and the risk of resistance to current direct-acting antiviral regimens," *J Hepatol,* vol. 75, pp. 462-473, Aug 2021.

- Work from this publication relates to HCV genetic diversity as described in section 2.3 and Figure 2-18 on page 68.
- RS collected and analysed data, created figures and assisted with manuscript writing, LA assisted with manuscript writing, MN and JS assisted with the figures, ET oversaw manuscript writing.

R. Shah, A. Agyei-Nkansah, F. Alikah, L. Asamoah-Akuoko, Y. C. O. Bagou, A. Dhiblawe, *et al.*, "Hepatitis C virus in sub-Saharan Africa: a long road to elimination," *Lancet Gastroenterol Hepatol,* vol. 6, pp. 693-694, Sep 2021.

- Work from this publication relates to the challenges of HCV elimination in SSA as described in section 2.10, including data shown in Table 2-3.
- RS co-ordinated survey responses, collected and analysed data and wrote the manuscript. ET and PO reviewed and edited the manuscript. The remaining authors are members of the HCV SSA network responding to the survey.

R. Shah, S. T. Barclay, E. S. Peters, R. Fox, R. Gunson, A. Bradley-Stewart, *et al.*, "Characterisation of a Hepatitis C Virus Subtype 2a Cluster in Scottish PWID with a Suboptimal Response to Glecaprevir/Pibrentasvir Treatment," *Viruses,* vol. 14, Jul 29 2022.

- Work from this publication relates to the investigation of HCV sub-genotype 2a in a cohort of Glasgow PWID and is presented in section 4.4.

- RS analsyed sequence data, conducted the *in-vitro* experiments, constructed figures and wrote the manuscript. SB, EP and RF provided clincal data. RG, ABS, SJS and AM provided clinical samples. LT, VJE, MNCB and AF sequenced HCV from clincial samples and generated whole genome sequences. ET and CD supervised the study and edited the manuscript.

R. Shah, P. Boucheron, K. Mandaliya, A. Kattamaiyo, S. Chevaliez, Y. Shimakawa, E. Songok, M. Lemoine,, "Hepatitis C virus infection in people who inject drugs in Africa," *Lancet Infect Dis,* vol. 20, pp. 282-283, Mar 2020.

- Work from this publication relates to the clinical and demographic characteristics of the Kenyan PWID cohort as described in sections 3.2.1 and 4.1.

- RS designed and implemented the study, collected and analysed data and wrote the manuscript. KM and AK provided laboratory support for sample preparation and storage. SC conducted HCV qPCR and genotyping. PB and YS oversaw statistical analysis .ES and ML supervised the study and revised the manuscript.

Lucrèce Ahovègbé*, Rajiv Shah*, Aboudou Raïmi Kpossou, Chris Davis, Marc Niebel, Ana Filipe, Emily Goldstein, Khadidjatou S Alassan, René Keke, Jean Sehonou, Nicolas Kodjoh, Sossa Edmond Gbedo, Surajit Ray, Craig Wilkie, Sreenu Vattipally, Lily Tong, Pakoyo F Kamba, S Judith Gbenoudon, Rory Gunson, Patrick Ogwang, Emma C Thomson. Hepatitis C virus diversity and treatment outcomes in Benin: a prospective cohort study, The Lancet Microbe (published online June 14, 2024 https://doi.org/10.1016/S2666-5247(24)00041-7)

- Work from this publication relates to the diversity of HCV in Benin, presence of RASs and the *in-vitro* effectiveness of NS5A inhibitors against diverse genotype 2 subtypes, as described in section 4.3.

- LA and RS are joint first authors. RS conducted bioinformatic analysis to generate whole genomes, constructed phylogenetic trees for genotyping and subtyping, conducted the *in-vitro* assays and created the figures and assisted with manuscript writing. LA designed the study and wrote the manuscript. ARK, KSA, RK, JS, NK, SEG, PFK, and SJG recruited and provided clinical data on the cohort members. EG and RG conducted HCV qPCR on serum samples. CD, LT and AF sequenced HCV from clinical samples. SR and CW assisted with statistical analysis. CD assisted with sequence analysis. SV wrote the bioinformatic pipelines. PO and ET supervised the study and revised the manuscript.

Heffernan A, Barber E, Cook NA, Gomaa AI, Harley YX, Jones CR, Lim AG, Mohamed Z, Nayagam S, Ndow G, **Shah R**, Sonderup MW, Spearman CW, Waked I, Wilkinson RJ, Taylor-Robinson SD. Aiming at the Global Elimination of Viral Hepatitis: Challenges Along the Care Continuum. Open forum infectious diseases. 2018;5(1):ofx252

- This publication relates to the challenges of HCV elimination. I contributed to the section on HCV elimination in key populations , in particular the text relating to PWID. The Kenya HCV in PWID study contributed to this statement paper. AH co-ordinated manuscript writing and the remaining authors contributed their relevant expertise.

1.2 Manuscripts in preparation

Rajiv Shah, Alex Kattam Maiyo, Joyceline Kinyua, Chris Davis, Lily Tong, Ana Filipe de Silva, Elijah Songok, Emma Thomson. The introduction of epidemic HCV subtypes 1a and 4a among highly connected PWID networks in Coastal Kenya.

- Related to sections 4.1.2, 4.1.3 and 4.1.4.

- I designed this study, collected and analysed the data, created figures and will write the manuscript. AM and JK provided laboratory support with sample storage and preparation in Kenya. CD, LT, AS conducted library preparation and sequencing. CD assisted with bioinformatic analysis. ES

and ET provided supervision. ET supervised bioinformation analysis and will edit the manuscript.

Rajiv Shah, Lucrece Ahovegbe, Lily Tong, Ana Filipe de Silva, Sreenu Vattipally, Ponsiano Ocama, Chris Davis, Emma Thomson. Diversity and origins of HCV genotype 4 in Uganda.

- Related to sections 4.2.1, 4.2.2 and 4.2.3.
- I conducted the wet laboratory work and bioinformatic analysis, created figures and will write the manuscript. LA conducted sample collection. LT and AS oversaw library preparation and sequencing. SV wrote the bioinformatic pipelines. PO oversaw sample collection, storage and transport and provided overall supervision. CD and ET are the senior authors with overall supervision.

HCV Drug Resistance in resource limited settings. Current knowledge, research gaps, and public health implications.

- A multiple author list contribution to a statement paper from WHO that I will be co-ordinating.

Acknowledgements

Firstly, I would like to thank the Medical Research Council for giving me the opportunity and funds to carry out a PhD at the Centre for Virus Research. It has been an incredible opportunity to work with talented researchers across several basic science disciplines.

I would like to thank my supervisor Professor Emma Thomson whose work ethic, dedication to, and passion for science is immense and has served as an inspiration to me. Negotiating the COVID-19 pandemic was incredibly challenging but she still found time to encourage and support me through my PhD despite being extremely busy with the COVID-19 public health response.

I would also like to thank Thomson lab members Dr Chris Davis and Dr Marc Niebel who were both instrumental in me getting through my experiments and data analysis, as well as always providing a shoulder to lean on. I would have not completed this thesis without their support. I also appreciate the support from the rest of the Thomson lab group including Dr Patawee Asamaphan, Dr Shirin Ashraf, Dr James Shepherd, Dr Ben Talbot (I appreciate the guidance and support with the use of Phyloscanner), Dr Stella Atim, Martin Mayanja, Prossy Namuwulya, Dr Elen Vink, Marina Kugler and Paula Olmo.

I would also like to thank my secondary supervisor Dr Sreenu Vattipally who was extremely patient with guiding me through the bioinformatic analysis parts of the thesis. He was always approachable and took the time to explain concepts that I struggled to understand. I am grateful for the support provided by my assessors Professor Arvind Patel and Dr Antonia Ho.

I would also like to thank my tertiary supervisor Professor Elijah Songok and his team members in KEMRI, Nairobi; Bernard Langat, Joyceline Kinyua and Alex Kattam Maiyo. They were instrumental for the initiation of the Kenya HCV in PWID project that started in 2015 through an ISSF Wellcome Trust Fellowship at Imperial College, London. At the same time, I would like to thank Dr Maud Lemoine, Professor Mark Thurz and Professor Simon Taylor-Robinson for guiding and supporting me through that fellowship. A significant part of this thesis would not have been possible without the ground work done during that fellowship. I

also appreciate the staff at the drop-in-centres in Mombasa (Reachout Centre Trust, Old Town and Muslim Education & Welfare Association Drop-In-Centre, Kisauni) and Watamu (KANCO Drop-In-Centre). I am indebted to them for helping me recruit PWID into the study and ensuring the field work ran smoothly. I am grateful to all PWID who agreed to be part of the study. Finally, I am grateful to Diana and Munga who accompanied me on field visits and provided an excellent phlebotomy service.

I am immensely grateful to Lucrece Ahovegbe for sharing her Benin data with me. We crossed paths at the CVR for six months and she is an inspirational young person.

For the Uganda data I am grateful to Professor Ponsiano Ocama and his team at Makerere University, Kampala, Uganda for providing plasma samples

I would like to especially mention Dr Pradhib Venkatesan, who is my clinical supervisor and has been a mentor to me since I was a medical student. His immense work ethic, passion for science and extensive knowledge-base, combined with a very humble personality has served as an inspiration. His encouragement and support have allowed me to take time out of my clinical training program to pursue academic interests.

I am immensely grateful to my parents and grand-parents for providing me with an education and always being unconditionally supportive of my career choices. Lastly, I am forever indebted to my wife, Bhavisha, for providing unconditional and unwavering support. I am incredibly lucky to have her in my life and none of this would have been possible for me without her.

Author's Declaration

This work was completed at the MRC-University of Glasgow Centre for Virus Research between August 2019 and December 2022 and has not been submitted for another degree. All work presented in this thesis was obtained by the author's own efforts, unless otherwise stated.

Next generation sequencing was carried out by Dr Lily Tong (NGS Genomics Team).

The bioinformatic pipeline used for creating HCV consensus sequences was developed by Professor Emma Thomson using in house scripts developed by Dr Sreenu Vattipally.

The bioinformatic pipeline used for investigating mixed infections and removal of cross-contamination was developed by Professor Emma Thomson.

BEAST analysis was optimised and completed by Professor Emma Thomson.

The HCV-GLUE framework used for investigating deep sequencing data was developed by Dr Josh Singer with further functionality developed in conjunction with Dr Marc Niebel.

Plasmids pSGR-JFH1 and pJFH-1/GND, for the *in-vitro* experiments, were supplied by Dr Connor Bamford from Professor John McLauchlan's laboratory.

Definitions/Abbreviations

A	Alanine
AASLD	American association for the study of liver diseases
APRI	AST to platelet ratio index
BAM	compressed Binary version of SAM file
C	Cysteine
CI	Confidence interval
CLDN-1	Claudin-1
CLD	Cytoplasmic lipid droplet
DAA	Directly Acting Antivirals
DGAT1	Diacylglycerol acyltransferase-1
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EASL	European Association for the Study of the Liver
EIA	Enzyme immunoassay
F	Phenylalanine
FAM	Fluroescin amidites
FIB-4	Fibrosis-4
G	Glycine
H	Histidine
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HIC	Higher income country
HIV	Human immunodeficiency virus
I	Isoleucine
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
IRES	Internal ribosome entry site
K	Lysine
kDa	Kilodalton
L	Leucine
LIC	Lower income country
LDL	Low-density lipoprotein
M	Methionine
MASLD	Metabolic dysfunction-associated steatotic liver disease
MGB	Minor groover binder
MSM	Men who have sex with men
N	Asparagine
NCR	Noncoding region
dNTP	Deoxynucleoside triphosphate
OCLN	Occludin
ORF	Open reading frame
P	Proline
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
PEG	Polyethylene glycol
POC	Point-of-care
PWID	People who inject drugs

Q	Glutamine
QC	Quality control
R	Arginine
RAS	Resistance associated substitution
RDP	Recombination detection program
RdRp	RNA-dependent RNA polymerase
RBV	Ribavirin
RLU	Relative light units
S	Serine
SAM	Sequence alignment map
SGR	Sub-genomic replicon
SPRI	Solid phase reversible immobilisation
SR-B1	Scavenger receptor B type I
SSA	Sub-Saharan Africa
SVR12	Sustained virological response at 12 weeks
SVR24	Sustained virological response at 24 weeks
T	Threonine
TMRCA	Time to most recent common ancestor
TRIS	Tris(hydroxymethyl)aminomethane
USA	United States of America
V	Valine
VLDL	Very-low-density lipoprotein
W	Tryptophan
WHO	World Health Organisation
Y	Tyrosine

2 Introduction

2.1 Overview

Hepatitis C Virus (HCV) is a bloodborne virus that predominantly infects the liver. It is a leading cause of chronic liver disease worldwide with an estimated 58 million suffering with chronic HCV infection and approximately 1.5 million new infections occurring each year [1]. It was estimated in 2019 that 290,000 people lost their lives from the complications of HCV related chronic liver disease, namely cirrhosis and hepatocellular carcinoma (HCC). The World Health Organization (WHO) has set ambitious goals to eliminate viral hepatitis by 2030 [2]. A key strategy behind these goals is the discovery of Direct Acting Antivirals (DAAs), which have revolutionised the treatment of HCV infection. Before the advent of DAAs, treatment for HCV was not very effective, involved long treatment durations, was not well tolerated, and was dependent on HCV sub-genotype. DAAs have transformed these issues. They are all oral regimens with short treatment durations of 8 to 12 weeks, very well tolerated and effective against multiple genotypes thus allowing simplification of treatment programs.

However, most countries are currently off-track for achieving these goals with an estimated 78.6% of HCV infections thought to be undiagnosed [3]. Furthermore, most clinical trials and real world DAA treatment programs have been conducted in high income countries (HICs) where the predominant circulating sub-genotypes are epidemic lineages. However, in lower income countries, for example in Sub-Saharan Africa (SSA), the predominant circulating sub-genotypes are very different and have not been well characterised. There is growing evidence that certain sub-genotypes are more challenging to treat, for example sub-genotypes 1l and 4r [4, 5].

HCV is an incredibly diverse virus with 8 known genotypes and over 90 known subtypes [6]. Yet it is likely that what we know represents a very small proportion of the true diversity of HCV given that most published whole HCV genomes are from HICs. This is, in part, explained by the need for expensive and resource intensive sequencing techniques to produce high quality genetic data.

This thesis explores the diversity of HCV in SSA and the implications of this diversity on treatment with DAAs.

2.2 Virology

HCV was discovered in 1989 following a series of cases of hepatitis not caused by Hepatitis A Virus (HAV) or Hepatitis B Virus (HBV) [7-9]. The HCV genome was then cloned by *Choo et al,* following which diagnostic tests were developed [10].

HCV is a member of the genus *Hepacivirus* within the virus family *Flaviviridae* [11, 12]. Its phylogenetic relatedness to other members of the family is shown in Figure 2-1. The origins of HCV remain unclear. Hepaciviruses have been discovered in several mammals including horses, rodents, bats, colobus monkeys and cows, however, the equine hepaciviruses remain the most genetically closely related to HCV [13]. Natural HCV infection only occurs in humans, but experimental infection of chimpanzees is possible. While this may suggest that the HCV pandemic originated from a non-human primate reservoir, similar to the Human Immunodeficiency Virus (HIV) [14], no evidence for such a source has been found [15, 16].

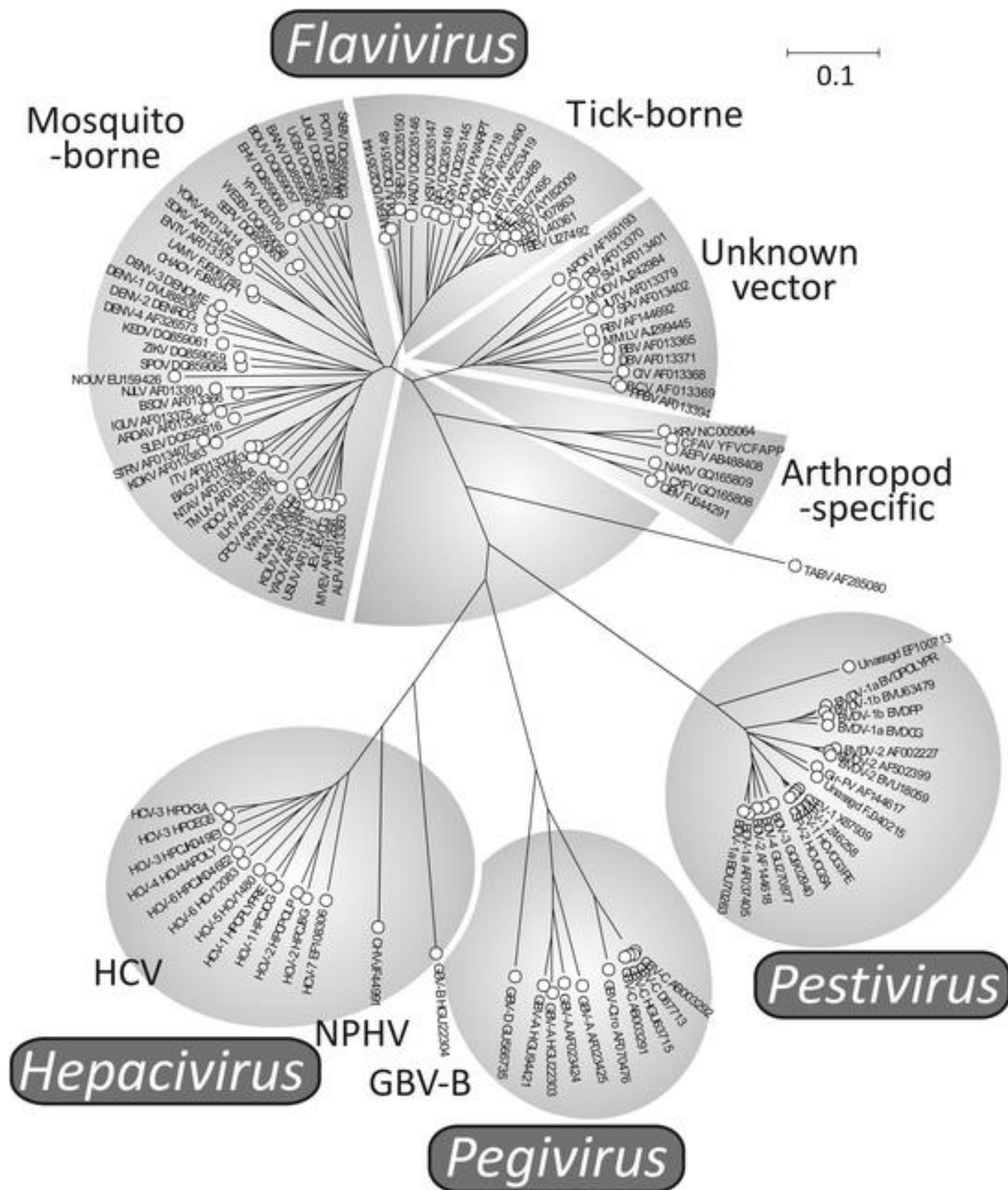


Figure 2-1: Phylogenetic tree of the members of the *Flaviviridae* family
The unrooted tree was constructed using sequences of conserved regions of the RNA polymerase gene, using a neighbour-joining method of amino acid p-distances. This figure is reproduced with permission from *Simmonds et al* [17], through the terms and conditions provided by Springer Nature and Copyright Clearance Centre (licence number 5725961136180).

2.2.1 Genome Organisation

HCV contains a 9.6 kb positive-strand RNA genome, with a 5’ noncoding region (NCR), a single open reading frame (ORF) that encodes three structural proteins and seven non-structural proteins, and a 3’ NCR. This is shown diagrammatically in Figure 2-2. The HCV polyprotein is just over 3000 amino acids, with some

variation between genotypes. Structural proteins are Core and the envelope proteins, E1 and E2. Non-structural proteins include the p7 viroporin, NS2 protease, NS3-NS4A protease/helicase complex, NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (RdRp).

The 5' NCR contains 341 nucleotides made up of four highly structured domains, numbered I to IV. These contain numerous stem-loops and a pseudoknot [18, 19]. Domains II, III and IV make up the internal ribosome entry site (IRES) [20]. The HCV IRES can form a stable pre-initiation complex by directly binding the 40S ribosomal unit without the need of canonical translation initiation factors. The 5' NCR is the most conserved region of the HCV genome. Its nucleotide sequence shares approximately 50% sequence identity with pestiviruses [21, 22].

The 3' NCR contains 225 nucleotides made up of three regions, which include a variable region of 30-40 nucleotides, a long internal poly(U)-poly(U/C) tract and a highly conserved X region, which contains three stem-loop structures [23-25]. The 3' NCR interacts with the NS5B RdRp [26, 27]. The X region and the 52 nucleotides upstream of the poly(U/C) tract were found to be essential for RNA replication, while the rest of the 3'NCR appears to enhance viral replication [28-31].

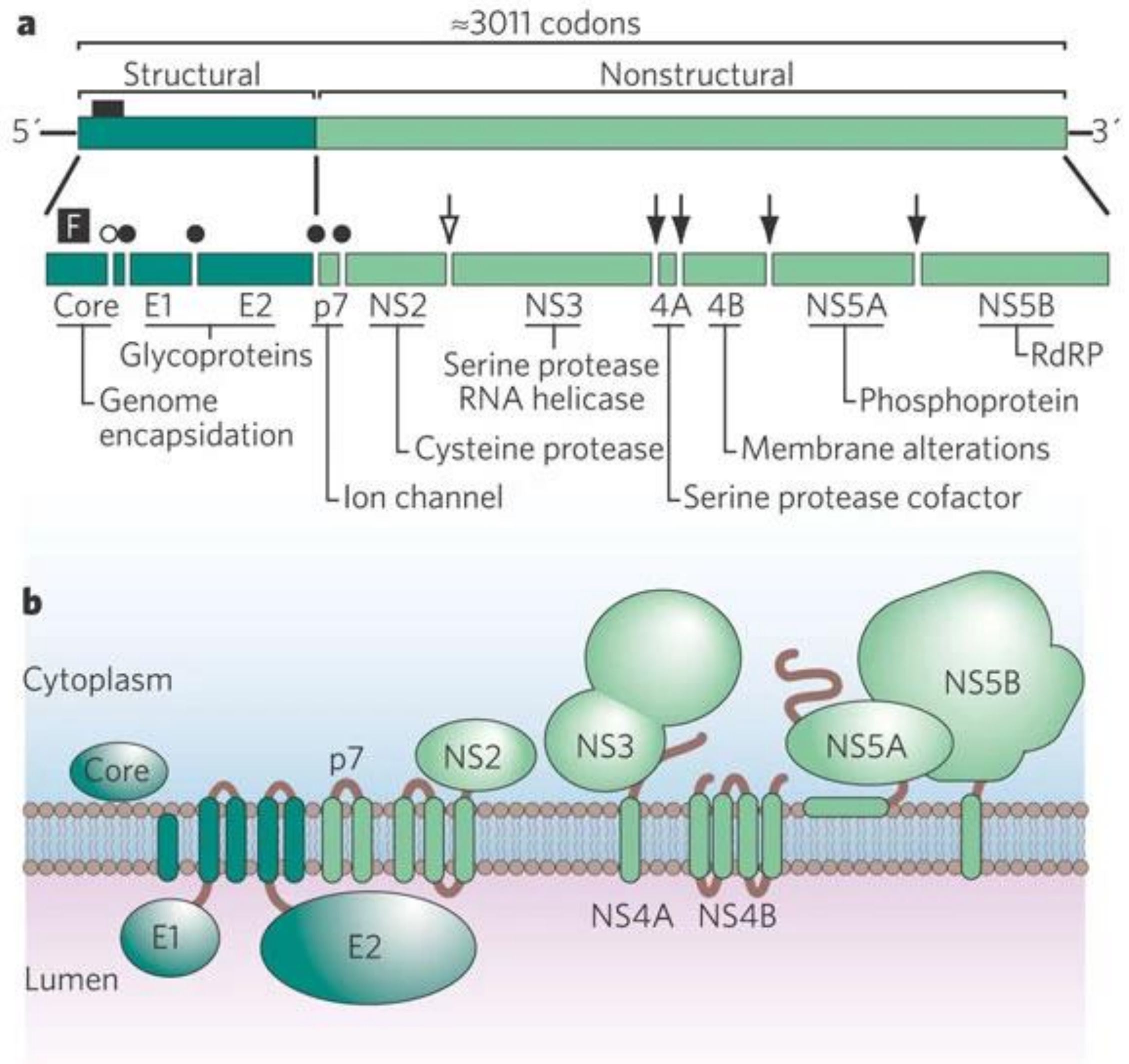


Figure 2-2: HCV genome organisation and structure
a, structure of the viral genome including non-coding regions and the long open reading frame encoding the HCV polyprotein. Circles denote signal peptidase cleavage sites and arrows refer to sites cleaved by NS3-NS4A protease. **b,** topology of HCV proteins in relation to the cellular membrane. This figure is reproduced with permission from *Lindenbach et al [32]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (http://creativecommons.org/licenses/by-nc-sa/3.0/).

2.2.1.1 Structural Proteins

The first structural protein encoded by the HCV ORF is the core protein, which is 191 amino acids and has a molecular weight of 21 kilodaltons (kDa). This protein forms the viral nucleocapsid. It is a highly basic, RNA binding protein. It consists of two domains. The N-terminal domain (D1) is hydrophilic and is involved in

nucleocapsid assembly [33]. This domain is also involved in numerous interactions with cellular factors and therefore may contribute to alterations of host cell function [34]. When expressed in transgenic mice it can induce hepatocellular carcinoma (HCC) [35, 36]. Core D1 contains a helix-loop-helix motif from amino acids 15-41, creating an immunodominant antigenic site, as well as containing residues that are critical for function [37]. The C-terminal domain (D2) is hydrophobic. D1 folds on interaction with D2 and this allows association with lipid droplets [38]. This interaction plays a role in steatosis formation [39, 40].

The envelope glycoproteins, E1 and E2, are essential components of the HCV life cycle, including virion assembly, virus entry and fusion with the endosomal membrane [41, 42]. They are both type I transmembrane glycoproteins with molecular weights of 33-35 and 70-75 kDa, respectively. They are highly glycosylated, E1 containing 5 glycosylation sites and E2 containing 11. E2 contains several hypervariable regions, which are extremely diverse and the amino acid sequences can differ by up to 80% between genotypes as well as between subtypes of the same genotype [43]. The most important of these is hypervariable region 1 (HVR1), which is a 27 amino acid peptide that is a major HCV neutralising epitope [44, 45].

The frameshift (F) protein is a result of a -2/+1 ribosomal frameshift in the N-terminal core-encoding region of the HCV polyprotein. Antibodies to this protein detected in individuals with chronic HCV infection suggest that it is produced during infection [46], however, its exact role in the HCV lifecycle is not known.

2.2.1.2 Non-structural Proteins

P7 is a small 63 amino acid polypeptide that is an integral membrane protein [47]. It comprises two transmembrane α-helices connected by a positively charged cytosolic loop. Mutations in this loop suppress infectivity of liver transfection of HCV cDNA in chimpanzees, thus suggesting p7 is an essential protein [48].

NS2 is a non-glycosylated transmembrane protein with a molecular weight of 21-23 kDa. Together with the N-terminal domain of NS3, NS2 constitutes a zinc-

dependent metalloprotease that cleaves the site between NS2 and NS3 [49-51]. NS2 is short lived, getting degraded following self-cleavage from NS3 [52]. Beyond its protease activity, NS2 plays a central role in organising virus assembly and interacts with host cellular proteins [53, 54].

The NS3-NS4A complex comprises of the multifunctional NS3 protein and cofactor NS4A. NS3 has 631 amino acids with a molecular weight of 70 kDa and contains a serine protease domain in its N-terminal third, and a helicase/NTPase domain in its C-terminal two-thirds. The NS4A cofactor is 54 amino acids. It contains a β-strand in its central portion that is incorporated into the N-terminal β-barrel of NS3. The N-terminal part of NS4A forms a transmembrane α-helix required for integral membrane association of the NS3-NS4A complex [55]. The C-terminal portion of NS4A forms a highly negatively charged α-helix that is involved in HCV RNA replication and virus particle assembly [56]. The NS3-NS4A complex is an important antiviral target.

The NS3 serine protease domain catalyses HCV polyprotein cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions. The catalytic triad is formed by amino acids Histidine, Aspartic Acid and Serine at positions 57, 81 and 19, respectively [57-59]. The NS3 helicase-NTPase is a member of the superfamily 2 DExH/D-box helicases [60]. It has multiple functions including, RNA binding and unwinding of RNA regions that have extensive secondary structure by coupling and NTP hydrolysis [61, 62]. The NS3-NS4A complex also plays roles in the persistence and pathogenesis of HCV. It has been shown to cleave toll-like receptor 3 (TLR3) molecule toll/IL-1R domain-containing adaptor inducing interferon (TRIF) as well as T cell protein tyrosine phosphatase [63, 64].

NS4B is an integral membrane protein of 261 amino acids with a molecular weight of 27 kDa. It has four transmembrane domains and an N-terminal amphipathic helix, which allow it to serve as a membrane anchor for the replication complex [65-67]. NS4B also modulates NS5B RdRp activity [68] and induces interleukin 8 [69].

NS5A is a 56-58 kDa, 447 amino acid membrane associated phosphoprotein. It associates with membranes through an N-terminal amphipathic helix [70, 71] and contains three distinct domains [71]. Domain I and II are required for genome

replication whereas domain III is involved in assembly. Domain I contains the conserved tetracysteine zinc-binding motif and shares high sequence similarity to primate and non-primate hepaciviruses [72-75]. Domains II and III, in contrast, have very low homology. NS5A is known to be extensively phosphorylated, with multiple phosphorylation sites across the protein, and can interact with over 100 host cellular proteins. This explains how it influences cell growth and cellular signalling pathways [76].

The NS5B protein is the RdRp, which is the key enzyme that facilitates HCV replication by synthesis of a complementary negative-strand RNA using the genome as a template. It is a 68 kDa, 591 amino acid protein. The N-terminal 530 amino acids form the catalytic domain, and contains motifs that are shared by all RdRps, including the trademark GDD sequence, and the classical fingers, palm, and thumb organisation of a right hand (Figure 2-3) [77-79]. Interactions between the fingers and thumb subdomains create an encircled catalytic site that ensures the synthesis of positive- and negative-strand RNA. The short C-terminal region of 21 amino acids forms an α-helical transmembrane domain that allows cytosolic orientation of the catalytic domain [80, 81].

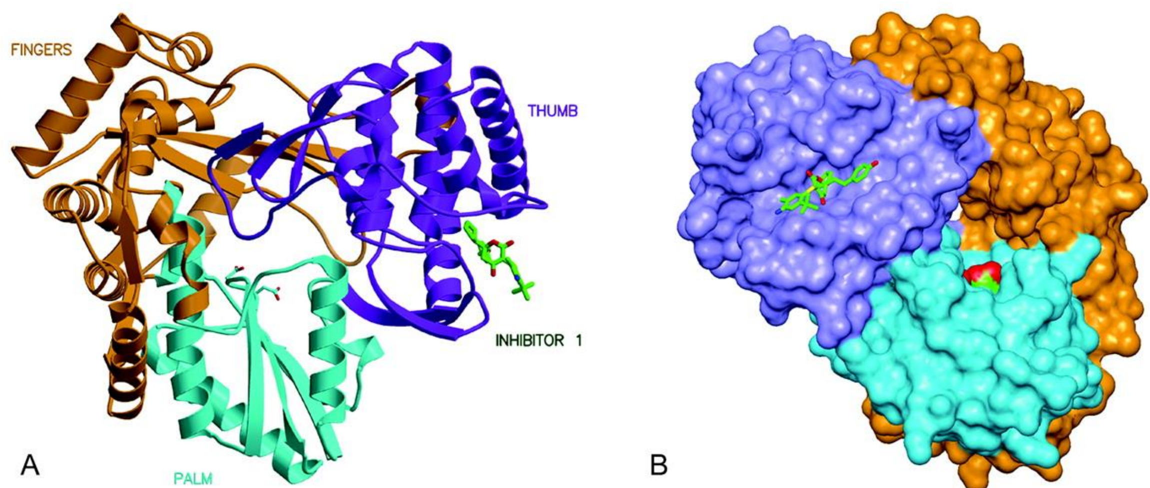


Figure 2-3: Overall views of HCV NS5B with bound inhibitor 1
(A) Ribbon representation of NS5B with domains coloured according to thumb (purple; residues 371 to 563), palm (blue; residues 188 to 227 and 287 to 370), and fingers (orange; residues 1 to 187 and 228 to 286). Active site aspartic acids 318 and 319 are visible at the centre of the molecule in the palm domain. (B) Molecular surface of NS5B with bound inhibitor 1, rotated ~90° from view in panel A. This figure is reproduced with permission from *Love et al* [82], through the terms and conditions provided by the American Society for Microbiology and Copyright Clearance Centre (licence ID 1449499-1).

2.2.2 Viral Life Cycle

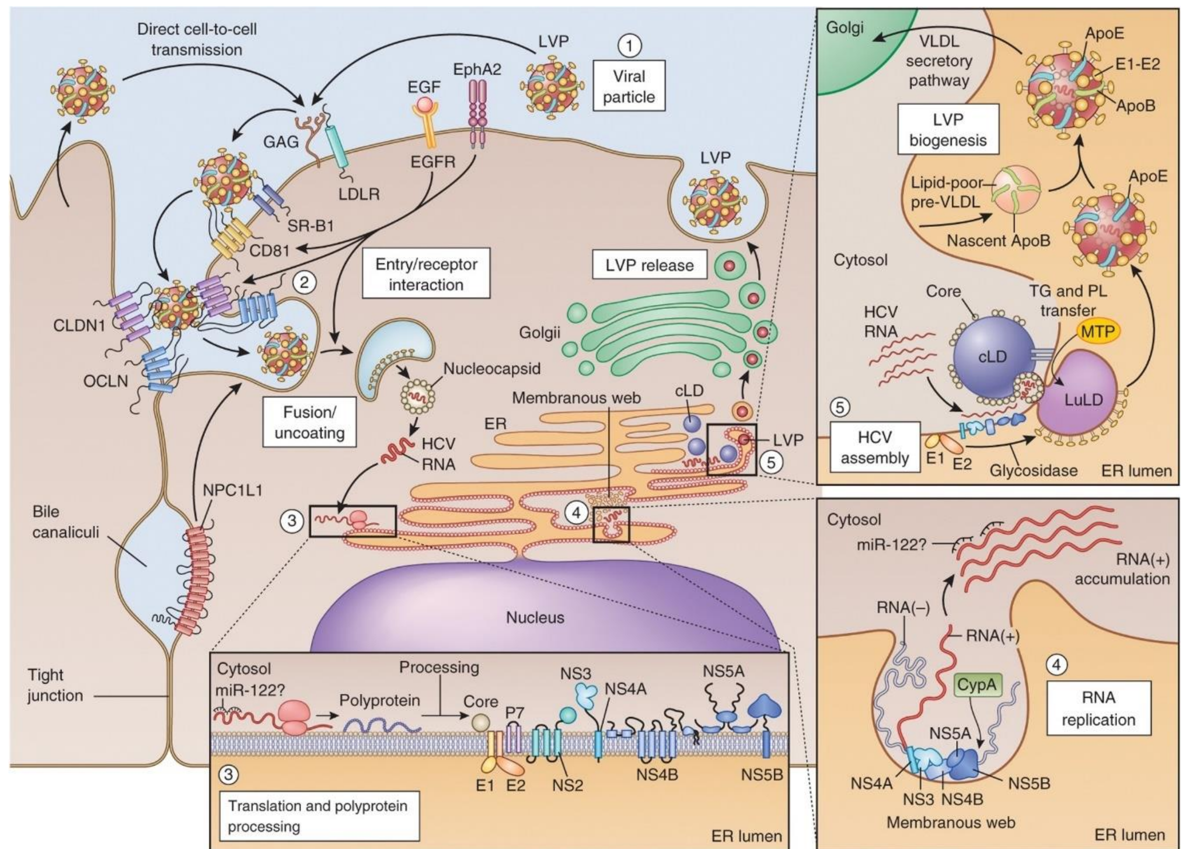
The HCV virion is enveloped and 50-80 nm in diameter. The envelope glycoproteins are embedded in a lipid bilayer surrounding the nucleocapsid made up of core protein and encasing the single-stranded RNA genome [83, 84]. HCV virions exist as lipoviroparticles and are associated with low-density and very-low-density lipoproteins (LDL and VLDL). This allows them to be pleomorphic and may allow them to escape neutralisation [85]. The life cycle of HCV is summarised in Figure 2-4, with a focus on sites for intervention.

Several receptors are involved in HCV entry into hepatocytes. The LDL receptor and glycosaminoglycans are thought to be initially involved in cell binding [86, 87], followed by interaction between E1, E2, and co-receptors scavenger receptor B type I (SR-B1) and CD81 [88, 89]. Other receptors, claudin-1 (CLDN1) and occludin (OCLN) are also required for entry [90, 91]. Clathrin-mediated endocytosis allows uptake of the virus particle into the cell [92], followed by fusion and uncoating. Endoplasmic reticulum-associated translation is initiated by an IRES located in the HCV 5' NCR [93]. The resulting HCV polyprotein is cleaved to release three structural and seven non-structural proteins, as described in section 2.2.1.

RNA replication takes place in association with endoplasmic reticulum derived membrane induced by NS4B and NS5A [65, 94]. NS5B is the main protein involved in genomic replication, as described earlier. The phosphorylation state of NS5A regulates the balance between RNA replication and downstream processes [95].

Virus assembly and disassembly is tightly regulated and coupled to host cell lipid synthesis [85]. After cleavage, core protein relocates from endoplasmic reticulum membranes to cytoplasmic lipid droplets (CLDs), assisted by diacylglycerol acyltransferase-1 (DGAT1) [38, 96, 97]. The HCV RNA genome is probably delivered to the sites of nucleocapsid assembly, facilitated by NS2-coordinated virion assembly through interaction with E1, E2, p7, NS3 and NS5A [98, 99]. Signal and stop-transfer sequences coordinate endoplasmic reticulum translocation of the envelope glycoproteins. After folding, heterodimerisation and addition of N-linked sugars, the E1 and E2 glycans are trimmed by glycosidases I and II [100]. In the later stages of assembly HCV embraces the

VLDL pathway [101, 102]. Nucleocapsid containing lipid droplets fuse with apoB containing VLDL particles to form lipoviroparticles. These acquire apoC and apoE [85, 102, 103] and exit through the Golgi apparatus [104].



Points of intervention in the HCV life cycle

1. The viral particle (neutralizing antibodies, virocidal peptides)
2. Entry and receptor interaction (antibodies and small molecules targeting receptors, kinase inhibitors)
3. Translation and polyprotein processing (NS3-NS4A protease inhibitors)
4. HCV RNA replication (NS5B polymerase and NS5A inhibitors, miR-122 antagonists, cyclophilin inhibitors, statins, PI4KIIIα inhibitors)
5. Assembly and virion morphogenesis (NS5A inhibitors, DGAT1 inhibitors, glycosidase inhibitors, MTP inhibitors)

Figure 2-4: The HCV lifecycle and points of intervention
Points of intervention in the HCV life cycle are marked with numbered circles, and types of inhibitors of the individual steps are indicated in the legend. Interaction of extracellular HCV lipoviroparticles (LVPs) (1) with cellular surface receptors initiates the entry process (2), which can also occur from direct cell-to-cell transmission. After pH-dependent fusion and uncoating, the incoming HCV genome is translated and the resulting polyprotein processed (bottom inset, (3)). Replication takes place in ER-derived membrane spherules (membranous web, bottom right inset, (4)), the architecture of which remains to be fully defined. The spatiotemporal contribution of miR-122 binding to the HCV genome is not yet fully understood, and miR-122 presence is indicated with '?'. In the assembly and release process (top right inset, (5)), core protein is transferred from cytoplasmic lipid droplets (cLDs) to form nucleocapsids that, assisted by NS5A, are loaded with RNA. Replicase proteins supposedly bind HCV RNA during transfer from replication to packaging, the intracellular sites of which might converge. It is not clear whether the RNA is transiently located on the cLD. The p7, NS2 and NS3-NS4A proteins are also involved in coordination of assembly. HCV virion morphogenesis is coupled to the VLDL pathway, and particles are produced as LVPs.

EphA2, ephrin receptor type A2; GAG, glycosaminoglycans; PL, phospholipids; TG, triglycerides. This figure is reproduced with permission from *Scheel et al* [105], through the terms and conditions provided by Springer Nature and Copyright Clearance Centre (licence number 5726660429736).

2.3 Genomic Diversity

HCV is a genetically diverse RNA virus, with eight genotypes, and over ninety subtypes, known to infect humans. This is depicted in the phylogenetic tree in Figure 2-5. There is approximately 30% sequence difference between HCV genotypes and about 15% sequence difference between subtypes of the same genotype [6]. The high error rate of the HCV RdRp and selection pressure exerted by the host immune system has contributed to global diversity, yet there are clear geographical patterns that have emerged over time. For example, genotypes 1, 2, 3 and 4 are likely to have originated in SSA given the number of endemic subtypes found in this region. A similar pattern is seen with genotypes 3 and 6 in South Asia and East Asia, respectively. However, the true genetic diversity of HCV has not yet been fully characterised due to under-sampling mainly in LICs (Figure 2-6). The most heavily sampled HCV genomes represent genetically conserved epidemic subtypes (1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d and 6a), but regions with the highest HCV diversity are the least sampled.

There are two differing schools of thought on the origins of HCV. One is the hypotheses that HCV is about 2000 years old and may have entered humans multiple times for each HCV genotype lineage [106]. The other hypotheses is that HCV entered humans over 400,000 years ago, a time that precedes the migration of humans out of Africa [107].

The following sections describe the geographical distribution of known HCV genotypes with a focus on endemic subtypes. This work formed part of a review that we have published in the *Journal of Hepatology* [108].

Tree scale: 0.1

Figure 2-5: Maximum likelihood phylogenetic tree of all known HCV reference sub-genotypes

A RAxml constructed tree with 1000 bootstraps, using a GTR model of inference, on the ICTV HCV alignment of all known reference sub-genotypes [109].

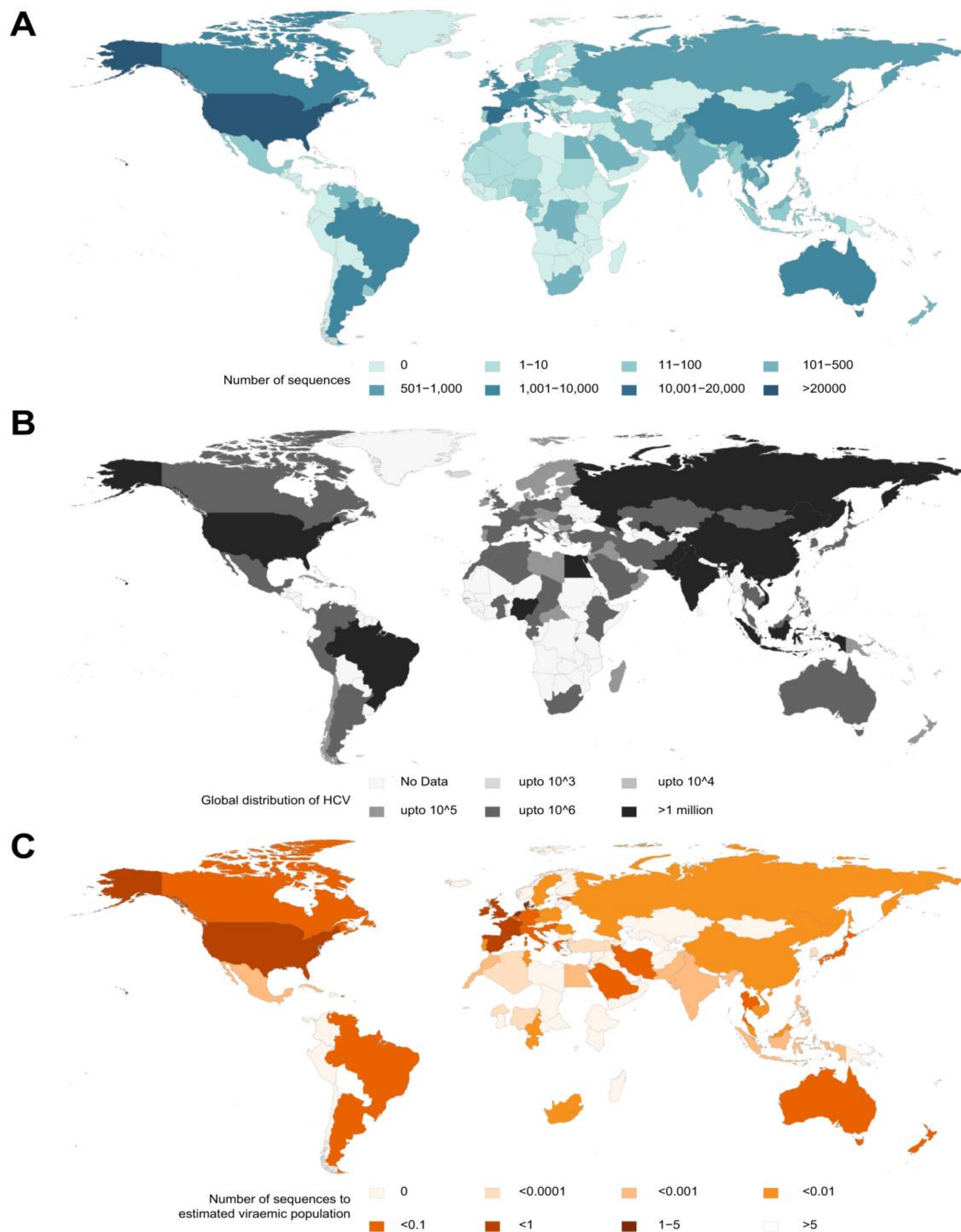


Figure 2-6: Global maps illustrating availability of sequence data, distribution of viraemic population and ratio of sequence data to infected population
(A) HCV genetic sequences greater than 500 nucleotides in length uploaded to GenBank and curated using HCV GLUE (http://hcv-glue.cvr.gla.ac.uk) by country (accessed on 4th August 2020). (B) Global distribution of estimated HCV viraemic population [110]. (C) Ratio of the number of published HCV genetic sequences to the estimated viraemic population per country. This figure is reproduced with permission from *Shah et al [108]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (https://creativecommons.org/licenses/by/4.0/).

2.3.1 Genotype 1

Sub-genotypes 1a and 1b are the most widespread genotype 1 lineages. They have a worldwide distribution due to a founder effect, through transmission in healthcare settings and injections in PWID [111], and to a lesser extent, through sexual and vertical transmission [112, 113]. Genotype 1 is likely to have originated in Central-West Africa where high levels of HCV sub-genotypic diversity are found (Figure 2-7).

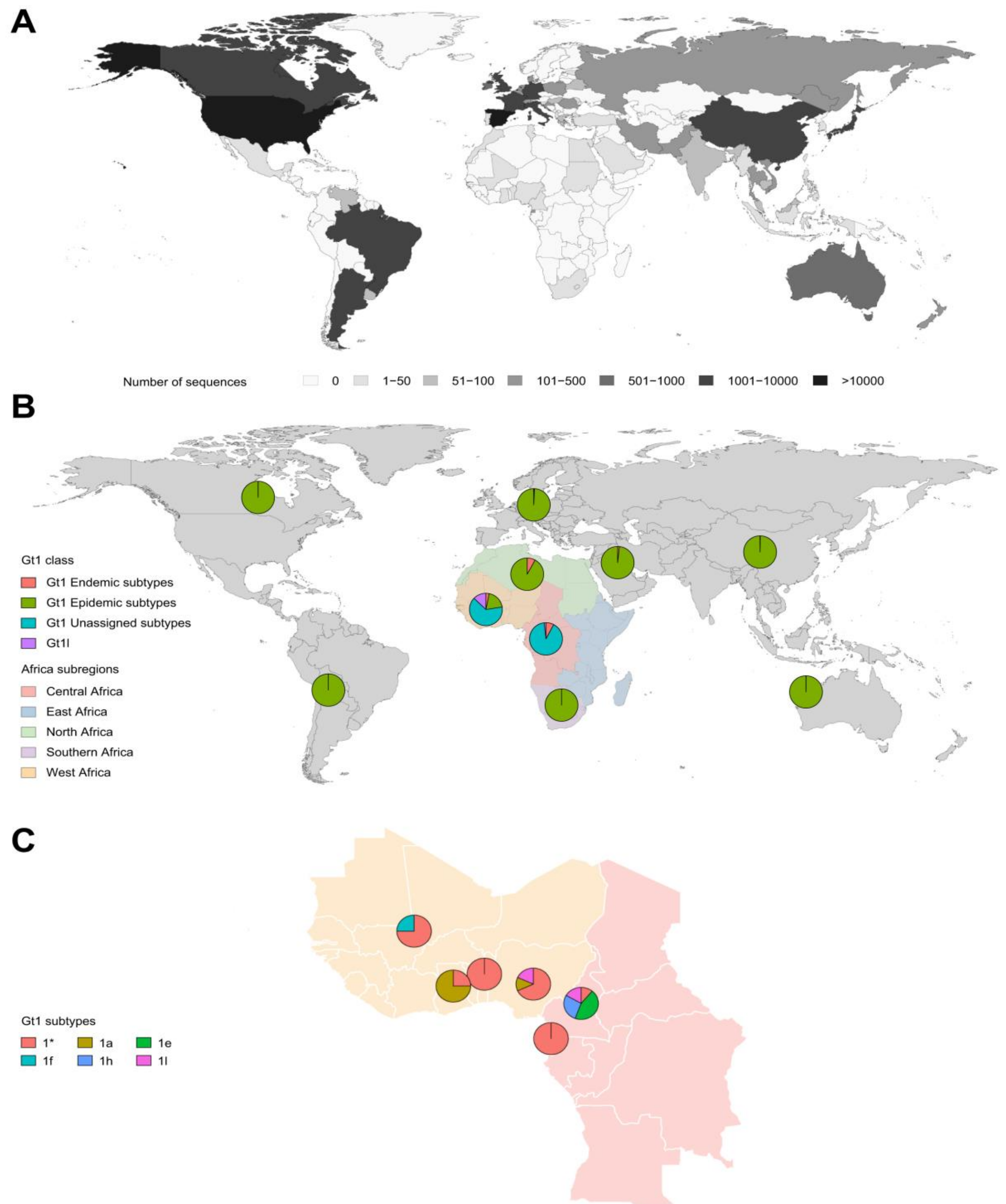


Figure 2-7: Genotype 1 and sub-genotype distribution
(A) Global distribution of genotype 1 HCV. (B) Genotype 1 subtype distribution with a focus on the subregions of Africa where genotype 1 is most diverse. Epidemic genotype 1 subtypes include 1a and 1b. Endemic genotype 1 subtypes include 1c, 1d, 1e, 1f, 1g, 1h, 1j, 1k, 1l and 1m. (C) Genotype 1 subtype diversity within West Africa (Benin, Ghana, Mali and Nigeria) and Central Africa (Cameroon and Equatorial Guinea). 1∗ refers to unassigned Genotype 1 subtypes. This figure is reproduced with permission from *Shah et al [108]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (https://creativecommons.org/licenses/by/4.0/).

2.3.2 Genotype 2

Genotype 2 is likely to have originated in West Africa, where it is endemic (Figure 2-8). It spread through this region most probably through iatrogenic blood-borne transmission, for example, through mass injected treatment campaigns in Cameroon between 1921 and 1957 for several infections including African trypanosomiasis, syphilis, yaws and leprosy. Genotype 2 is also globally distributed. It was dispersed to the Caribbean through the transatlantic slave trade, as evidenced by genotype 2 sequences sampled in Martinique closely resembling those sampled in Benin and Ghana, and the people of Martinique share ancestry with those in West Africa [114]. The Dutch run slave trade had a role in spreading genotype 2 from South America to Asia, in particular, from Suriname to Indonesia. For example, subtype 2e is found in both Suriname and Indonesia [115]. Other genotype 2 subtypes found in the Americas are also found in Asia. For example, 2a, 2b and 2c are found in Argentina, Brazil, Venezuela and, USA, as well as in China (2a), Japan (2a, 2b), Thailand and Vietnam (2a) [116].

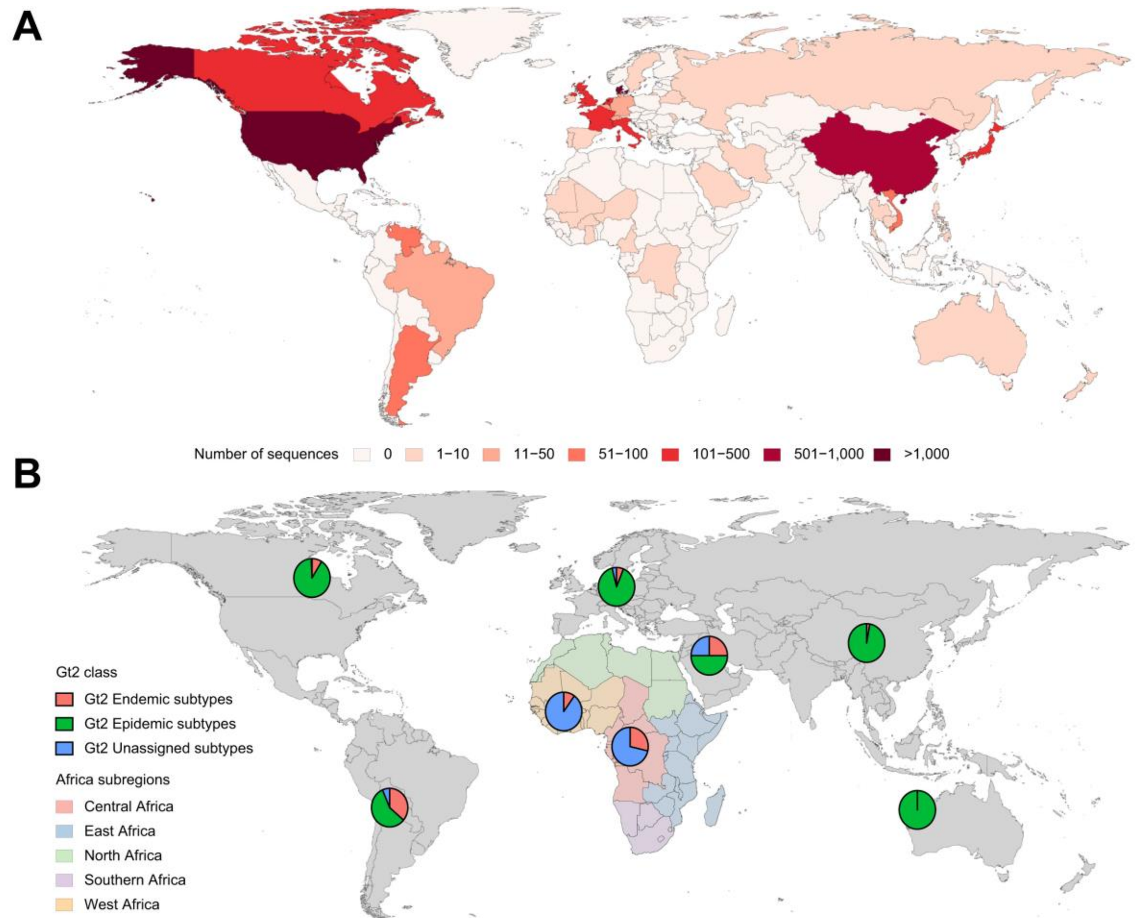


Figure 2-8: Genotype 2 and sub-genotype distribution
(A) Global distribution of Genotype 2 HCV. (B) Genotype 2 subtype distribution with a focus on the subregions of Africa where it is most diverse. Epidemic Genotype 2 subtypes include 2a, 2b and 2c. Endemic Genotype 2 subtypes include 2d, 2e, 2f, 2i, 2j, 2k, 2l, 2m, 2n, 2o, 2q, 2r, 2s, 2t and 2u. This figure is reproduced with permission from *Shah et al [108]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (https://creativecommons.org/licenses/by/4.0/).

2.3.3 Genotype 3

Genotype 3 is widely distributed around the world, but the most diversity is seen in the Indian sub-continent and Southeast Asia (Figure 2-9). Subtype 3a is the best characterised and is highly prevalent in South Asia and has successfully entered populations in HICs. Subtype 3b is widely distributed in Asia, namely India, China, Malaysia and Thailand [117]. Less well characterised subtypes 3c, 3d and 3e have been found in Nepal, 3f, 3g and 3i in India, and 3k in Indonesia [118]. While genotype 3 is not commonly found in Central and South America and SSA, they have been found in Somalia and in individuals originating from this region [119].

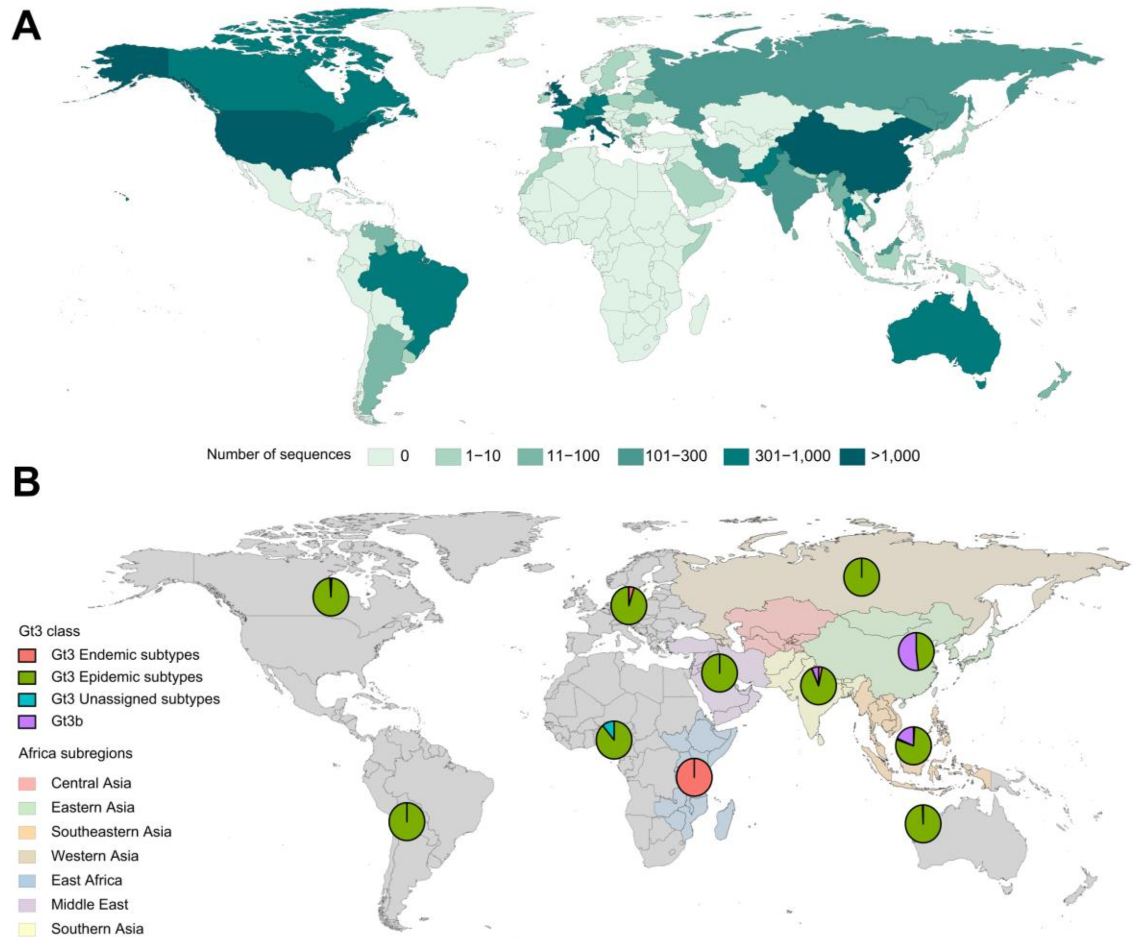


Figure 2-9: Genotype 3 and sub-genotype distribution
(A) Global distribution of Genotype 3 HCV. (B) Genotype 3 subtype distribution with a focus on the subregions of Asia and East Africa where it is most diverse. The epidemic Genotype 3 subtype is 3a. Endemic Genotype 3 subtypes include 3d, 3e, 3f, 3g, 3h, 3i and 3k. This figure is reproduced with permission from *Shah et al [108]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (https://creativecommons.org/licenses/by/4.0/).

2.3.4 Genotype 4

Genotype 4 is believed to have its origins in Central and East Africa, where diverse endemic strains are predominant (Figure 2-10). Over time, several lineages of this genotype have spread into North Africa and beyond. Among these, genotypes 4a and 4d have shown the widest dispersion. Genotype 4a, in particular, is the most widespread strain globally. Its transmission was significantly amplified in Egypt, due to unsafe injection practices within healthcare settings [120].

Clinical trials have primarily concentrated on genotype 4a, with some attention given to genotype 4d. The latter strain was introduced to Saudi Arabia during the

early 20th century [121], likely originating from countries in the Horn of Africa, such as Ethiopia [122]. Recently, it has emerged among PWID in Southern Europe and found a niche as a sexually transmitted infection among men who have sex with men (MSM) in Northern Europe. Furthermore, crossover from PWID to MSM is likely to have occurred in urban centres like Amsterdam, London, Berlin, and Paris [123]. While the globally dispersed subtypes 4a and 4d have been well-characterised, other strains prevalent in SSA have only recently come under scrutiny.

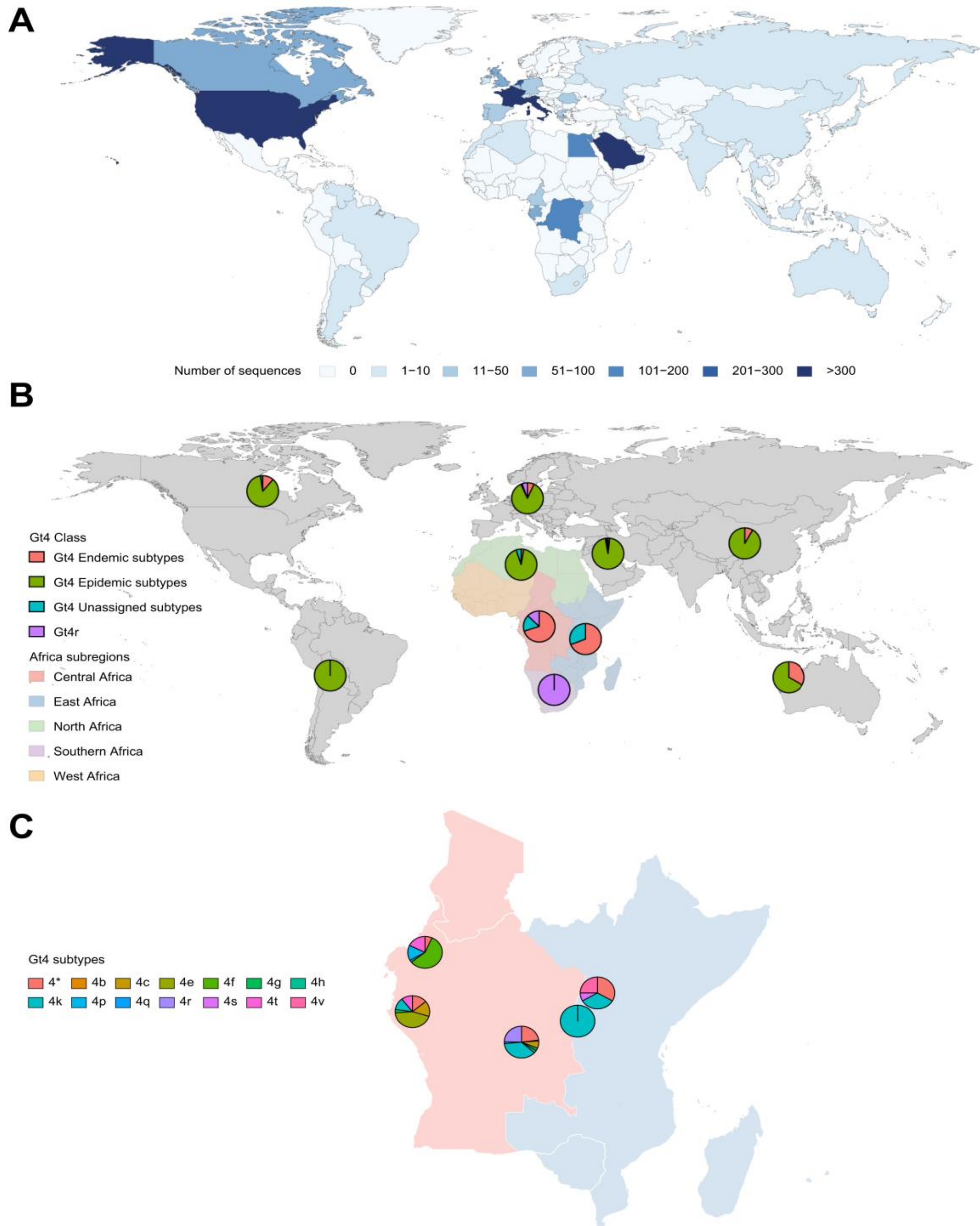


Figure 2-10: Genotype 4 and sub-genotype distribution
(A) Global distribution of Genotype 4 HCV. (B) Genotype 4 subtype distribution with a focus on the regions of Africa where it is most diverse. Epidemic Genotype 4 subtypes include 4a and 4d. Endemic Genotype 4 subtypes include 4b, 4c, 4e, 4f, 4g, 4h, 4i, 4k, 4l, 4m, 4n, 4o, 4p, 4q, 4s, 4t, 4v and 4w. (C) Genotype 4 subtype diversity within Central (Cameroon, Democratic Republic of Congo and Gabon) and East Africa (Rwanda and Uganda). 4∗ refers to unassigned Genotype 4 subtypes. This figure is reproduced with permission from *Shah et al [108]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (https://creativecommons.org/licenses/by/4.0/).

2.3.5 Genotype 5

This is the most common genotype in South Africa [124], and in rare cases has been exported to Asia, Europe and North America (Figure 2-11). There is only one official subtype, 5a, however, a divergent lineage in Burkina Faso has been identified [125], suggesting that genotype 5 may be more widespread in SSA than previously thought.

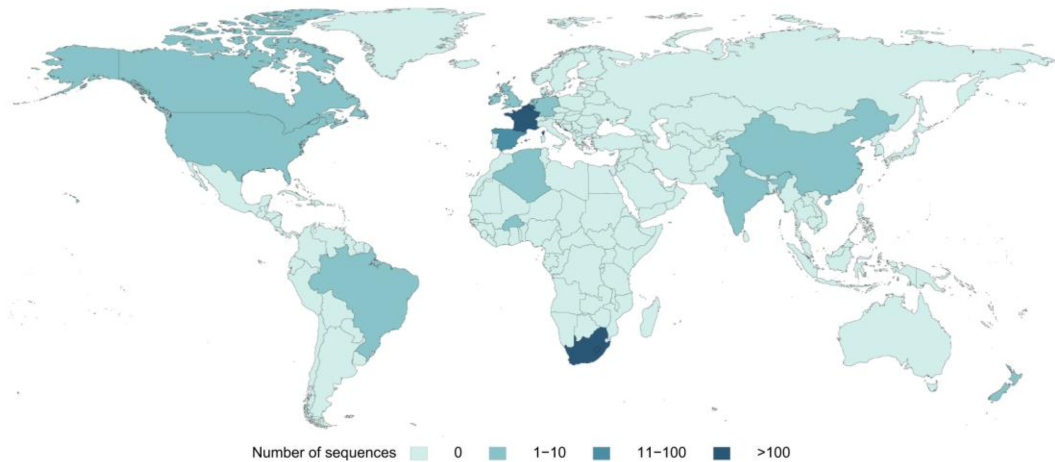


Figure 2-11: Genotype 5 distribution
Global distribution of Genotype 5 HCV. The shading in Asia represents a full length and one partial HCV sequence from China[51] as well as one full length HCV sequence from India (unpublished), reflecting how rare Genotype 5 HCV is in Asia. This figure is reproduced with permission from *Shah et al [108]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (https://creativecommons.org/licenses/by/4.0/).

2.3.6 Genotype 6

Genotype 6 is the most prevalent genotype in Southeast Asia and is the most diverse HCV genotype (Figure 2-12). Epidemic subtype 6a has a global distribution, however, other subtypes have distinct geographical distributions within the region. For example 6u is found in Laos [126] and Vietnam [127], whereas 6v is found in China and Thailand [128, 129].

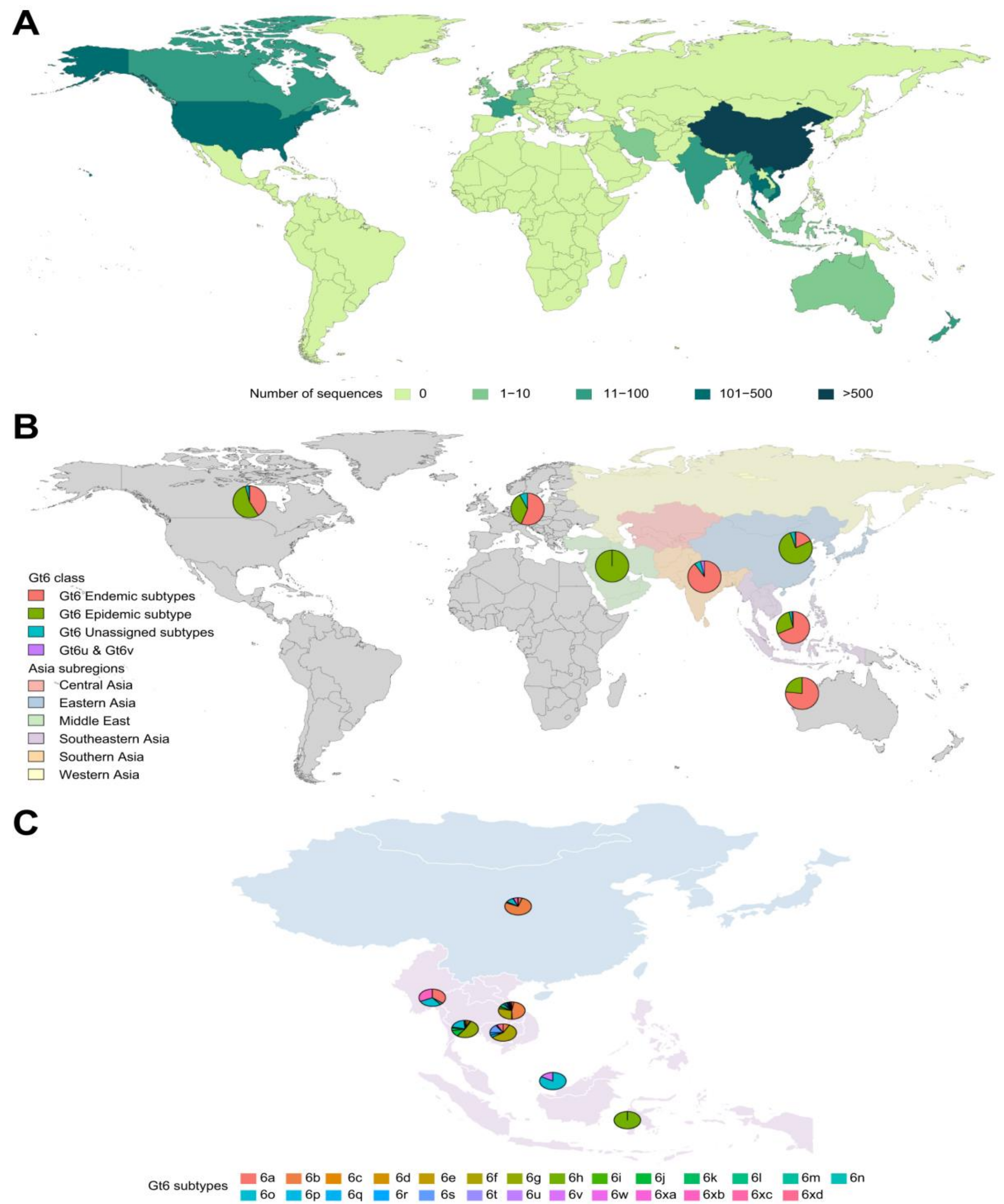


Figure 2-12: Genotype 6 and sub-genotype distribution

(A) Global distribution of Genotype 6 HCV. (B) Genotype 6 subtype distribution with a focus on the regions of Asia where they are most diverse. The epidemic Genotype 6 subtype is 6a. Endemic Genotype 6 subtypes include 6b, 6c, 6d, 6e, 6f, 6g, 6h, 6i, 6j, 6k, 6l, 6m, 6n, 6o, 6p, 6q, 6r, 6s, 6t, 6w, 6xa, 6xb, 6xc, 6xd, 6xe. (C) Genotype 6 subtype diversity within Eastern (China) and South-eastern Asia (Cambodia, Thailand, Vietnam, Myanmar, Malaysia and Indonesia). 6∗ refers to unassigned Genotype 6 subtypes. This figure is reproduced with permission from *Shah et al [108]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (https://creativecommons.org/licenses/by/4.0/).

2.3.7 Genotypes 7 and 8

Genotypes 7 and 8 have recently been discovered in individuals from DRC, Uganda [130] and India [131].

2.4 Epidemiology

Globally 80% of all HCV infections occur in 31 countries, among which China, Pakistan, Nigeria, Egypt, India and Russia account for more than 50% of all infections (Figure 2-13). As a blood-borne virus HCV spread rapidly throughout the world during the twentieth century fuelled by parenteral transmission routes such as medical treatments, including blood transfusions and immunisations. Injecting drug use has become a more prominent transmission route in recent times, particularly in HICs. Iatrogenic transmission through poor infection control and inadequate screening of donated blood products remains a significant risk in some LICs [132]. PWID represent a continuing reservoir of the global HCV epidemic [133]. Sexual transmission of HCV, not previously thought of as a high-risk transmission risk, has emerged as a significant transmission risk factor, particularly among HIV positive MSM [112]. Outside of HIV co-infection, sexual transmission of HCV is seldom observed in serodiscordant couples. In one study of monogamous heterosexual couples, HCV prevalence was estimated at 0.6% [134]. Vertical transmission of HCV occurs in 6% of mothers, but this rises to 11% in those with HIV co-infection [113]. Mode of delivery and feeding type do not influence HCV transmission from infected mothers. Renal haemodialysis units with inadequate infection control practices and prisons (several routes of transmission) are also important risk factors for HCV transmission [135]. While the burden of HCV related liver disease in SSA is significant, detailed data on epidemiology, prevalence and estimates on the proportion of the population diagnosed, treated, and cured, are lacking [136]. Seroprevalence and viraemic prevalence estimates have been calculated in modelling studies and these are summarised by SSA region and country in Table 2-1.

Table 2-1: Seroprevalence and viraemic prevalence by region and country in SSA
Data estimated HCV seroprevalence and 95% prediction interval (PI) as predicted by a model for median age of the adult population in each country [137]. Viraemic prevalence and 95% uncertainty interval (UI) [110].This table is adapted with permission from *Sonderup et al [136]*, through the terms and conditions provided by the Lancet Gastroenterology & Hepatology and Copyright Clearance Centre (licence number 5733360996837).

	Estimated seroprevalence (95% PI)	Viraemic prevalence (95% UI)
Western Africa		
Burkina Faso	6·1% (1·3-14·2)	1·3% (1·0-1·4)
Benin	3·8% (0·7-9·2)	··
The Gambia	2·4% (0·0-9·7)	0·8% (0·5-1·3)
Ghana	3·2% (0·5-8·1)	1·4% (1·1-3·4)
Guinea	1·5% (0·5-9·5)	··
Côte d'Ivoire	2·2% (0·3-6·1)	··
Mali	1·9% (0·3-10·6)	··
Nigeria	3·1% (0·1-10·0)	1·4% (1·0-1·4)
Senegal	1·0% (0·0-4·6)	··
Central Africa		
Angola	3·9% (0·6-10·1)	··
Burundi	3·1% (0·2-9·1)	1·0% (0·8-4·0)
Cameroon	4·9% (0·9-11·9)	0·7% (0·5-0·8)
Democratic Republic of the Congo	2·1% (0·4-12·0)	··
Gabon	4·9% (1·0-11·5)	7·0% (5·1-7·3)
Congo (Brazzaville)	2·9% (0·0-11·7)	··
Rwanda	3·1% (0·3-12·0)	··
Eastern Africa		
Ethiopia	2·7% (0·1-9·2)	0·6% (0·4-0·7)
Kenya	2·8% (0·4-7·3)	0·2% (0·1-0·3)
Madagascar	1·7% (0·0-7·7)	0·2% (0·2-0·3)
Malawi	2·0% (0·0-7·0)	··
Mozambique	1·3% (0·1-6·9)	··
Somalia	2·6% (0·1-8·5)	··
Tanzania	2·7% (0·2-7·8)	··
Uganda	2·7% (0·4-7·0)	··
Southern Africa		
Namibia	1·6% (0·0-7·3)	··
South Africa	1·1% (0·0-5·8)	0·7% (0·4-0·9)
Zambia	1·1% (0·0-3·7)	··
Zimbabwe	1·6% (0·0-5·9)	··

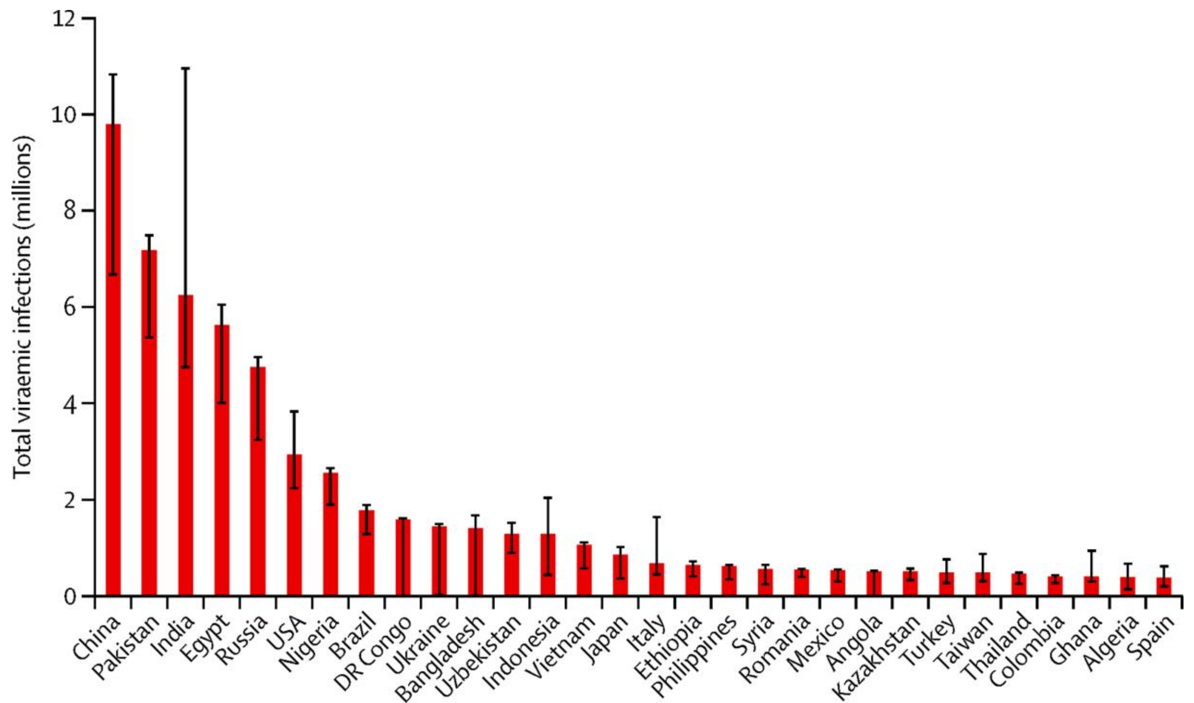


Figure 2-13: Countries accounting for 80% of total viraemic HCV infections
This figure is reproduced with permission from *Blach et al [110]*, through the terms and conditions provided by the Lancet Gastroenterology & Hepatology and Copyright Clearance Centre (licence number 5733321291661).

2.5 Natural History and Clinical Consequences

75-80% of HCV infected individuals go on to develop chronic infection, of whom 10-20% develop complications, including decompensated cirrhosis and HCC (Figure 2-14). These complications can take 20-30 years to develop following established chronic infection but certain risk factors can speed up this progression. These include higher age at infection, male gender, obesity, excessive alcohol consumption, HIV co-infection and immunosuppression [138]. The risk of developing HCC is 1% in people with no liver fibrosis to 13% in those with established cirrhosis, over a 5-year period [139]. Hepatitis B virus (HBV) co-infection, diabetes mellitus, metabolic dysfunction-associated steatotic liver disease (MASLD), infection with HCV genotype 3, excessive alcohol consumption, advanced age, lower platelet counts and male gender all increase the risk of developing HCC [140].

Acute HCV infection is usually asymptomatic. Less than 25% of cases have clinical symptoms and less than 1% develop fulminant hepatitis. If clinical symptoms do occur, they become apparent 2-26 weeks after infection and can

last for 2-12 weeks. HBV co-infection and HIV co-infection increase the risk of fulminant hepatitis [141].

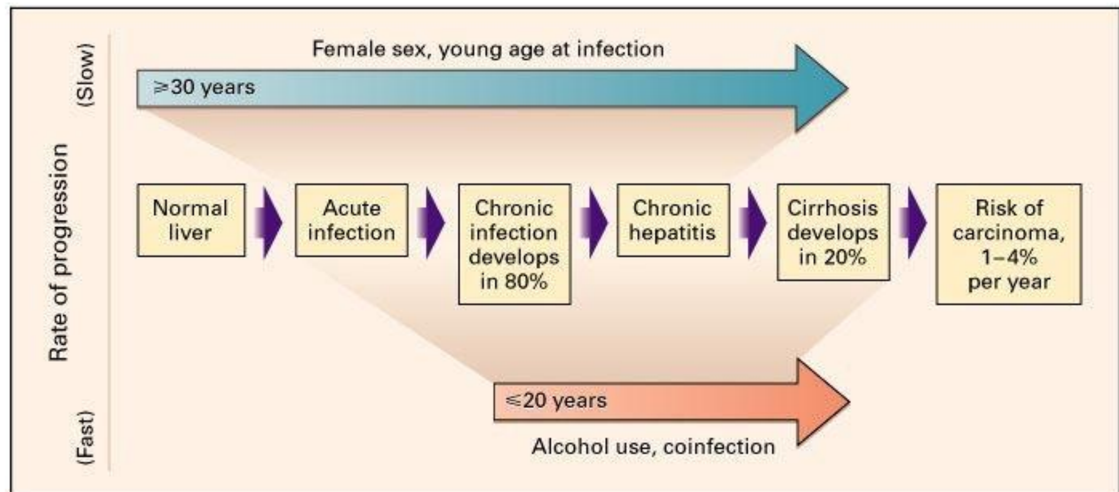


Figure 2-14: The natural history of HCV infection and its variability from person to person
The course of infection varies widely among persons. Factors that decrease the risk of progression include female sex and a younger age at infection Factors that increase the risk include alcohol intake, an older age at infection, male sex, and coinfection with other viruses. Persons with a favourable risk profile often do not have progressive liver disease until 30 or more years after infection. In contrast, 20 percent of persons with chronic hepatitis C will eventually have cirrhosis, and this can occur 20 years or less after infection, especially in those with alcohol abuse or coinfection with human immunodeficiency virus type 1 or hepatitis B virus. Once cirrhosis is established, the risk of hepatocellular carcinoma is 1 to 4 percent per year. This figure is reproduced with permission from *Lauer et al [142]*, through the terms and conditions provided by the New England journal of Medicine and Copyright Clearance Centre.

Immune or inflammatory related extrahepatic manifestations can occur in up to 75% of individuals with chronic HCV infection. These include mixed cryoglobulinaemia vasculitis, atherosclerotic cardiovascular disease, renal disease (type 1 membranoproliferative glomerulonephritis, focal segmental glomerulosclerosis, interstitial nephritis), type 2 diabetes mellitus, skin disease (porphyria cutanea tarda, lichen planus, necrolytic acral erythema), thyroid disease (Hashimoto's thyroiditis and Graves' disease), eye disease (Mooren's ulcers and Sjogren's syndrome), and lymphoproliferative disease (non-Hodkin lymphoma and T-cell lymphoma). These are summarised in Figure 2-15. Individuals with chronic HCV infection have a lower quality of life than that of the general population.

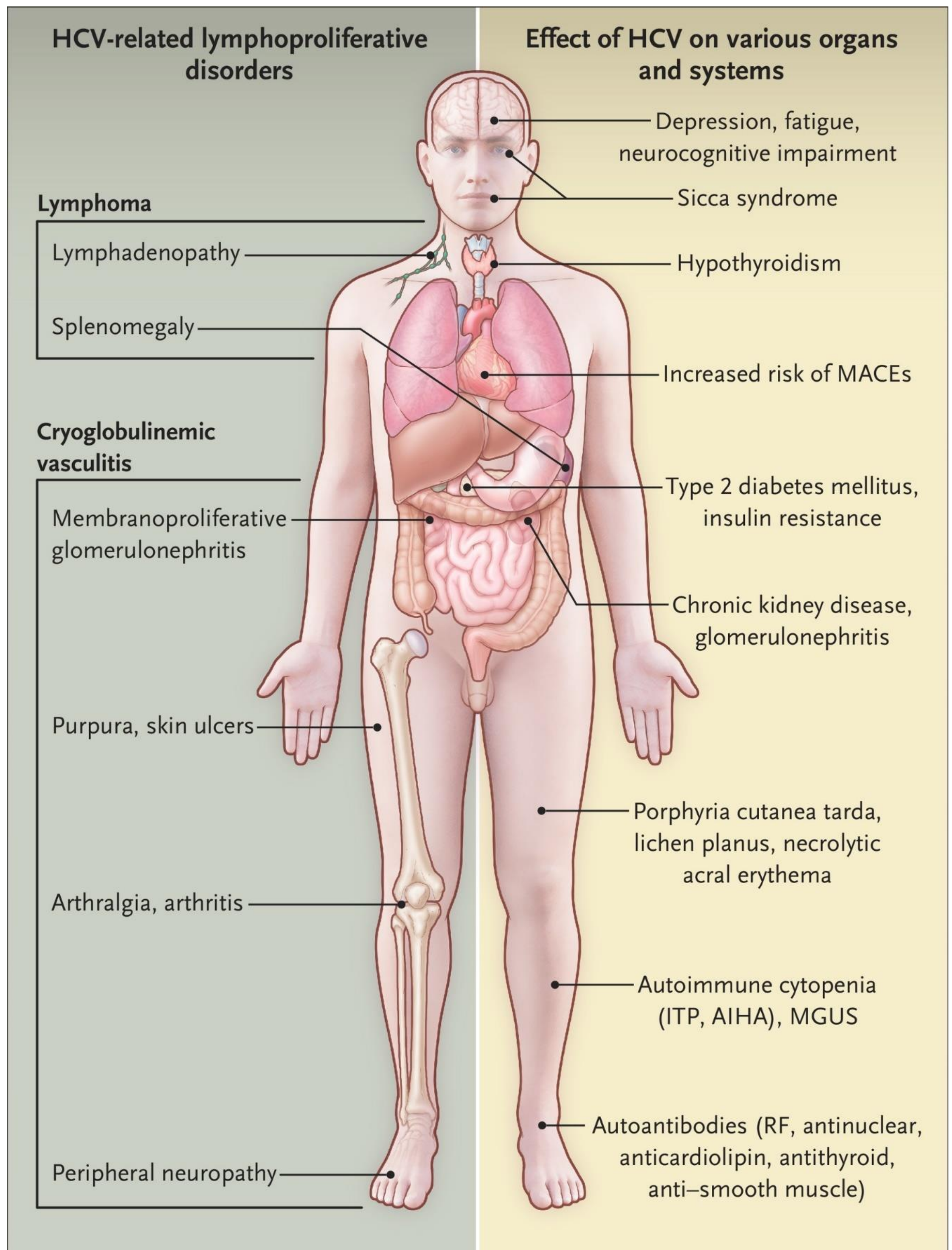


Figure 2-15: Extrahepatic manifestations of chronic HCV infection
The left side shows HCV-related lymphoproliferative disorders, which can be benign (cryoglobulinemic vasculitis) or malignant (lymphoma). The right side shows the effect of HCV infection on various organs and systems. AIHA denotes autoimmune hemolytic anaemia, ITP immune thrombocytopenia, MACE major adverse cardiovascular event, MGUS monoclonal gammopathy of undetermined significance, and RF rheumatoid factor. This figure is reproduced with permission from *Cacoub et al [143]*, Copyright Massachusetts Medical Society.

2.6 Diagnosis

The standard HCV testing algorithm is two-step process to confirm exposure to the virus by serological methods, followed by qualitative or quantitative nucleic acid testing, to indicate active infection. HCV antibodies are detectable 6-12 weeks following exposure [144], although in individuals who are immunocompromised, antibody detection can be delayed or completely absent [145]. HCV RNA is detectable two weeks after exposure. Conventional HCV RNA testing requires blood sample collection by venepuncture and then transport to and processing in a centralised laboratory. The involvement of multiple steps of testing, and therefore multiple visits to a healthcare facility, before diagnosis and treatment presents a barrier to large scale, and accessible, diagnostics. This algorithm also allows for multiple points at which individuals can be lost to follow-up, summarised in Figure 2-16. Furthermore, the costs of HCV diagnostics present another major barrier to large scale testing.

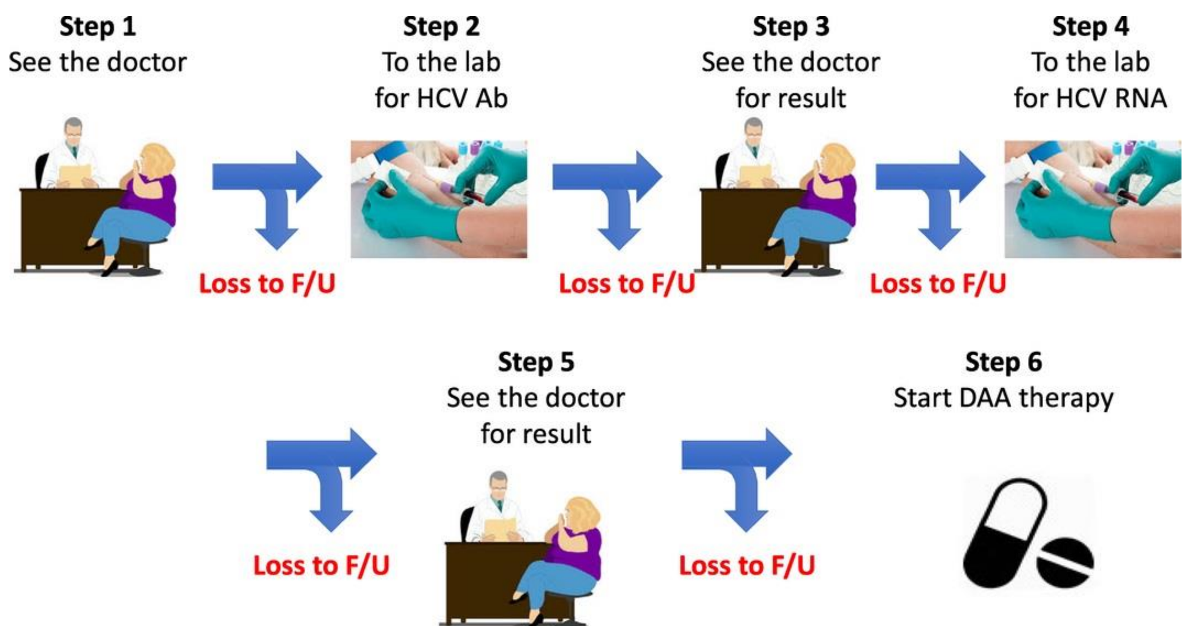


Figure 2-16: Steps in current HCV diagnostic pathway
Each of the steps in the algorithm for HCV diagnosis provides potential for loss to follow-up. This figure is reproduced with permission from *Feld et al [146]*, through the terms and conditions provided by Clinical Liver Disease and Copyright Clearance Centre (licence number 5733661324661).

Various methods have been developed to simplify the diagnostic algorithm. For example, the use of assays that use samples that are easier to obtain and involve minimal processing as well as the ability to perform the test on site. Point-of-care (POC) tests for HCV antibody detection have been developed. Some of these only require a finger prick blood sample and provide rapid results [147, 148]. Dried blood spot sampling has provided an easier way to collect samples appropriate for HCV RNA detection and quantification [149]. In more recent times, POC tests for HCV RNA detection have been developed, using either finger prick blood or whole blood samples [150, 151]. Where nucleic acid testing is not available and/or too costly, immunoassays to detect HCV core antigen may be used as an alternative, as recommended by WHO. These assays have shown high sensitivity and specificity with HCV viral loads of 500-3000 IU/ml, depending on genotype [152-155]. HCV core antigen correlates well with HCV RNA.

POC HCV antibody and HCV RNA assays have been shown to have useful sensitivity and specificity in a variety of populations and settings, including community health centres, opiate substitution treatment centres, prisons and homelessness settings [156].

While several commercial genotyping assays are available, genotyping is not routinely recommended in simplified diagnostic algorithms, as this adds an extra barrier to large scale testing and treatment. However, in certain situations HCV genotyping may be required, as specified by regional guidelines [157, 158].

An assessment of liver fibrosis is required prior to therapy because the presence of liver cirrhosis may require an adjustment to the treatment regimen and will also require post treatment surveillance for HCC. Non-invasive methods for liver fibrosis, using either liver stiffness measurements or serum biomarkers, are recommended. Both methods show good performance in identifying cirrhosis and no fibrosis, but are less accurate in determining intermediate degrees of fibrosis. Comprehensive guidance is provided by the AASLD, EASL and WHO [157-159].

2.7 Treatment

The primary goal of HCV infection treatment is to achieve cure, which is defined as a sustained virological response (undetectable HCV RNA in whole blood or plasma) at 12- or 24-weeks after completion of therapy. Most individuals who achieve SVR12 have no evidence of HCV infection for years following therapy, hence its use as a surrogate marker for cure [160]. Clearance of HCV is possible as the virus does not integrate into the host genome, unlike HBV and HIV. An SVR is associated with reductions in all-cause mortality, liver-related mortality, liver-related and non-liver related morbidity (cirrhosis, hepatocellular carcinoma, decompensated liver disease, liver transplantation, and extrahepatic manifestations), improvements in liver fibrosis and quality of life [161-169] and also prevents transmission.

The first therapeutic drug approved for HCV treatment was interferon (IFN)-α, in 1991. This was administered as a subcutaneous injection three times a week for up to 48 weeks. Cure rates with IFN-α were less than 16% [170, 171]. In 2001, recombinant IFN-α was conjugated with polyethylene glycol (PEG) creating a longer acting version of the drug that could be administered once a week. The first oral agent to be approved for treatment of HCV infection was the oral guanosine nucleoside analogue ribavirin (RBV), in 1998. Treatment with IFN-α and RBV increased cure rates up to 28% in individuals infected with genotype 1, and 66% in individuals infected with genotype 2 or 3 [172, 173]. Combining RBV with PEG IFN-α improved cure rates in individuals infected with genotype 1 to 41% [174, 175]. Regrettably, treatment with IFN was hindered by its toxicity, which encompassed flu-like symptoms such as muscle and joint pain, fever, depression, neutropenia, and a range of other side effects affecting virtually all body systems. RBV, when used in combination therapy, induced haemolytic anaemia and posed genotoxic and teratogenic risks. Consequently, the effectiveness of combination therapy was sub-optimal due to its adverse effects and contraindications [176, 177].

The development of oral DAAs eventually led to IFN-α and RBV free, all oral treatment regimens, which revolutionised the treatment landscape for HCV infection. The first two DAAs to be approved were protease inhibitors boceprevir and telaprevir, in 2011. These were administered in combination with PEG IFN-α

and RBV. However, they were restricted to use in individuals infected with genotype 1, and also carried the added burden of adverse effects such as severe anaemia and, for telaprevir, serious rash and gastrointestinal symptoms. Nonetheless, they improved cure rates to 75% [178-183]. In 2013, the first NS5B inhibitor DAA was approved, sofosbuvir. This drug had potent antiviral activity against all HCV genotypes. In the subsequent four years, more protease inhibitors were developed and a new class of DAAs, NS5A inhibitors, emerged, leading to all oral, IFN-α and RBV free treatment regimens. The treatment lengths were significantly shorter than IFN-α/RBV containing regimens, much better tolerated and highly effective. Furthermore, the development of pan-genotypic regimens (sofosbuvir/velpatasvir and glecaprevir/pibrentasvir), with broad and potent antiviral activity (cure rates of over 95% in clinical trials), has markedly reduced treatment complexity. This has allowed the successful provision of HCV treatment by a range of different providers in diverse clinical settings [184-191]. A summary of genotype specific and pan-genotypic DAA phase III clinical trials is provided in Table 2-2.

2.7.1 Protease Inhibitors

These compounds are directed against the NS3/NS4A protease complex. Solving the crystal structure of the NS3 protease domain, complexed with a synthetic NS4A cofactor peptide, allowed the design of specific inhibitors of the enzyme [192-195]. These compounds are peptidomimetic inhibitors that compete for natural NS3 serine-protease substrates thereby preventing HCV polyprotein cleavage. First generation protease inhibitors include asunaprevir, paritaprevir and simeprevir. They are highly potent but have a low genetic barrier (the number of mutations required to overcome drug-selective pressure) to resistance. Second generation protease inhibitors such as grazoprevir, glecaprevir and voxilaprevir have a higher genetic barrier to resistance, are even more potent and have a broader range of activity against HCV genotypes.

2.7.2 NS5A Inhibitors

All NS5A inhibitors are thought to bind to domain I of NS5A, reducing hyperphosphorylation and blocking its dimerization [196]. By targeting the multifunctional NS5A protein, inhibitors inhibit several stages of the virus life

cycle [197]. Specific NS5A inhibitors have been discovered in cell-based replicon systems through screening of compound libraries. Daclatasvir was the first NS5A inhibitor to be discovered [198]. Along with ledipasvir and ombitasvir, they are first generation NS5A inhibitors that are highly potent, but have limited activity against genotypes 2 and 3. Elbasvir, velpatasvir and pibrentasvir are second generation NS5A inhibitors that have pan-genotypic activity. NS5A inhibitors have a low genetic barrier to resistance.

2.7.3 NS5B Inhibitors

The NS5B protein is an RdRp. The solving of its crystal structure revealed distinct subdomains, referred to as thumb, fingers and palm (Figure 2-3), and provided essential data for development of compounds that target it [77-79, 199]. NS5B inhibitors are classified into non-nucleotide and nucleotide inhibitors and they act at specific stages of RNA synthesis [200]. Dasabuvir is an approved non-nucleotide inhibitor that targets the palm site of NS5B, but only has activity against genotype 1 and has a low genetic barrier to resistance. Thus, it has limited clinical use. Sofosbuvir is an approved nucleotide inhibitor. It mimics natural NS5B substrates and causes direct chain termination by targeting the NS5B active site. Sofosbuvir is a phosphoramidate prodrug that yields a monophosphate following intracellular hydrolysis, followed by further modification to its diphosphate and then active triphosphate form, which binds to the NS5B active site. Sofosbuvir has a high genetic barrier to resistance and is highly potent and has therefore formed the backbone of many DAA treatment regimens.

Table 2-2: Efficacy of genotype specific and pan-genotypic DAA regimens in phase III clinical trials

DCV – Daclatasvir, DSV – Dasabuvir, EBR – Elbasvir, GLE – Glecaprevir, GZR – Grazoprevir, LDV – Ledipasvir, OBV – Ombitasvir, PIB – Pibrentasvir, PTV/r – Paritaprevir/Ritonavir, SOF – Sofosbuvir, VEL – Velpatasvir, VOX – Voxilaprevir, mITT - Modified intention-to-treat, SVR - Sustained virological response. Adapted with permission from *Brzdek et al [201]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (https://creativecommons.org/licenses/by-nc/4.0/).

Study	Number of participants	Regimen	Genotype	SVR (mITT analysis) *n/N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
ION-1 [202]	865	LDV/SOF, 12 wk (*n* = 214)	1	179/179 (100)		32/33 (97.0)	
		LDV/SOF + RBV, 12 wk (*n* = 217)		178/178 (100)		33/33 (100)	
		LDV/SOF, 24 wk, (*n* = 217)		181/182 (99.5)		31/32 (96.9)	

Study	**Number of participants**	**Regimen**	**Genotype**	**SVR (mITT analysis) *n/N* (%)**			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
		LDV/SOF + RBV, 24 wk (*n* = 217)		179/179 (100)		36/36 (100)	
ION-2 [203]	440	LDV/SOF, 12 wk (*n* = 109)	1		83/87 (95.4)		19/22 (86.4)
		LDV/SOF + RBV, 12 wk (*n* = 111)			89/89 (100)		18/22 (81.8)
		LDV/SOF, 24 wk, (*n* = 109)			86/87 (98.9)		22/22 (100)

Study	**Number of participants**	**Regimen**	**Genotype**	**SVR (mITT analysis) *n/N* (%)**			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
		LDV/SOF + RBV, 24 wk (*n* = 111)			88/89 (98.9)		22/22 (100)
ION-3 [204]	647	LDV/SOF, 8 wk (*n* = 215)	1	202/214 (94.4)			
		LDV/SOF + RBV, 8 wk (*n* = 216)		201/211 (95.3)			
		LDV/SOF, 12 wk (*n* = 216)		206/209 (98.6)			

Study	**Number of participants**	**Regimen**	**Genotype**	**SVR (mITT analysis) *n/N* (%)**			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
SAPPHIRE-I [205]	473	OBV/PTV/r + DSV + RBV, 12 wk	1	455/473 (96.2)			
SAPPHIRE-II [206]	297	OBV/PTV/r + DSV + RBV, 12 wk	1		286/297 (96.3)		
PEARL-III [207]	419	OBV/PTV/r + DSV + RBV, 12 wk (*n* = 210)	1b	209/210 (99.5)			
		OBV/PTV/r + DSV + placebo, 12 wk (*n* = 209)		207/207 (100)			

Study	Number of participants	Regimen	Genotype	SVR (mITT analysis) *n/N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
PEARL-IV [207]	305	OBV/PTV/r + DSV + RBV, 12 wk (*n* = 100)	1a	97/99 (98.0)			
		OBV/PTV/r + DSV + placebo, 12 wk (*n* = 205)		185/204 (90.7)			
GARNET [208]	163	OBV/PTV/r + DSV, 8 wk	1b	160/162 (99.0)			
AGATE-I [209]	120	OBV/PTV/r + RBV, 12 wk (*n* = 59)	4			57/58 (98.0)	

Study	Number of participants	Regimen	Genotype	SVR (mITT analysis) *n/N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
		OBV/PTV/r + RBV, 16 wk (*n* = 61)				60/60 (100)	
AGATE-I Part II [210]	64	OBV/PTV/r + RBV, 24 wk	4			60/60 (100)	
AGATE-II [211]	160	OBV/PTV/r + RBV, 12 wk (*n* = 131)	4	94/98 (96.0)	30/31 (97.0)		
		OBV/PTV/r + RBV, 24 wk (*n* = 29)				27/28 (96.0)	

Study	**Number of participants**	**Regimen**	**Genotype**	**SVR (mITT analysis) *n/N* (%)**			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
C-EDGE treatment naïve [212]	421	GZR/EBR ± RBV, 12 wk	1, 4, 6	68/70 (97.0)		231/246 (94.0)	
C-EDGE treatment-experienced [213]	420	GZR/EBR ± RBV, 12 or 16 wk	1, 4, 6		255/264 (96.6)		135/144 (93.8)
FISSION [214]	256	SOF + RBV, 12 wk	1-3	147/204 (72.1)		23/49 (46.9)	

Study	**Number of participants**	**Regimen**	**Genotype**	**SVR (mITT analysis) *n/N* (%)**			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
FUSION [215]	201	SOF + RBV, 12 wk (n = 103)	2, 3		25/26 (96.2), 14/38 (36.8)		6/10 (60.0), 5/26 (19.2)
		SOF + RBV, 16 wk (n = 98)	2, 3		23/23 (100), 25/40 (62.5)		7/9 (77.8), 14/23 (60.9)
POSITRON [215]	207	SOF + RBV, 12 wk	2		85/92 (92.0%)		16/17 (94.0%)
			3		57/84 (68.0%)		3/14 (21.0%)
VALENCE [216]	323	SOF + RBV, 12 wk (n = 73)	2	29/30 (96.7)	30/32 (93.8)	2/2 (100)	7/9 (77.8)

Study	Number of participants	Regimen	Genotype	SVR (mITT analysis) *n/N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
		SOF + RBV, 24 wk (n = 250)	3	87/92 (94.6)	85/98 (86.7)	12/13 (92.3)	29/47 (61.7)
Omata et al [217]	153	SOF + RBV, 12 wk	2	80/82 (95.0)	52/54 (100)	8/8 (100)	8/9 (89.0)
BOSON [218]	363	SOF + RBV, 16 wk	3	58/70 (83.0)	41/54 (76.0)	12/21 (57.0)	17/36 (47.0)
		SOF + RBV, 24 wk		65/72 (90.0)	44/54 (81.0)	18/22 (82.0)	26/34 (76.0)
Satsangi et al [219]	105	SOF + RBV, 24 wk	3	49/49 (100)	1/1 (100)	22/23 (95.6)	3/3 (100)
ALLY-3 [220]	152	DCV + SOF, 12 wk	3	105/109 (96.0)		20/32 (63.0)	

Study	Number of participants	Regimen	Genotype	SVR (mITT analysis) *n/N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
				73/75 (97.0)	32/34 (94.0)	11/19 (58.0)	9/13 (69.0)
ASTRAL-1 [221]	624	SOF/VEL, 12 wk	1, 2, 4, 5, 6	496/501 (99.0)		120/121 (99.2)	
ASTRAL-2 [222]	266	SOF/VEL, 12 wk (n = 134)	2	133/134 (99.0)			
		SOF + RBV, 12 wk (n = 132)		124/132 (94.0)			
ASTRAL-3 [222]	552	SOF/VEL, 12 wk (n = 277)	3	160/163 (98.0)	31/34 (91.0)	40/43 (93.0)	33/37 (89.0)

Study	Number of participants	Regimen	Genotype	SVR (mITT analysis) *n/N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
		SOF + RBV, 24 wk (n = 275)		141/156 (90.0)	22/31 (71.0)	33/45 (73.0)	22/38 (58.0)
ASTRAL-4 [223]	267	SOF/VEL, 12 wk (n = 90)	1-6			75/89 (84.3)	
		SOF/VEL + RBV 12 wk (n = 87)				82/87 (94.0)	
		SOF/VEL, 24 wk (n = 90)				77/87 (88.5)	

Study	Number of participants	Regimen	Genotype	SVR (mITT analysis) *n/N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
EXPEDITION-1 [224]	146	GLE/PIB, 12 wk	1, 2, 4, 5 or 6			145/146 (99.0)	
ENDURANCE-1 [216]	703	GLE/PIB, 8 wk (n = 351)	1	343/344 (99.7)			
		GLE/PIB, 12 wk (n = 352)		345/345 (100)			
ENDURANCE-3 [225]	505	GLE/PIB, 12 wk (n = 233)	3	214/217 (99.0)			

Study	Number of participants	Regimen	Genotype	SVR (mITT analysis) *n/N* (%)			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
		SOF + DCV, 12 wk (n = 115)		110/111 (99.0)			
		GLE/PIB, 8 wk (n = 157)		143/149 (96.0)			
ENDURANCE-2 [226]	202	GLE/PIB, 12 wk	2	192/192 (100)			
ENDURANCE-4 [226]	121	GLE/PIB, 12 wk	4-6	120/120 (100)			

Study	**Number of participants**	**Regimen**	**Genotype**	**SVR (mITT analysis) *n/N* (%)**			
				Patients without cirrhosis		Patients with cirrhosis	
				Treatment-naïve	Treatment-experienced	Treatment-naïve	Treatment-experienced
EXPEDITION-8 [227]	343	GLE/PIB, 8 wk	1-6			334/335 (99.7)	
EXPEDITION-4 [228]	104	GLE/PIB, 12 wk	1-6	102/104 (98.0)			
POLARIS-1 [229]	263	SOF/VEL/VOX, 12 wk	1-6	253/263 (96.0)			
POLARIS-4 [229]	182	SOF/VEL/VOX, 12 wk	1-4	178/182 (98.0)			

2.8 Treatment Failure

The failure to achieve SVR12 following DAA therapy can be due to multiple reasons including non-adherence, drug-drug interactions, inability to achieve sufficient drug concentrations due to malabsorption and impaired cellular uptake due to cirrhosis. Another important reason is the presence of resistance associated substitutions (RASs) that reduce virus susceptibility to DAAs. RASs may be preexisting or may be selected out by previous DAA therapy. The next section discusses HCV resistance to DAAs in more detail.

2.9 HCV Resistance to DAAs

Identifying clinically relevant RASs is challenging. RASs detected in cell culture studies with administration of individual DAAs are not always identical to RASs selected in individuals who fail to achieve SVR12 after DAA therapy. Likewise, not all RASs detected by genotypic resistance analysis are relevant for prediction of response to DAA therapy. In addition, HCV exists as a population of variants due to its error prone viral polymerase and high replication rate. Population-based sequencing shows existence of viral variants at a frequency of 15-20% within the viral population yet deep sequencing strategies can detect RASs that are present at a frequency of 1%. At present it is considered that RASs present below 15% may not be important [230, 231]. Furthermore, the prevalence of naturally occurring RASs varies by genotype and subtype [232], however most studied RASs are those present in epidemic subtypes, mainly prevalent in HICs. Figure 2-17 summarises the main RASs that have been seen associated with DAAs [233].

Panel A

NS3-protease (1-181 aa)

Asunaprevir (1st generation): 36, 43, 54, 55, 56, 80, 122, 168

Glecaprevir (2nd generation): 36, 56, 80, 155, 156, 166, 168

Grazoprevir (2nd generation): 36, 43, 56, 80, 155, 156, 158, 168

Paritaprevir (1st generation): 36, 56, 80, 155, 156, 168

Simeprevir (1st generation): 36, 41, 43, 80, 122, 155, 156, 168, 170

Vaniprevir (1st generation): 36, 80, 155, 156, 168

Voxilaprevir (2nd generation): 36, 41, 43, 56, 80, 122, 155, 156, 168, 170, 175

Panel B

NS5A domain I (1-213 aa)

Daclatasvir (1st generation): 24, 28, 30, 31, 32, 58, 62, 92, 93

Elbasvir (2nd generation): 28, 30, 31, 58, 93

Ledipasvir (1st generation): 24, 28, 30, 31, 32, 38, 58, 92, 93

Ombitasvir (1st generation): 24, 28, 30, 31, 58, 92, 93

Pibrentasvir (2nd generation): 24, 28, 30, 31, 58, 93

Velpatasvir (2nd generation): 24, 28, 30, 31, 32, 58, 92, 93

Panel C

NS5B-polymerase (1-591 aa)

NI

Sofosbuvir: 159, 237, 282, 316, 320, 321

NNI

Dasabuvir: 314, 316, 368, 414, 448, 553, 554, 556, 558, 559

Panel D

Resistance figure notation

RASs detected *in vivo* in DAA failing patients are underlined, independently of *in vitro* data information.

For NS5B-inhibitors and 1st generation NS3- and NS5A-inhibitors, substitutions detected only *in vitro* with fold-change ≥100 are shown, and if detected also *in vivo* are in bold. Substitutions associated only *in vitro* with fold-change >1000 are represented in bold.

For 2nd generation NS3- and NS5A-inhibitors, *in vivo* and/or *in vitro* substitutions with fold-change >2.5 are shown, and with fold-change ≥10 are in bold.

156 — amino acid position

L S L T L T T
T T T V T V V
V V V V

HCV genotypes and subtype: 1a – red 1b – blue 2a/b/c – green 3a – purple 4a/d – yellow 5-light blue 6-brown

Figure 2-17: Summary of main RASs to DAAs

Summary of substitutions associated with resistance to protease inhibitors (A), NS5A inhibitors (B), and nucleoside and non-nucleoside NS5 B inhibitors (C). As summarized in panel D: HCV genotypes and subtypes are represented by different colours. Amino acid substitutions detected *in vivo* in DAA failing patients are underlined, independently of *in vitro* data information. In addition, RAS detected only *in vitro* but associated with fold-change in drug activity compared to the wild-type replicons>100 (1st generation NS3-inhibitors, 1st generation NS5A-inhibitors, and NSB-inhibitors) or>2.5 (2nd generation NS3-inhibitors and 2nd generation NS5Ainhibitors) are also reported. For 1st generation NS3- and NS5A-inhibitors and NS5B-inhibitors, *in vivo* substitutions with fold-change>100, and substitutions associated only *in vitro*
with fold-change>1000 are represented in bold. For 2nd generation NS3- and NS5A-inhibitors, *in vivo* and/or *in vitro* substitutions with fold-change>10 are represented in bold. This figure is reproduced with permission from *Sorbo et al [233]*
through the terms and conditions provided by Elsevier and Copyright Clearance Centre (licence number 5737111496704).

2.9.1 Resistance to Protease Inhibitors

NS3 protease inhibitor RASs are found at baseline, the most frequently observed RAS being Q80K in genotype 1 [234]. Among first generation protease inhibitors there is significant cross-resistance (resistance to all drugs within the class) with mutations at amino acid positions 36, 54, 155, 156 and 168. The RAS A156T confers high level resistance, but is associated with a lack of fitness when drug is not present and therefore is almost never found at baseline. Genotype 3 is difficult to treat with both first and second generation protease inhibitors, most likely due to naturally occurring polymorphisms in the active site, including R123T, I132L and D168Q [235]. However, glecaprevir does have good activity against genotype 3. NS3 associated RASs generally make for less fit virus and can therefore disappear from peripheral blood within weeks to months.

2.9.2 Resistance to NS5A Inhibitors

RASs associated with NS5A inhibitors are often seen at baseline and are highly stable thus contributing to viral fitness and they can persist indefinitely [236]. NS5A inhibitors tend to have a low barrier to resistance and RASs are rapidly selected, mainly between amino acid positions 28 and 93. The most common sites involving RASs are 24, 28, 30, 31, 58, 92 and 93, with varying prevalence across genotypes and subtypes, but the most clinically relevant RAS across all

NS5A inhibitors is Y93H [237-239]. The presence of multiple NS5A RASs at baseline signifies high level resistance and are frequently described in individuals failing to achieve SVR12 [238, 239].

2.9.3 Resistance to NS5B Inhibitors

Baseline RASs to dasabuvir or sofosbuvir are rarely detected at baseline. The most important RAS associated with sofosbuvir treatment is S282T and has been identified in *in vitro* studies of all genotypes [240], but is rarely reported in clinical trials (1%) of individuals that fail sofosbuvir based regimens [241]. Pooled data from clinical trials and real life, involving individuals with genotype 1 and 3 infection failing sofosbuvir based regimens, have reported the emergence of the RAS L159F, sometimes in combination with C316N, L320F or V321A [242-244]. However, these RASs are not associated with reduced sofosbuvir susceptibility in *in vitro* studies [245, 246]. Retreatment with sofosbuvir has been associated with selection of S282T and an increased risk of repeated treatment failures [247, 248]. RASs associated with dasabuvir treatment of individuals with genotype 1a or 1b infection are highlighted in panel C of Figure 2-17. The most frequently reported RAS was S556G [242, 244, 249].

2.10 Challenges of HCV Elimination in SSA

As described in section 2.3, the diversity of HCV is vast yet most available genomic data comes from HICs where the predominant circulating genotypes and subtypes are epidemic lineages. These are the same regions where most DAA based clinical trials have been conducted (Figure 2-18). DAA regimens such as sofosbuvir/velpatasvir and glecaprevir/pibrentasvir have been termed “pan-genotypic” based on effectiveness against these epidemic lineages. International guidelines, including those provided by WHO [159], are based on these data. However, there is a lack of clinical trial and real-world data of DAA effectiveness against endemic HCV lineages highly prevalent in SSA. Furthermore, poor health system infrastructure in many parts of SSA makes diagnosis and treatment unattainable for many individuals infected with chronic HCV. Simplification of the diagnostic algorithm has been discussed in section 2.6.

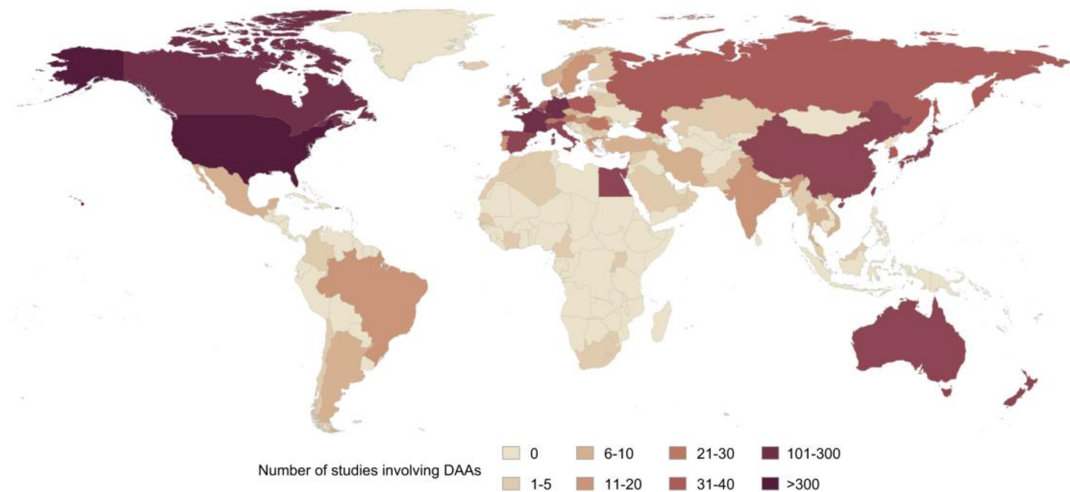


Figure 2-18: Map showing locations of DAA-related studies involving HCV-infected individuals

Details of registered clinical trials were downloaded from https://clinicaltrials.gov/. This figure is reproduced with permission from *Shah et al [108]*, under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence (https://creativecommons.org/licenses/by/4.0/).

We did a survey of the membership in the HCV sub-Saharan Africa Network, consisting of blood transfusion service experts and lead physicians for HCV treatment to gain an understanding of the size of the challenge that sub-Saharan Africa faces. This collaboration resulted in the development of a network of 25 senior treating physicians across 13 countries, many of whom are head of their national viral hepatitis programmes, and 13 blood transfusion service experts across 12 countries. Physicians and blood transfusion service experts responded to surveys covering questions on the availability of diagnostic tests, direct-acting antivirals, and associated costs. Survey responses were received from members representing ten countries. A summary of the survey findings is shown in Table 2-3.

All responding countries had access to serology tests; however, the type of tests available varied between and within countries. For example, in Uganda, serology tests often depended on what kits the laboratory had in stock. The cost of serological tests also differed in each country, ranging from US$0·60 to $13 per test in Zimbabwe to $25 in Benin. In Zimbabwe, the cheaper serology tests are available only in public health facilities that often have a short supply of kits, thus forcing patients to seek tests in private facilities where costs are inevitably

higher. PCR tests to detect viral RNA were available in only 60% (6/10) of countries.

Availability of direct-acting antivirals varied, but the most commonly available regimens among countries participating in this survey included the first generation NS5A inhibitors, daclatasvir (90%) and ledipasvir (70%). Five (50%) countries reported having access to the second generation NS5A inhibitor velpatasvir, but none of the countries had access to protease inhibitor-based regimens or alternative NS5A inhibitors, such as pibrentasvir.

The costs of diagnosis and treatment are largely borne by the patient and only in Rwanda are funds provided for patients to be tested and treated, with treatment costs borne by private sector fundraising. Treatment costs per patient are substantial, ranging from $500 in Uganda to $2400 in the Democratic Republic of the Congo. In Malawi, direct-acting antivirals are not readily available.

National HCV protocols were available in 80% (8/10) of surveyed countries, whereas a protocol for direct linkage to care from national blood transfusion services was only available in 60% (6/10) of countries.

Countries such as Rwanda have shown an incredible drive to develop their national HCV management programmes, to improve the chance of achieving WHO elimination targets. However, most countries in sub-Saharan Africa have multiple barriers to overcome before they can reach these targets. Substantial investment and political commitment will be essential to overcome these challenges.

Table 2-3: Summary of diagnostics and treatment costs, and availability, by country

This table is reproduced with permission from *Shah et al [250]*, through the terms and conditions provided by Elsevier and Copyright Clearance Centre (licence number 5737191262856).

Country	Are serology tests available?	Are serology test costs provided for patients?	Range of costs of serology tests ($USD per test)	Are PCR tests available?	Are PCR test costs provided for patients?	Range of costs of PCR tests ($USD per test)	Are treatment costs provided for patients?	Is there direct linkage to care following diagnosis from transfusion services?	Is a National HCV protocol available?	Approximate cost of DAA treatment ($USD per patient)
Benin	yes	no	25	yes (mainly private laboratories)	no	104-128	no	no	yes	960
DRC	yes	no	10-25	yes	no	80-225	no	no	yes	750-2400
Ghana	yes	no	10	yes	no	110-140	no	yes	yes	800-1100
Malawi	yes	yes	19-26	no	n/a	n/a	no	yes	yes	N/A
Nigeria	yes	no	2.5-4	yes	no	125-150	no	yes	yes	750-1050
Rwanda	yes	yes	0.65	yes	yes	10	yes (private sector fundraising)	yes	yes	60
Sierra Leone	yes	no	5-10	no	n/a	n/a	no	yes	yes	1300

Uganda	yes	no	2-4	yes	no	55-120	no	yes	yes	500-1200
Zimbabwe	yes	no	0.6-13	no	n/a	n/a	no	no	no	627
Somalia	yes	no	10	no	n/a	n/a	no	no	no	560

2.11 Aims and Hypotheses

Three sets of clinical samples were obtained from three countries (Kenya, Uganda and Benin) from distinct regions in SSA and two distinct populations (PWID and general community population). The following hypotheses were explored:

- Chronic HCV infection and associated liver disease is highly prevalent among PWID in Coastal Kenya
- PWID networks in Coastal Kenya are closely related with well-defined transmission clusters
- The introduction of HCV into the PWID community in Coastal Kenya coincides with the epidemic of injecting drug use
- HCV in Uganda and Benin is highly diverse and has a high prevalence of baseline RASs
- Glasgow PWID infected with HCV 2a and failing treatment with Glecaprevir/Pibrentasvir is due to RASs.

The work presented in this thesis aims to do the following:

1. Determine the prevalence of HCV exposure and viraemia among PWID in Coastal Kenya
2. Determine the prevalence of HCV associated liver disease among PWID in Coastal Kenya using serum biomarkers
3. Investigate PWID networks using HCV genomic data
4. Estimate the year of introduction of HCV into the PWID community in Coastal Kenya using molecular clock analysis

5. Investigate the genetic diversity of HCV in Uganda and Benin using next generation sequencing and phylogenetics

6. Determine the prevalence and explore the nature of baseline RASs in HCV from Kenya, Uganda and Benin using bioinformatics approaches and functional *in vitro* assays

7. Investigate the presence of known and unknown RASs in HCV 2a circulating among Glasgow PWID, using bioinformatic approaches and functional *in vitro* assays.

8. Generate and publish high quality full-length HCV genomes

3 Materials and Methods

3.1 Materials

3.1.1 Commonly used Reagents/Chemicals

Absolute ethanol (VWR Chemicals BDH)

NEBNext multiplex oligos (New England Biolabs)

Deoxyribonucelotide triphosphates (dNTPs) (New England Biolabs)

Random Hexamers (ThermoFisher Scientific)

Isopropranol (ThermoFisher Scientific)

Phosphate Buffered Saline (Tissue Culture Grade) (Sigma)

Dimethyl sulfoxide (DMSO) (Sigma)

Trypan blue stain (0.4%) (Gibco)

HEPES Buffer Solution (1M) (Gibco)

Penicillin 10000 units/ml (Gibco)

Streptomycin 10000 units/ml (Gibco)

Milli-Q Q-POD Ultra-Pure Water (Biopak)

Ambion™ DNase I (ThermoFisher Scientific)

SuperScript III Reverse Transcriptase (ThermoFisher Scientific)

DTT (ThermoFisher Scientific, R0861)

RNaseOUT™ Recombinant Ribonuclease Inhibitor (ThermoFisher Scientific)

Tris(hydroxymethyl)aminomethane chloride, pH 8.0

Formaldehyde solution (Fisher Scientific, 10630813)

Coomassie brilliant blue R powder (Sigma-Aldrich, B0149-500G)

3.1.2 Kits

RNAdvance Blood (Beckman Coulter, A35604)

SuperScript III Reverse Transcriptase kit (ThermoFisher Scientific, 18080044)

Superscript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (ThermoFisher Scientific, 12574026)

FastDigest XBAI (ThermoFisher Scientific, FD0684)

Monarch DNA Gel Extraction Kit (New England Biolabs, T1020S)

RNeasy Mini Kit (Qiagen, 74104)

QIAPrep Spin Miniprep Kit (Qiagen, 27104)

T7 RiboMAX Express Large Scale RNA Production System (Promega, P1320)

Pierce™ Gaussia Luciferase Flash Assay Kit (ThermoFisher Scientific, 16159)

Cell Proliferation Reagent WST-1 (Merck, 5015944001)

NEBNext® mRNA Second Strand Synthesis Module (New England Biolabs, E6111L)

KAPA LTP Library Preparation Kit (Kapa Biosystems, Roche)

USER® enzyme (New England Biolabs, M5505L)

Qubit® dsDNA HS assay kits (ThermoFisher Scientific)

3.1.3 Equipment

KingFisher Flex Purification System (ThermoFisher Scientific)

Fast 7500 Real-Time PCR Machine (Applied Biosystems)

Qubit 2.0 Fluorometer (ThermoFisher Scientific)

4200 TapeStation (Agilent Technologies)

MiSeq System instrument (Illumina)

NextSeq System instrument (Illumina)

Chameleon II plate reader (Hidex)

PHERstar FS microplate reader (BMG Labtech)

Gene Pulser XCell Electroporation System (Bio-Rad)

TC20 Automated Cell Counter (Bio-Rad)

NanoDrop™ One (ThermoFisher Scientific)

Biomek FXP Liquid Handler (Beckman Coulter)

Celigo Image Cytometer (Nexcelom)

4mm cuvettes (Molecular BioProducts, ThermoFisher Scientific, 5540-11PK)

3.1.4 Cell Line

Table 3-1 Cell line used

Cells	Description	Source
Huh7	Human hepatoma cell line	Jean Dubuisson (CNRS, Institut de Biologie de Lille, Lille, France)

3.1.5 Tissue Cell Culture

Table 3-2 Tissue culture reagents

Solution	Constituents
Dulbecco's modified Eagle's medium (DMEM)(Gibco) supplemented with GlutaMAX	10% heat inactivated (56°C for 30 minutes) foetal calf serum, 1M HEPES buffer solution (5mls), penicillin + streptomycin (5mls)
Dulbecco's modified Eagle's medium (DMEM)(Gibco) supplemented with GlutaMAX	2% heat inactivated (56°C for 30 minutes) foetal calf serum, 1M HEPES buffer solution (5mls), penicillin + streptomycin (5mls)

3.1.6 Drug Compounds

Ombitasvir (MedChemExpress)

Ledipasvir (MedChemExpress)

Daclatasvir (MedChemExpress)

Dasabuvir (MedChemExpress)

Paritaprevir (MedChemExpress)

Grazoprevir (MedChemExpress)

Asunaprevir (MedChemExpress)

Simeprevir (MedChemExpress)

Glecaprevir (AmBeed Inc)

Pibrentasvir (Cambridge Bioscience Ltd)

Velpatasvir (Cambridge Bioscience Ltd)

Drug compounds were reconstituted in 100% DMSO and stored as stock solutions at -20°C.

3.1.7 Software

Name of Software	Link	Author/Reference
TrimGalore	https://github.com/FelixKrueger/TrimGalore	Felix Krueger, Cambridge
CREATE_KMERS	https://github.com/vbsreenu	Sreenu Vattipally, MRC-University of Glasgow Centre for Virus Research
Tanoti	https://github.com/vbsreenu/Tanoti	Sreenu Vattipally,

		MRC-University of Glasgow Centre for Virus Research
SAM2CONSENSUS	https://github.com/vbsreenu/Sam2Consensus	Sreenu Vattipally, MRC-University of Glasgow Centre for Virus Research
SAM_STATS	https://github.com/lparsons/galaxy_tools/tree/master/tools/sam_stats	L. Parsons, New Jersey, USA
HCV_ORF_mapping_pipeline_top5.sh	Housed on server at MRC-University of Glasgow Centre for Virus Research	Emma Thomson, MRC-University of Glasgow Centre for Virus Research
GapFiller	//git://git.code.sf.net/p/gapfiller/code	[251]
De novo Assembly pipeline	https://github.com/vbsreenu/ContigsMerger	Sreenu Vattipally, MRC-

		University of Glasgow Centre for Virus Research
SPAdes	https://github.com/ablab/spades	[252]
IDBA_UD	https://github.com/loneknightpy/idba	[253]
ClusterPicker version 1.3	https://code.google.com/archive/p/cluster-picker-and-cluster-matcher/	[254]
MAFFT version 7.313	https://mafft.cbrc.jp/alignment/software/	[255]
RAxML	https://cme.h-its.org/exelixis/web/software/raxml/	[256]
IQ-TREE	http://www.iqtree.org/	[257]
HCV GLUE	http://hcv-glue.cvr.gla.ac.uk/#/home	[258, 259]
Phyloscanner	https://github.com/BDI-pathogens/phyloscanner	[260]
Geneious Prime 2023.2.1	https://www.geneious.com/	
BEAUti version 1.10.4 and BEAST version 1.8.2	https://beast.community/beauti	[261]
Tracer	https://beast.community/tracer	[262]

TreeAnnotator	https://beast.community/treeannotator	Marc Suchard, UCLA
FigTree version 1.4.3	http://tree.bio.ed.ac.uk/software/figtree/	Andrew Rambaut, The University of Edinburgh

3.2 Clinical Samples

3.2.1 Kenya Cohort

A field study was conducted in Coastal Kenya during an Institutional Strategic Support Fund Wellcome Trust Fellowship at Imperial College, London between 2015 and 2016, under the supervision of Dr Maud Lemoine and Professor Elijah Songok. This study has contributed to the chapter on HCV in Kenyan PWID.

3.2.1.1 Ethical Approval

Ethical approval was provided by the Scientific and Ethics Review Unit, Kenya Medical Research Institute (KEMRI) on 13th October 2015 followed by a couple of 1 year extension approvals on 25th February 2020 and 18th February 2021 (protocol number 2209).

3.2.1.2 Study Sites

Three community Drop-In-Centres (DICs) along the coast of Kenya were involved in participant recruitment. These were supported by Kenya AIDS NGOs Consortium (KANCO). DICs were safe places set up in the community to provide harm reduction services for the public. The following sites were involved in the study:

1. Mombasa Drop-In-Centre, Reachout Centre Trust, Old Town, Mombasa

2. Muslim Education & Welfare Association Drop-In-Centre, Kisauni, Mombasa

3. KANCO Drop-In-Centre, Watamu

3.2.1.3 Participant Recruitment and Procedures

The study proposal set out to recruit 400 PWID. The inclusion criteria were:

a) Adults 18 years or older who could provide verbal and written consent

b) Past or active history of injecting drug use

Participants who were unable to provide consent were not included in the study.

Participants were informed of the study and encouraged to engage with the study team by peer support workers who were involved in welfare of PWID in the local areas. All participants were sensitised to the consequences of HCV infection and were informed of the aims and objectives of the study prior to recruitment.

Peer support workers recruited participants in the community, and then study participants would sensitise fellow peers for ongoing recruitment, i.e. a snowball sampling strategy. Once a total number of 200 recruited participants per geographic region (split evenly between the 2 sites in Mombasa) was reached, recruitment stopped. Participants were recruited consecutively daily between Monday and Friday.

Each recruited participant was administered a comprehensive epidemiological questionnaire to assess risk factors for HCV infection. They also underwent a clinical examination and blood sampling to assess haematological, liver and renal biochemistry. Extra plasma and serum samples were taken for HCV serological and molecular tests. Each participant was assigned an anonymised study identification number. 50 µl of whole blood was also added to 12 mm dried blood spot cards.

3.2.1.4 Clinical Samples

Whole blood samples were spun down on site using a portable centrifuge and then transported, within 4 hours, on ice to Pathcare Kenya Limited Laboratory in Mombasa, where plasma and serum was separated. Some of the plasma and serum was used for basic biochemical tests, as described above, while the rest was aliquoted into 1.5 ml screw-cap cryovials and stored at -20°C. Once recruitment and sample collection were complete, the stored samples were shipped, on dry ice, to the KEMRI laboratory in Nairobi for storage at -80°C. Dried blood spot cards were packed individually in plastic sealable bags with a desiccant pack. These were stored together with the plasma and serum samples.

All samples underwent a screening enzyme immunoassay (EIA) test for HCV antibodies (Bioelisa HCV 4.0, Biokit S.A). All positives were confirmed by another EIA test (INNO-LIA HCV Score, Fujirebio). Dried blood spot cards of sero-positive samples were packed and shipped to Professor Stephane Chevaliez (Hôpital Henri-Mondor, Créteil, France), who conducted HCV qPCR. In brief, HCV RNA was extracted from dried blood spot cards and then quantified using the Cobas Ampliprep/Cobas TaqMan HCV version 2 (CAP/CTM; Roche Molecular Systems Pleasanton, California) assay according to the manufacturer's instructions.
Liver disease was assessed using APRI [263, 264] and FIB-4 [265] scores. Ideally, I would have used transient elastography to assess liver fibrosis, however, this was not available.
My PhD project was initially set up to sequence HCV from these clinical samples in Kenya at the KEMRI-Wellcome Trust laboratories in Kilifi, Kenya. However, the onset of the COVID-19 pandemic disrupted these plans. Instead, the samples were shipped to the MRC-University of Glasgow Centre for Virus Research for sequencing.

3.2.1.5 Statistical analysis

Association between pre identified risk factors and HCV infection were analysed by univariable analysis. Those risk factors that showed an association with HCV infection with a p value of <0.1 were taken forward for multivariable analysis using logistic regression. Age and sex were adjusted for as standard. This analysis was conducted by Dr Yusuke Shimakawa (Pasteur Institute, Paris)

3.2.2 Uganda Samples

2 groups of serum samples from Uganda were provided for HCV sequencing. The first group of samples numbered 69 and were provided by Professor Ponsiano Ocama, Makerere University, Kampala. These were all HCV RNA positive and were from a blood donor population. The second group of samples numbered 100 and were HCV seropositive samples provided by Lucrece Ahovegbe, from a blood donor population in North-East Uganda.

3.2.3 Benin Samples

HCV seropositive serum and plasma samples from Benin were collected by Lucrece Ahovegbe. The samples were collected from individuals recruited from two hospitals in Benin; Centre National Hospitalier Universitaire (CNHU) in Cotonou and Centre Hospitalier Departmental de Borgou-Parakou in Parakou. Samples were collected between June 2019 and December 2020 as part of the national screening program led by the society for viral hepatitis elimination in Benin “Société Beninoise d’Hepato-gastro-entérolgie (SBHGE)”. Serological screening was run using the InTec HCV Rapid Test. Ethical approval for the study was provided by the Comité National d’Ethique pour la Recherche en Santé (number 38, 15th October 2019).

3.2.4 Glasgow PWID genotype 2 cohort

An additional cohort of PWID, unusually infected with HCV genotype 2 infection, and managed by clinicians within NHS Greater Glasgow and Clyde (GGC), included individuals that had failed treatment with Glecaprevir and Pibrentasvir (G/P). Plasma samples from 15 of these individuals, including 2 from individuals that failed treatment with G/P, were made available for sequencing. Demographic and clinical data from this cohort were collected between 2013 and 2019. Ethical approval was granted by the National Research Ethics Service Committee, East Midlands (11/EM/0323) and from the West of Scotland Research Ethics Committee (12/WS/0002).

3.3 Next Generation Sequencing

3.3.1 RNA Extraction

All RNA extractions were carried out in a biosafety level 2 laboratory. RNA was extracted from 400 µl of plasma or serum following the RNAdvance Blood kit protocol (Beckman Coulter). This process is summarised in Figure 3-1. In brief, 400 µl of sample was added to a well of a KingFisher deep-well 96 plate (ThermoFisher Scientific). 300 µl of lysis buffer and 30 µl of proteinase K solution was added to each sample and the plate was then placed on a heat block at a temperature of 55°C for 15 minutes. The plate was then added to the KingFisher Flex Purification System (ThermoFisher Scientific) for automation of the following steps:

1. Addition of magnetic beads for binding (400 µl of isopropanol and 10 µl of binding beads per sample).

2. Washing with a mixture of isopropanol (320 µl per sample) and WBE wash buffer (540 µl per sample).

3. Washing with 80% ethanol (800 µl).

4. DNase treatment (5 µl enzyme + 10 µl buffer + 85 µl water per sample).

5. Rebinding to beads (200 µl of RBB solution per sample).

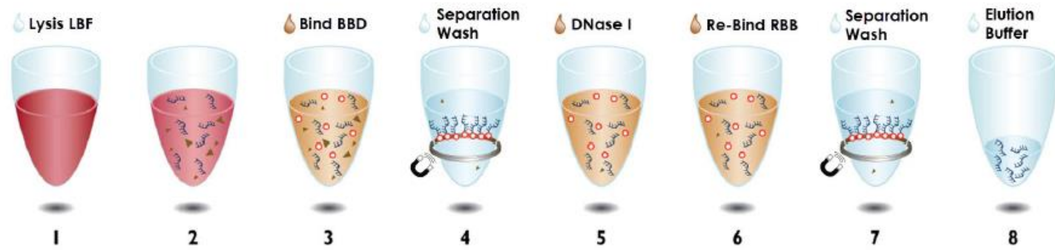
6. Washing with 80% ethanol (800 µl per sample).

7. Repeat washing with 80% ethanol (800 µl per sample).

8. Elution of RNA into 20 µl of nuclease-free water. The eluted RNA is placed into a new 96-well plate.

Eluted RNA was then immediately carried forward for library preparation or stored in a fridge at 4°C for a maximum of 12 hours, if library preparation was to be carried out at a later time. I did not extract and store RNA at -80°C for any length of time to avoid the risk of RNA degradation with a freeze-thaw cycle.

RNAdvance Blood



1. Add **Lysis** (LBF) to **PAXgene blood** and mix.
2. Lysis and **Proteinase K** digestion.
3. Addition of **Bind** (BBD).
4. Magnetic separation of beads from supernatant, wash with **Wash** (WBE) and **Ethanol**.
5. **DNase I** reaction.
6. Rebinding with **Re-Bind** (RBB).
7. Magnetic separation of beads from supernatant, wash with **Ethanol**.
8. Elution.

Figure 3-1: Workflow for RNA extraction.

This image is adapted from RNAdvance Blood protocol (Beckman Coulter).

3.3.2 HCV qPCR

HCV RNA quantification was carried out using Superscript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (ThermoFisher Scientific). Primers and probe were manufactured by ThermoFisher Scientific. Sequence details are shown in Table 3-3. A total reaction volume of 15 µl consisted of 2 µl of RNA template, 0.3 µl each of forward and reverse primers, 0.15 µl of probe, 0.03 µl of ROX dye, 0.3 µl of enzyme mix, 7.5 µl of 2x reaction mix and 4.42 µl of nuclease free water. Reagents and sample mixture were thoroughly mixed by pipetting and then briefly centrifuged before being added to the PCR machine with the following cycling conditions:

1. 50°C for 15 minutes

2. 95°C for 2 minutes

3. 40 cycles of 95°C for 8 seconds followed by 60°C for 34 seconds

Samples that had detectable HCV RNA were taken forward for DNA synthesis and library preparation.

Table 3-3: Sequence details of qPCR primers and probe
The reporter dye is FAM, and the quencher is MGB.

Primer (5’ to 3’)/Probe	Sequence
Forward primer	TCTGCGGAACCGGTGAGTAC
Reverse primer	GCACTCGCAAGCACCCTAT
Probe	FAM-AAAGGCCTTGTGGTACTG-MGB

3.3.3 cDNA Synthesis

Reverse transcription of extracted RNA was carried out using the SuperScript III reverse transcriptase kit (ThermoFisher Scientific). Starting with a template of 10 µl of RNA, a mixture of 1 µl dNTP and 1 µl random hexamers was added. The mixture was incubated at 65°C for 5 minutes and then immediately chilled on ice. To this mixture, 4 µl of reverse transcriptase buffer, 2 µl of SuperScript III reverse transcriptase, 1 µl of RNaseOUT enzyme and 1 µl of DTT was added. The reaction mixture was incubated as per the following cycle:

1. 25°C for 10 minutes.

2. 55°C for 60 minutes.

3. 70°C for 15 minutes.

4. 4°C hold.

After cDNA synthesis, libraries were prepared on a Biomek FXP automated liquid handling workstation (Beckman Coulter). The workflow and key reactions are described in detail below.

3.3.4 dsDNA Synthesis

dsDNA was synthesised using the NEBNext® mRNA Second Strand Synthesis Module (New England Biolabs). To 20 µl of cDNA sample, 8 µl of second strand synthesis buffer, 4 µl of second strand synthesis enzyme and 48 µl of water was added. The mixture was incubated at 16°C for 2.5 hours followed by an 8°C hold. dsDNA was cleaned and size selected by mixing to AMPure XP magnetic beads in a 1:1 ratio, followed by washing with 80% ethanol and elution in 25 µl of 10mM TRIS.

3.3.5 Library Construction

A KAPA LTP Library Preparation Kit (Kapabio Systems) and NEBNext Multiplex Oligos for Illumina (NEB) were used for the construction of indexed libraries.

To facilitate DNA clean-ups between reactions without transferring DNA, the reactions of the library construction process are with AMPure XP magnetic beads, which were added in the dsDNA clean-up step. In between reactions, SPRI (20% PEG, 2.5M NaCl) was added to the sample at a ratio of 1:1.4 volume of sample:SPRI, unless stated otherwise, to rebind DNA to beads. Bead bound DNA was then washed with 80% ethanol followed by elution in TRIS. This clean-up step was repeated in between reactions and thus will not be described again.

2 µl of end repair enzyme and 3 µl of 10X end repair buffer was added to each dsDNA cleaned sample, followed by incubation at 20°C for 30 minutes. A-tailing reaction was carried out by the addition of 1.5 µl of A-tail enzyme and 2.5 µl of 10X A-tail buffer to the flanked DNA and the mixture was incubated at 30°C for 60 minutes. The sample was then cleaned and washed before elution in 14 µl of TRIS. 1 µl was removed for A-tailed DNA quantification and determining the amount of adaptor to be added in the ligation reaction. Usually, the amount of Adaptor added is up to 20 times the amount of A-tailed DNA.

The sample then underwent adaptor ligation by the addition of 2.5 µl of T4 ligase, 5 µl of 5X buffer and 5 µl of diluted NEBNext Adaptor. This ligation reaction underwent incubation at 20°C for 60 minutes. The NEBNext adaptor is a U-shaped hairpin loop structure, which was cleaved by the addition of 0.75 µl USER® enzyme. This reaction was incubated at 37°C for 15 minutes. Opening the adaptor loop provides the substrate for subsequent PCR, during which barcodes (NEBNext® Multiplex Oligos for Illumina) are incorporated to the DNA, thus enabling multiplexing. Before the PCR reaction, the USER® enzyme treated ligation mixture was cleaned up, this time using a sample to SPRI ratio of 0.9:1, followed by elution in 11 µl of TRIS. This completed the construction of the library template. 10 µl was used as a template for library PCR amplification, by addition of 12.5 µl of 2X KAPA HF hot start mix and 2.5 µl of NEBNext® Multiplex Oligos for Illumina. The following cycling conditions were used:

1. 98°C for 45 seconds
2. 16 cycles of 98°C for 15 seconds followed by 65°C for 30 seconds followed by 72°C for 30 seconds
3. 72°C for 60 seconds
4. 4°C hold

Following amplification samples were made up to a volume of 50 µl by addition of TRIS. 45 µl of AMPure XP magnetic beads were then added at a beads to sample ratio of 0.9:1, and the mixture was incubated at room temperature for 5 minutes. Following 2 washes with 80% ethanol, samples were eluted in 20 µl of water. Samples were then quantified using Qubit fluorometer and qPCR (ABI® 7500 Fast qPCR machine, ThermoFisher).

3.3.6 Pooling of Libraries

Samples were pooled together based on their Ct values from qPCR. Each pool contained up to 16 multiplexes with up to a total of 1100 ng DNA. Within each pool, Ct differences between each library were no more than 3. For those

samples where Ct values were not obtained, pools were created based on the molecular mass of libraries.

3.3.7 Target Enrichment

Library pools were then enriched for HCV using a custom probe (KAPA HyperExpole mAX 5 Mb, IRN 1000009633) manufactured by Roche.

The process of enrichment was conducted using the KAPA HyperCap 3.0 workflow.

In brief, 4 µl of probe was hybridised to each pool followed by an overnight incubation at 55°C. The hybridisation reaction also contained 1 µl xGen™ Universal Blockers TS (IDT) and 20 µl COT Human DNA (Roche). These two reagents specifically block the adaptor and index regions of each library, thereby reducing non-specific binding to the probe. The probe contains biotinylated oligos. Following the hybridisation reaction, the target DNA-probe molecule is pulled out by incubating with streptavidin coated magnetic beads at 55°C for 15 minutes. Bead bound DNA was then washed sequentially with different salt gradient washing buffers (stringent wash buffer, wash buffer 1, 2 and 3) to remove non-biotin-streptavidin specific bound DNA. What remained was then resuspended in 20 µl of nuclease-free water. This was now the target enriched library and served as a template for amplification by PCR. The PCR reaction contained 25 µl of KAPA HiFi HotStart ReadyMix and 5 µl of primers (Post-Capture PCR Oligos). These primers are complementary to the Illumina adaptor sequences.

3.3.8 Illumina Sequencing

Target-enriched, amplified libraries were quantified by Qubit® dsDNA HS assay kits (ThermoFisher Scientific) through Qubit® Fluorometer (ThermoFisher Scientific). Size distribution of libraries was checked on the Agilent 4200 TapeStation using High Sensitivity D5000 ScreenTape and reagents (Agilent, 5067-5592). Further amplification and cleaning were performed where necessary. Finally, the libraries were pooled by equal molar concentration and sequenced on Illumina Nextseq 550 platform with paired ends for 2 x 150 cycles.

High-output cartridge kit v2.5 (300 cycles) was used (Illumina, 20024908). Raw data was output as bcl files, which were then converted to compressed fastq files using bcl2fastq software, followed by storage on the institute server.

A summary flowchart highlighting all the important steps in library preparation and HCV enrichment prior to sequencing is shown in Figure 3-2.

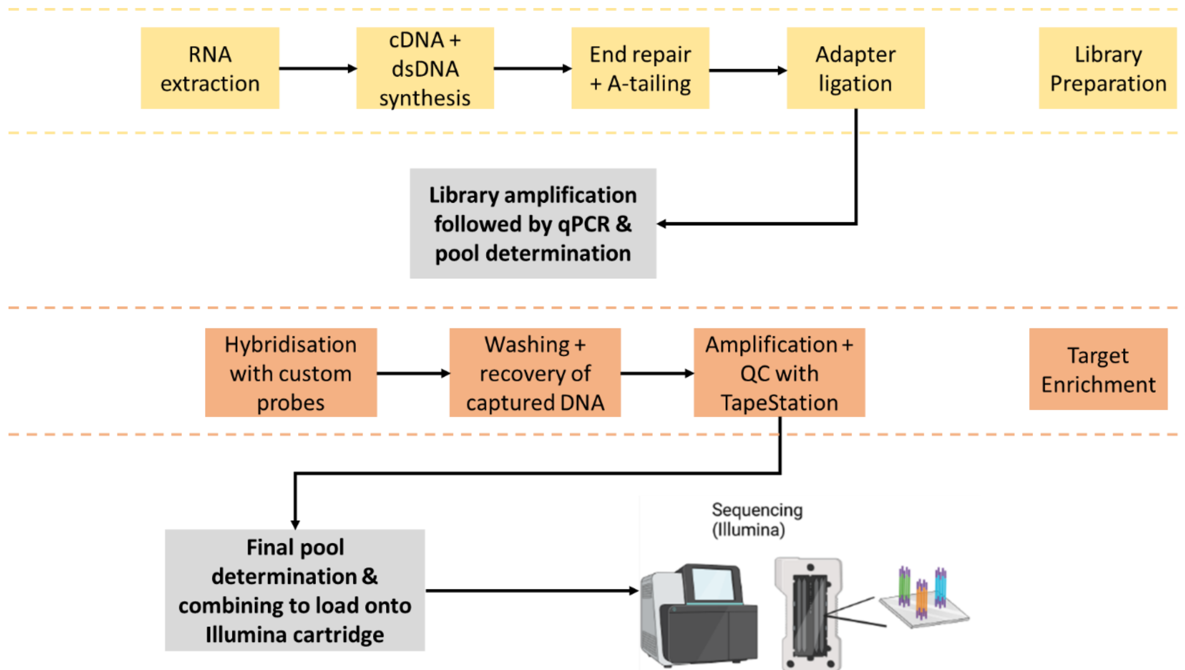


Figure 3-2: Summary workflow for library preparation
Summary flowchart highlighting important steps in library preparation and enrichment for HCV prior to sequencing.

3.4 Bioinformatic Analyses

3.4.1 Overview

Figure 3-3 summarises the workflow of bioinformatic analysis of raw reads from the Illumina sequencer. Whole HCV genomes were assembled using a combination of reference based and *de novo* assembly methods.

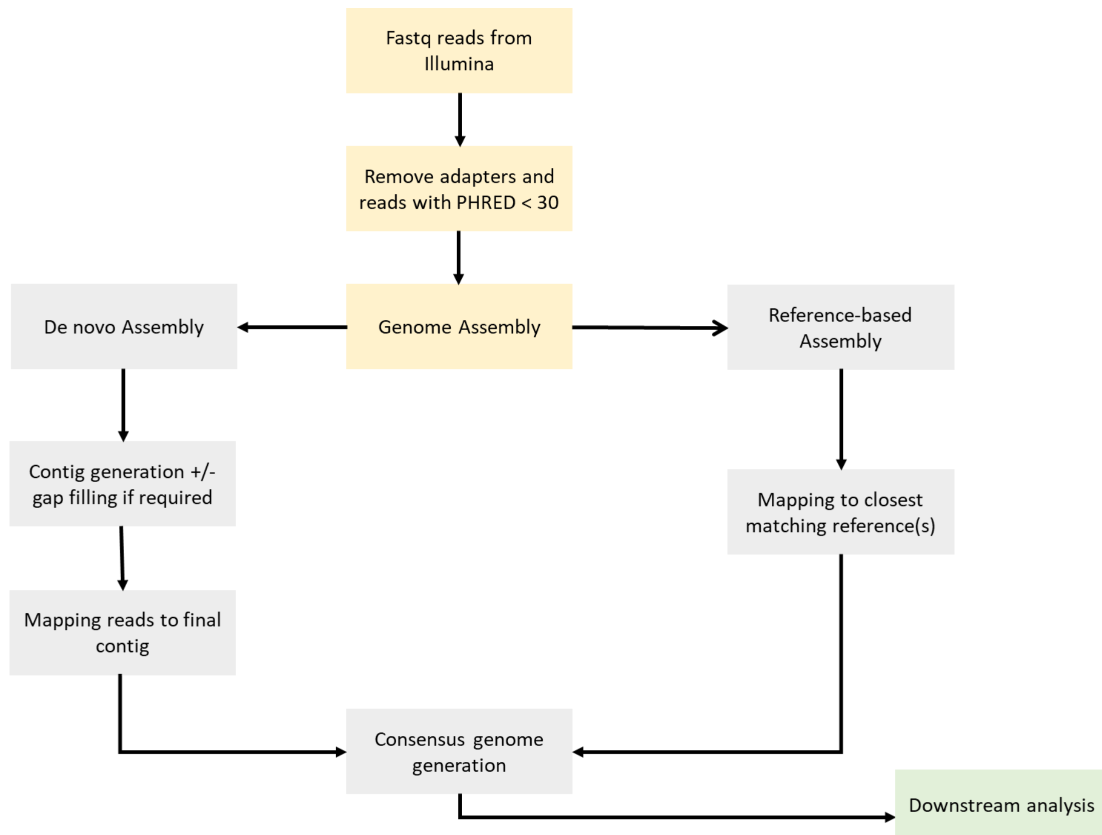


Figure 3-3: Bioinformatic analysis of raw NGS reads overview

3.4.2 Reference-based Assembly

TrimGalore (https://github.com/FelixKrueger/TrimGalore) was used to remove adapters and discard low quality (PHRED <30) reads. Reads for each sample were then fragmented into k-mers of random sizes (CREATE_KMERS, written by Sreenu Vattipally), which were then mapped to a comprehensive HCV genome database to identify the top 5 closest matching HCV reference genomes. Raw reads were then mapped to these HCV reference genomes using Tanoti (https://github.com/vbsreenu/Tanoti) to generate sequence aligned mapped (SAM) files, which were then used to generate consensus genome sequences using SAM2CONSENSUS (written by Sreenu Vattipally). Genomic statistics, including coverage and depth, were calculated for each SAM file using SAM_STATS (written by Sreenu Vattipally). This whole process was automated within a bash script written by Emma Thomson (HCV_ORF_mapping_pipeline_top5.sh). Figure 3-4 summarises the reference-based assembly process. Consensus genomes that had gaps in the sequence

underwent a gap filling process, using GapFiller [266]. Finally, only consensus genomes with a coverage of at least 90% were accepted.

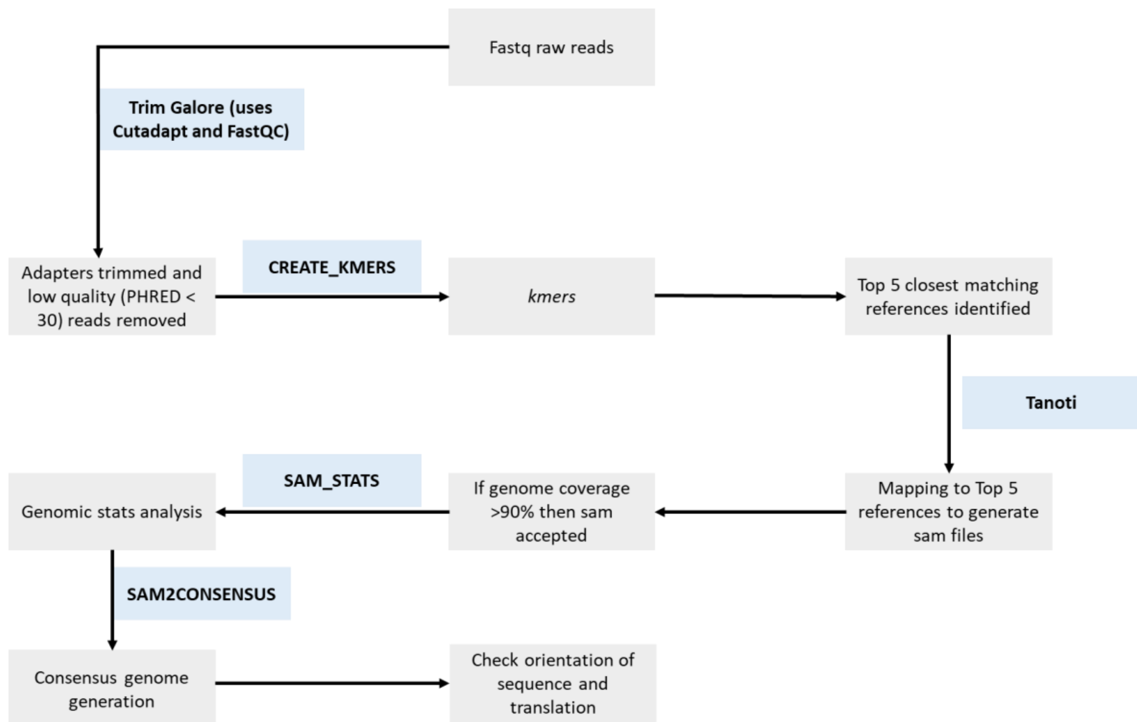


Figure 3-4: Reference-based assembly workflow
Blue shaded boxes reflect the programs that were used. CREATE_KMERS, Tanoti and SAM2CONSENSUS are programs written by Sreenu Vattipally.

3.4.3 *De novo* Assembly

Quality control process on raw reads for *de novo* assembly was the same as for reference-based assembly. Reads were then enriched for HCV *in-silico* by blasting against a comprehensive HCV specific database. Enriched reads were then run through either a single or dual *de novo* assembly pipeline. These were written by Sreenu Vattipally. For the single *de novo* assembly process, reads were run through SPAdes [267] iteratively until a contig with a length of >8500 nucleotides was generated or 100 runs had completed. Contigs that were <8500 nucleotides in length underwent a gap filling process, which was informed by the closest matching HCV reference genome. Full length contigs were then checked for correct orientation and that they translated to a polyprotein. Finally, they were used as reference for mapping raw reads to (using Tanoti, https://github.com/vbsreenu/Tanoti) followed by consensus genome generation. The dual *de novo* assembly process involved running both SPAdes and

IDBA_UD assemblers on enriched reads, iteratively until a contig of >8500 nucleotides was generated. Where contigs <8500 nucleotides were generated, they were merged and then gap filled by extension from raw reads. The remainder of the assembly process was as described for the single *de novo* assembly process. A summary of the *de novo* assembly workflow is shown in Figure 3-5.

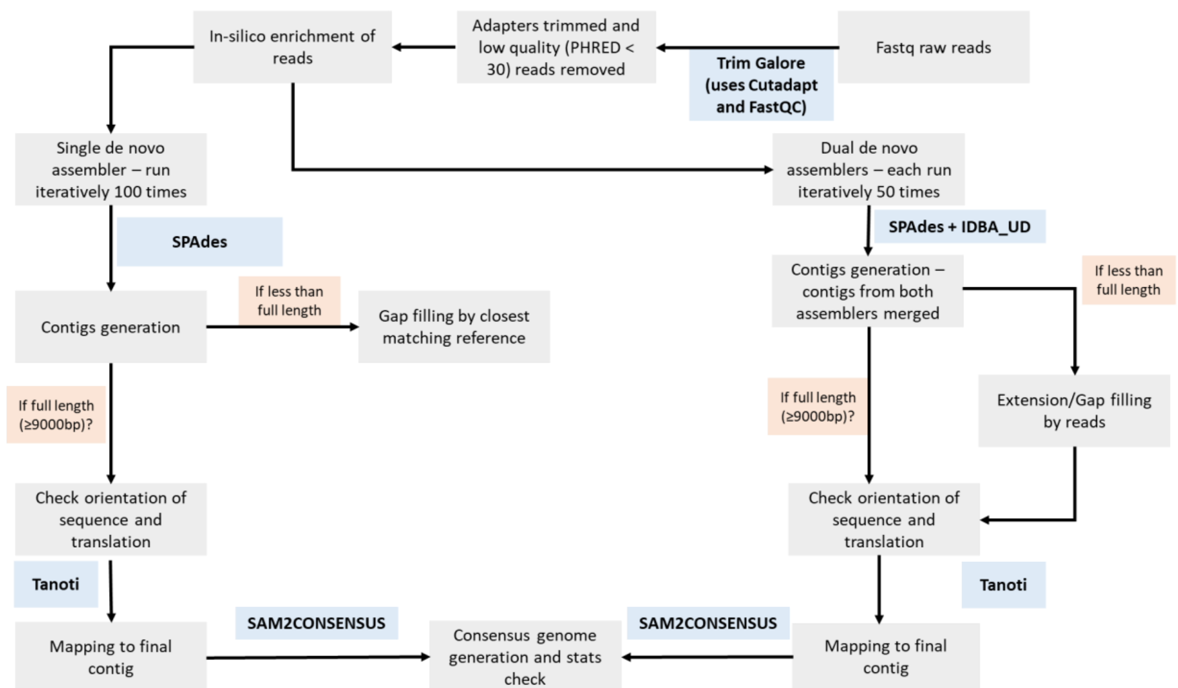


Figure 3-5: Denovo assembly workflow
Blue shaded boxes reflect the programs that were used. SPAdes [267] genome assembler version 3.11.1 was used and IDBA_UD [268] version 1.1.1 was used.

3.4.4 Cross-contamination Analysis

Genomes with a minimum of 90% coverage and a minimum of 10,000 mapped reads were taken forwards for further analysis. To ensure that assembled genomes were not influenced by cross-contamination from reads of other samples during the sequencing run, a phylogenetic tree, with 0.05 patristic distance boundary set to define clusters, was constructed from an alignment of all assembled genomes. This analysis was done using ClusterPicker version 1.3 [269]. An example of such a tree with colour coded clusters is shown in Figure. Then, within each cluster, all tips with mapped reads less than 10% of the tip with the highest number of mapped reads, were deemed as impossible to distinguish from cross-contamination and therefore discarded.

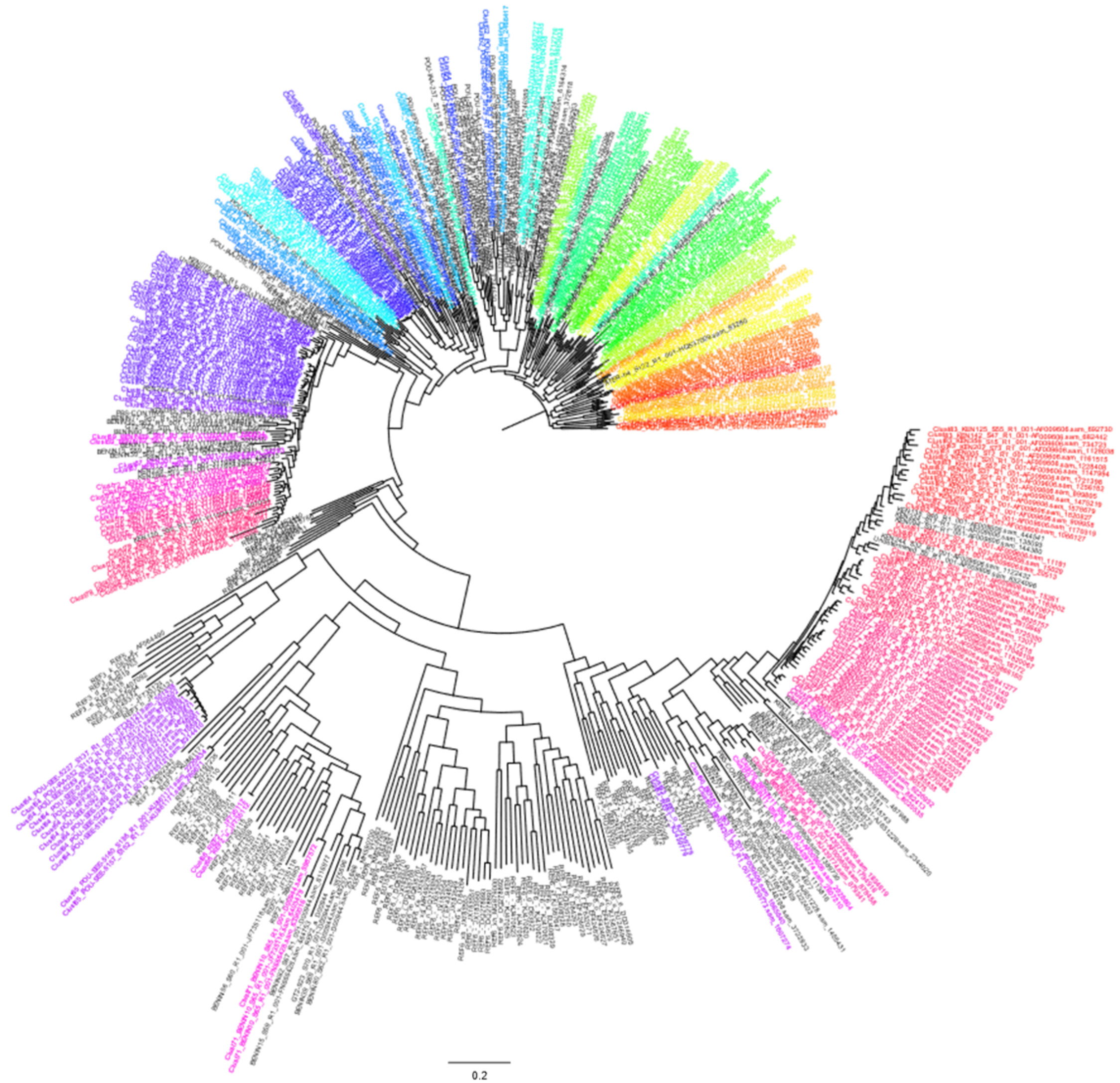


Figure 3-6: Full tree from ClusterPicker as part of cross-contamination analysis
Branches are coloured by cluster. Branches that are black are singletons, i.e. not part of any cluster.

3.4.5 Genotyping HCV

Genomes were genotyped by sequence comparison, using phylogenetics and genetic distance analysis, to known reference genotypes and subtypes, provided by ICTV (https://ictv.global/sg_wiki/flaviviridae/hepacivirus). These methods have been previously described ([6, 270]).

3.4.6 Analysis of Genetic Diversity

A combination of phylogenetics and genetic distance analysis was used to explore the diversity of whole HCV genomes. Multi sequence fasta file of consensus genomes were aligned using MAFFT version 7.313 [255]. Following manual inspection, and correction where necessary, of the alignment a test was conducted to select the most appropriate model of nucleotide sequence evolution to construct a maximum likelihood phylogenetic tree [271]. The general time reversible model was always the best model tested. Phylogenetic trees were constructed using either RAxML [272] or IQ-TREE [273], with a 1000 bootstraps. Where IQTREE was used, an ultra-fast bootstrap method was employed [274]. Genetic distance between sequences was calculated in MEGA X [275] using a bootstrap method for variance estimation and a partial deletion method to deal with gaps in the sequence data.

3.4.7 Resistance Associated Substitutions

Analysis of RASs was conducted within the HCV GLUE server using a script developed by Dr Marc Niebel. The program pulled out all the amino acids at known RAS sites within the NS3, NS5A and NS5B genes. This included looking at the deep sequencing data, including RASs that were present in at least 15% of the reads at the relevant site.

3.4.8 PWID Network Analysis

Phyloscanner [260] was used to analyse Kenyan PWID HCV sequence data to determine the dynamics of PWID networks. Details and methodology are provided in section 4.1.2.2.

3.4.9 Molecular Clock Analysis

After whole genome assembly of Kenyan HCV genomes, whole ORF sequences were aligned in frame at the nucleotide level using the mafft --auto setting and manually curated using Geneious Prime® 2023.2.1 (http://www.geneious.com/). A full genome maximum likelihood tree was created using IQ-TREE multicore version 1.6.12. Subclusters containing Kenyan sequences and near reference sequences of genotype 1a and genotype 4a were identified for further molecular

clock analysis. Root to tip analysis indicated a clock-like pattern. Separate subcluster alignments were generated by gene. For the E2 gene alignment, the E2 HVR region was removed. Alignments were uploaded to beauti v1.10.4 using the default settings to generate an xml BEAST file other than those listed here: (Partitions: unlinked substitution models, linked clock model and partition trees; Tips: year of collection was added from the year of collection or the year of submission to GenBank if year of collection unavailable; Sites: the HKY model with gamma site heterogeneity model was selected and partitioned into 2 partitions (1+2,3), following a method adapted from *Gray et al* [276]. Clocks: several clock models were tested including Strict, Relaxed Gamma, Exponential and Lognormal methods. The clock rate prior was selected at 0.001 (based on previously published rates [276]) with a normal distribution. An initial MCMC run of 10 million MCMCs was selected and adjusted as necessary before a rerun to achieve convergence (this varied between 500-1000 million MCMCs). A stepping stone marginal likelihood estimate was carried out for each run, in order to evaluate each model by Bayes Factor. The resulting xml script was run on a Linux server using beast version v1.8.2. Log files were examined using tracer v1.7.1 for convergence and to select an optimal burn in setting. ESS scores of >200 were obtained for all parameters on all final runs. The most representative tree was selected using treeannotator software v1.8.2. Trees were visualised in Figtree v1.4.3 and annotated using Adobe Illustrator 2024 software.

3.5 In-vitro Assay

3.5.1 Sub-genomic Replicon Constructs

pJFH-1 is a plasmid based on the pSGR-JFH1 (accession number AB114136.1), but the reporter gene has been changed to Gaussia Luciferase. The plasmid contains HCV non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B. The sequences of these non-structural proteins relate to HCV JFH-1, HCV sub-genotype 2a, which was isolated from a Japanese patient who died of fulminant hepatitis [277]. A map of the plasmid is shown in Figure 3-7.

pJFH-1/GND has a self-inactivating mutation in the NS5B gene and was thus used as a non-replicating control.

Sub-genomic replicon constructs for the Glasgow PWID genotype 2a study were manufactured by ThermoFisher Scientific.

Sub-genomic replicon constructs for the Benin *in-vitro* project were manufactured by CODEX DNA.

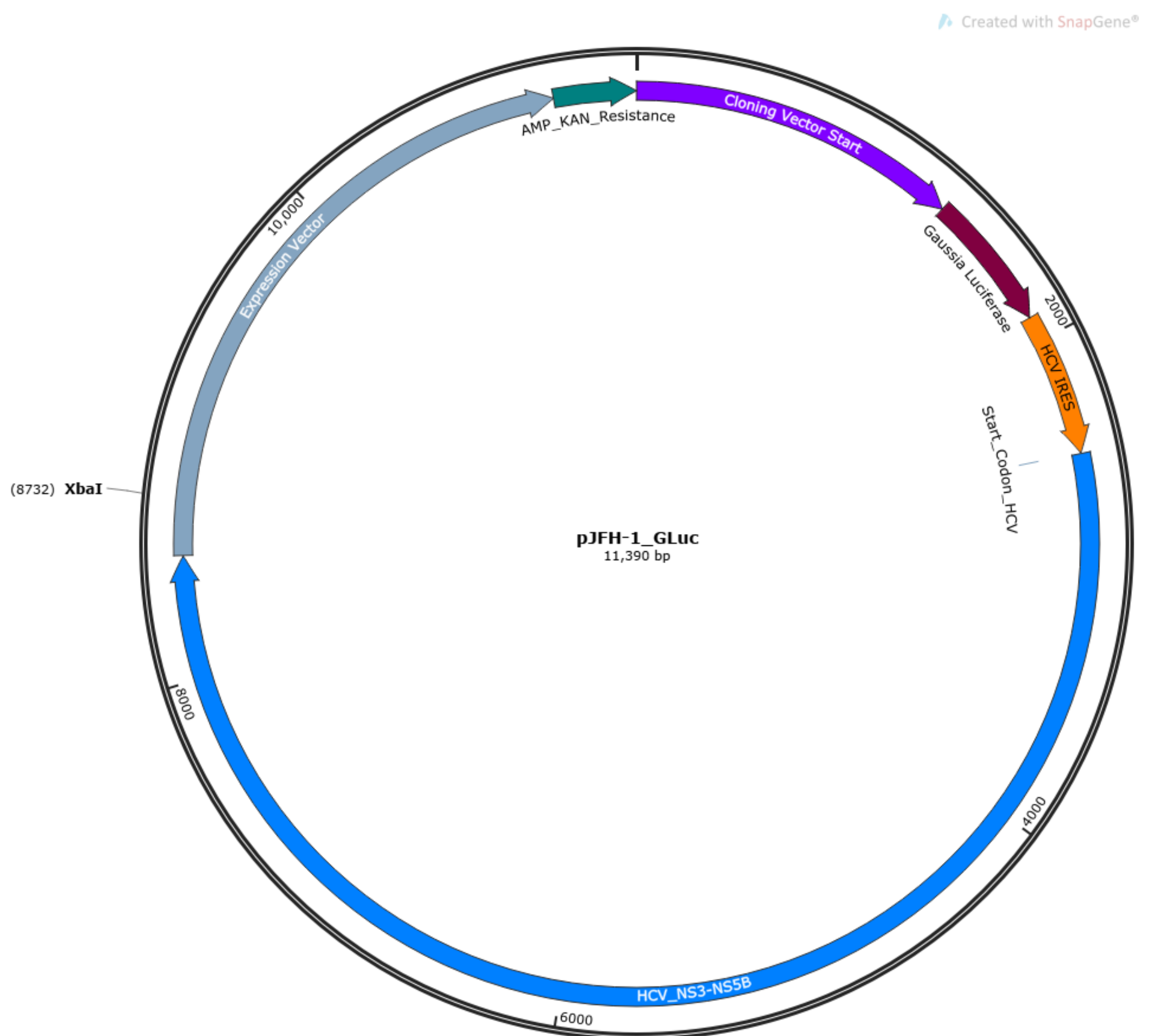


Figure 3-7: plasmid map of pJFH-1_Gluc

This map highlights the positions of the reporter gene, Gaussia Luciferase, HCV IRES and HCV non-structural proteins. Also highlighted is the XbaI restriction site, which is utilised to linearise the plasmid.

3.5.2 Drug Cell Toxicity

Before conducting the assay, it was important to check that the drug compounds themselves were not toxic to Huh7 cells. A 96-well plate was seeded with Huh7 cells at a density of 4×10^3 cells per well. The plate was incubated at 37°C for 4 hours followed by removal of media and addition of drug compounds in 10-fold dilutions starting with a concentration of 100µM. The highest drug concentration contained at least 10% DMSO. Each column of the plate consisted of a different drug compound and the last column was left untreated. A plate plan, and the various drug compounds tested, is shown in Figure 3-8. After addition of drugs, the plate was incubated at for 37°C 72 hours. The plate was then removed and carefully submerged in a trough with 8% formaldehyde, ensuring all media was removed and wells were coated with formaldehyde, for 40 minutes, to fix cells to the plate. This was followed by washing in 1% PBS and then a quick dry. Wells were then filled with 100µl of Coomassie brilliant blue 0.1% solution (0.1g of Coomassie brilliant blue R powder in 100mls of water, 50% methanol and 10% acetic acid), and the plate was placed on a shaker for 30 minutes. Following a final wash in PBS, to remove excess Coomassie brilliant blue stain, the plate was dried overnight before imaging on Celigo. Image acquisition and analysis settings are shown in Figure 3-9. Total light intensity readings were inverted, as a measure of cell viability, and plotted against different drug concentrations. The results are summarised in Figure 3-10. At concentrations of 1×10^5 nM (100µM) most cells showed a slight reduction in viability, however, this was also seen in the cells that were untreated with drugs, and therefore likely reflects toxicity from 10% DMSO. As the drug solutions (and media in untreated wells) were diluted down the column of wells, the toxic effect of DMSO was removed. Most cells were unaffected by drug compound toxicity, even at concentrations of 1000nM. The highest concentration of a drug compound used in the *in-vitro* assays was 100nM and so any effect on replication of SGRs was not due to drug toxicity to Huh7 cells.

	Ombitasvir	Ledipasvir	Daclatasvir	Pibrentasvir	Velpatasvir	Dasabuvir	Grazoprevir	Paritaprevir	Asunaprevir	Simprevir	Glecaprevir	Untreated
100µM												
10µM												
1µM												
100nM												
10nM												
1nM												
100pM												
10pM												

Figure 3-8: Plate plan for drug toxicity testing

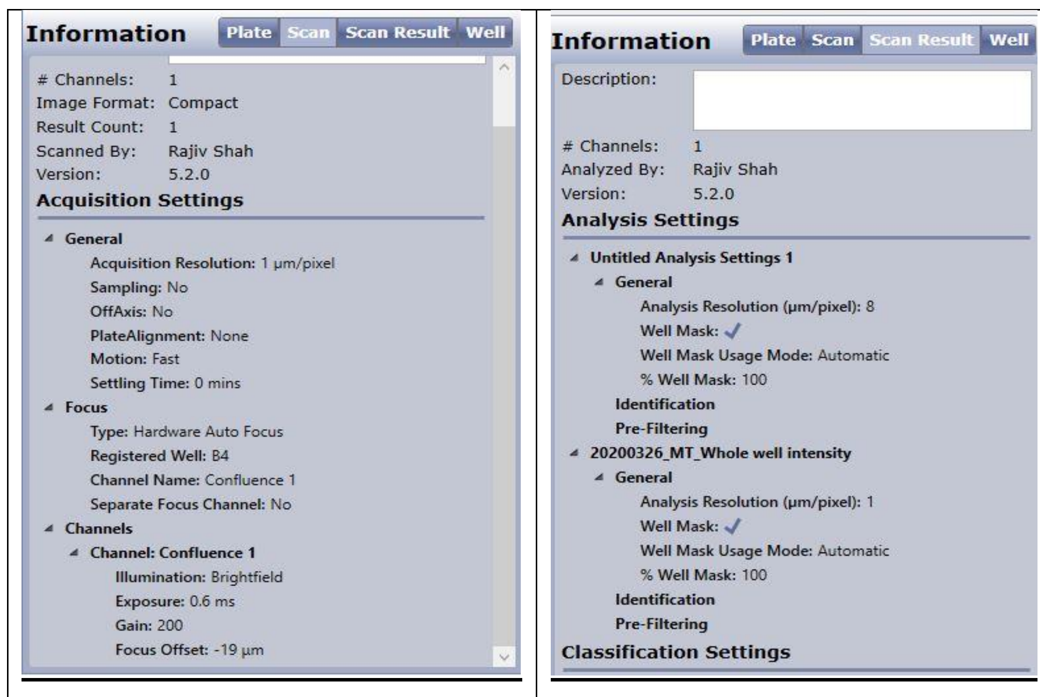


Figure 3-9: Celigo image settings

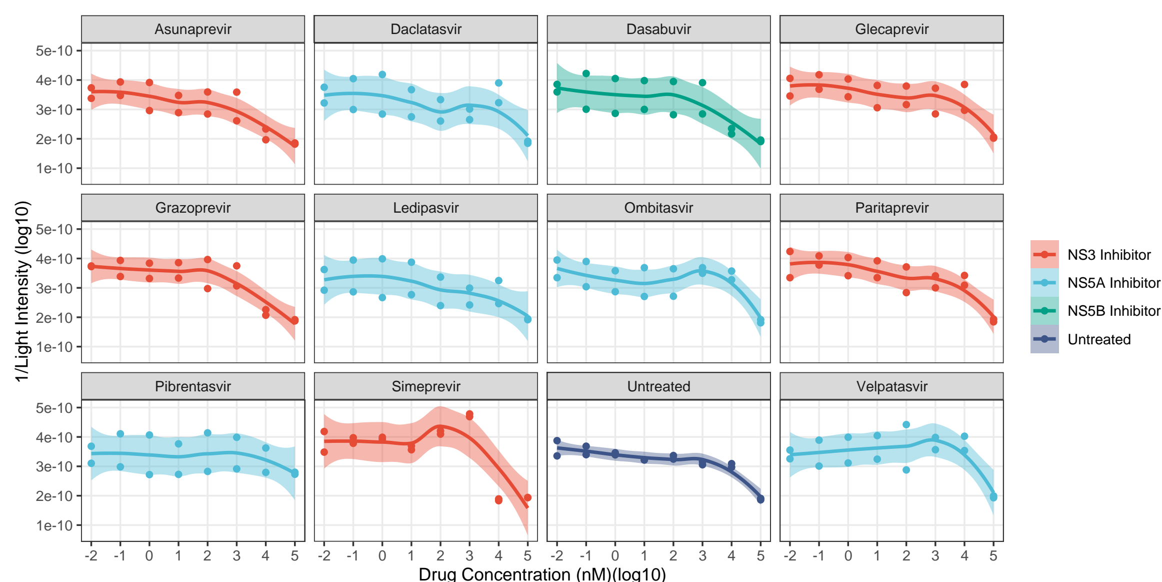


Figure 3-10: Cell viability at increasing drug concentrations.
Plots are shown for each drug compound tested and coloured by drug class.

3.5.3 RNA Transcription

SGR plasmids were linearised using FastDigest XBAI (ThermoFisher Scientific, FD0684), purified (Monarch DNA Gel Extraction Kit, New England Biolabs, T1020S) and then transcribed to RNA (T7 RiboMAX Express Large Scale RNA Production System, Promega, P1320). RNA was quantified on NanoDrop™ One (ThermoFisher Scientific) and then placed on ice.

3.5.4 Preparing Huh7 Cells for Electroporation

Huh7 cells were detached from cell storage flasks, to create a stock cell suspension, and counted using TC20 Automated Cell Counter (Bio-Rad). A final cell seeding density of 2 x 10^5 cells/ml was required. The appropriate volume of cells, from the stock cell suspension, was placed in a 15ml falcon tube, spun down at 1000G for 5 minutes, resuspended in PBS, spun down again at 1000G for 5 minutes and then resuspended in 400µl of PBS. This cell solution was then transferred to pre-chilled 4mm cuvettes (Molecular BioProducts, ThermoFisher Scientific, 5540-11PK), and placed on ice. 5µg of RNA was then added to the cuvette, and gently flicked to ensure incorporation of RNA into the cell solution, prior to electroporation.

3.5.5 Electroporation

Electroporation was carried out on the Gene Pulser XCell Electroporation System (Bio-Rad), using the following settings: 270V, 950 capacitance, ∞ resistance. Cuvettes were placed on ice immediately after electroporation to minimise cell death. The electroporated cells were then resuspended in pre-warmed media and transferred to a reservoir, from which a multichannel pipette was used to seed a 96-well plate with 100µl of cell solution thus ensuring a cell seeding density of 2 x 10^4 cells per well. Each SGR sample was run in triplicate and pJFH-1_Gluc and pJFH-1/GND were run with each experiment. An example of a plate plan is shown in Figure 3-11. Plates were incubated at 37°C for 4 hours, after which supernatant was removed and stored at -20°C, and drug compounds were added to cells in pre-specified dilutions. The final row of cells in the plate was left untreated. The plate was finally incubated at 37°C for 72 hours prior to harvesting supernatant for bioluminescence reading.

		SGR			SGR			pJFH-1_Gluc			pJFH-1_GND		
Drug Concentration (nM)	10000.00000												
	1000.00000												
	100.00000												
	10.00000												
	1.00000												
	0.10000												
	0.01000												
	0.000												

Figure 3-11: Example of plate plan for *in-vitro* assay

3.5.6 Luciferase Reporter Assay

A working solution of coelenterazine (100x) and gaussia luciferase flash assay buffer Pierce™ Gaussia Luciferase Flash Assay Kit (ThermoFisher Scientific, 16159) was made at a ratio of 1:100. 50µl of this solution was added to each well of an opaque, flat bottom 96-well plate (Greiner, 655083), followed by addition of 20µl of supernatant from electroporated cells. The plate was placed on a shaker for 10 minutes before being read in a luminescence plate reader (Chameleon II plate reader, Hidex).

3.5.7 Cell Viability following Electroporation

Electroporation is an effective way to get RNA into cells, however, it leads to a high rate of cell death. Thus, it was important to determine the optimal cell seeding density. This was assessed using a cell viability assay (WST-1, Merck, 5015944001). A working solution of WST-1 reagent and media was made up at a ratio of 1:10. Once the supernatant was harvested from the plate of electroporated cells after 72 hours incubation, 100µl of WST-1 working solution was added to each well and the plate was incubated at 37°C for 4 hours, followed by an absorbance read at a wavelength of 450nm (PHERAstar FS microplate reader, BMG Labtech).

3.5.8 Optimisation of Assay

The assay was optimised to determine:

1. The most appropriate quantity of RNA to electroporate into cells.
2. The most appropriate incubation time prior to addition of drugs.
3. The optimal cell seeding density.

As shown in Figure 3-12 the optimal RNA input quantity was approximately 5ng, and a 4-hour incubation prior to addition of drugs was adequate.

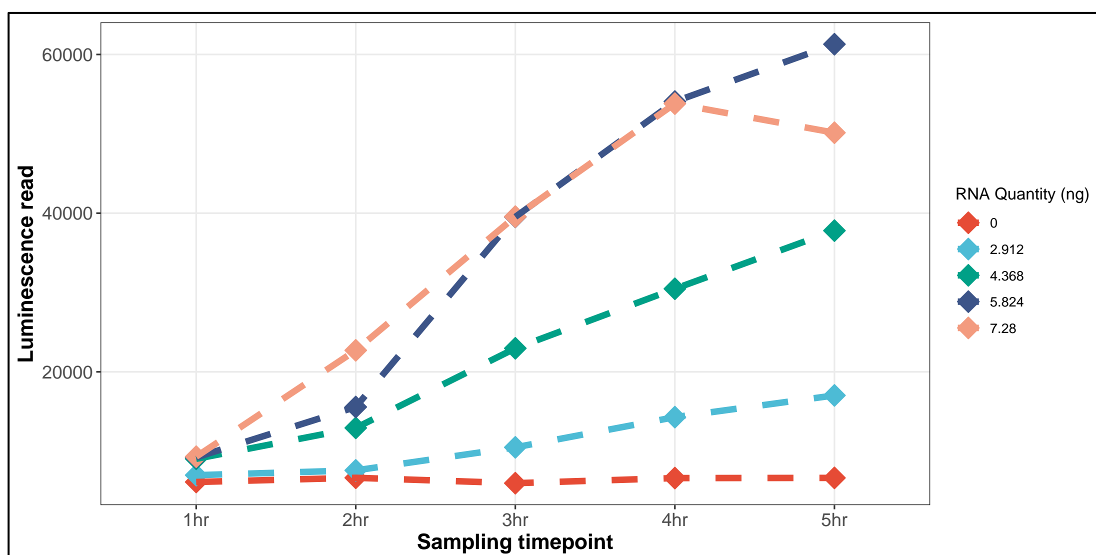


Figure 3-12: Optimal quantity of input RNA and incubation time prior to addition of drugs

A cell viability experiment, using WST-1 reagent, showed that a cell seeding density of 2 x 10^5 cells/ml was ideal (Figure 3-13).

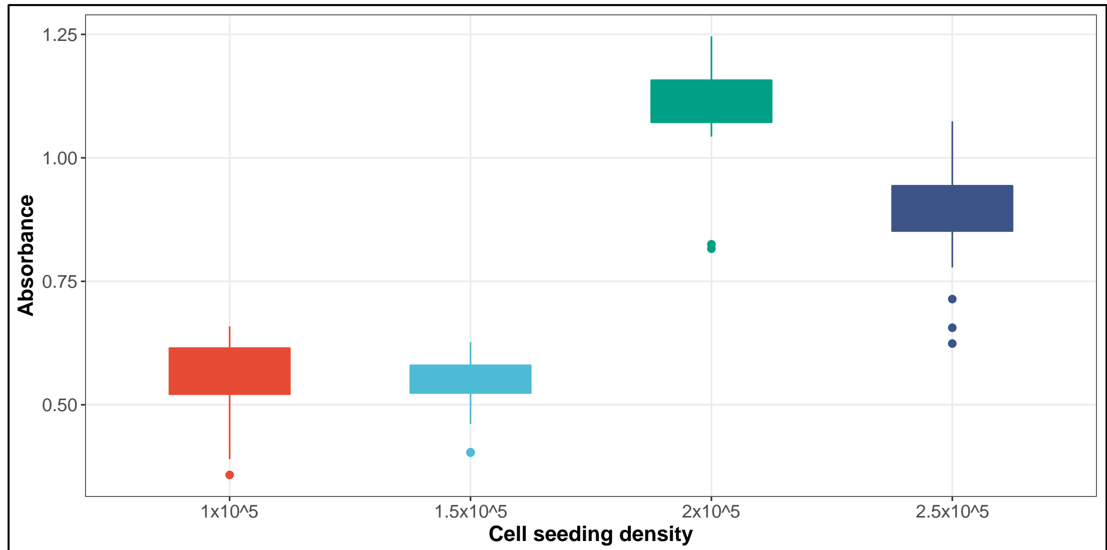


Figure 3-13: Optimal cell seeding density

3.5.9 Normalisation of Luciferase Assay Outputs

Both the 4-hour and 72-hour plates were read for luciferase activity. Relative light units (RLU) for each SGR were calculated as the 72hr reads divided by the 4hr reads.

3.5.10 Replication Capacity

Each SGR construct was assessed for replication efficiency. Supernatant was collected at 4, 24, 48 and 72 hours and growth curves were plotted. Then, their replication efficiency was compared to that of pJFH-1 using RLU readings in the following formula:

(SGR 72hr/SGR 4hr) / (pJFH-1 72hr/pJFH-1 4hr)

3.5.11 IC50 Analysis

After normalising RLU readings for each SGR, as described in section 3.5.9, the response of each SGR to drug treatment was expressed as a percentage where the maximum (100%) treatment response was set as the mean of readings in the wells where no drug was added. All RLU readings from drug treated wells for each SGR were normalised to the maximum response of the respective SGR. Resulting values were used to plot drug response curves and calculate IC50 values using non-linear regression in R (package drc) [278] or GraphPad Prism version 8.4.3.

4 Results

4.1 HCV in Kenyan PWID

This chapter describes the cohort of Kenyan PWID recruited between 2015 and 2016. Demographics and risk factors for HCV infection are described as well as the prevalence of advanced liver fibrosis. HCV diversity in this group is demonstrated as well as the interconnectedness of PWID networks. Molecular clock analysis to determine the year of introduction of HCV to Kenyan PWID is shown. Finally, the prevalence of RASs in HCV in this group is described.

4.1.1 HCV and Associated Liver Fibrosis in Kenyan PWID

Out of 400 recruited PWID, 36% (143/400) were HCV seropositive, of whom 62% (89/143) were viraemic. The median viral load (from dried blood spot samples) was 3.68 IU/ml (log converted). The prevalence of HIV-HCV co-infection was 34% (49/143) and HIV mono-infection was 7% (19/257). 76% (37/49) of co-infected individuals were HCV RNA positive. 3 HIV positive individuals who had tested negative for anti-HCV antibody, had complete HCV genomes identified through sequencing.

4.1.1.1 Demographic and Clinical Features

400 PWID were recruited into the study. 200 were from either of the two sites based in Mombasa, and 200 were from Watamu. The first phase of recruitment took place between 16/12/2015 and 22/01/2016. The second phase of recruitment took place between 06/06/2016 and 21/06/2016. Sociodemographic and clinical features of the group have been previously described and published [279], and are summarised in Table 4-1.

Most study participants were male (85%) and the median age of the whole group was 33 (range 19-56). 185 participants (48%) had achieved, at least, a secondary level of education and 247 participants (62%) reported having some form of work resulting in a relatively stable form of income. In this group 378 (95%) participants reported having being incarcerated at some point in their lifetime,

related to the possession of narcotic substances. All participants reported heroin as their injecting drug of choice. 130 (32%) reported needle sharing and 254 (64%) reported injecting in groups that would have new members each month. Most participants (93%, 372/398) reported a history of multiple sexual partners. Receipt of medicine via injection (84%) and a history of invasive procedures (47%) was also commonly reported in this group. Only 24 (6%) participants reported receiving a blood transfusion.

Table 4-1 Demographic and clinical features of Kenyan PWID

		Total	
		N or median	% or IQR
Sociodemographic Characteristics (N=400)			
		median	IQR
Age		33	28-39
		N	%
Sex	Male	340	85
	Female	60	15
Education*(N=386)	Primary school	201	52
	Secondary school or higher	185	48
Marital status	Married	95	24
	Single	305	76
Employed	Yes	247	62
	No	153	38
Jailed or imprisoned	Yes	378	95
	No	22	5
Number of sexual partners* (N=398)	1	26	7
	2-5	117	29
	6-10	81	20
	>10	174	44
Behaviours (N=400)			
Ever shared a needle	No	270	68
	Yes	130	32
Drug use network	Injections alone or in stable group	146	36
	Injections in groups with new members each month	254	64
Ever had a tattoo or piercing	No	313	78
	Yes	87	22
Medical History (N=400)			
HIV status	Negative	332	83
	Positive	68	17
Surgery	No	212	53
	Yes	188	47
Herbal medication	No	349	87
	Yes	51	13
Methadone	No	265	66
	Yes	135	34
Injected medication	No	66	16
	Yes	334	84
Blood transfusion	No	376	94
	Yes	24	6
Liver Biomarkers (N=399)			
		median	IQR
Haemoglobin (g/dL)		13.1	12.2-14.2

	Total	
	N or median	% or IQR
Platelet (x10^9/L)	213	171-254
Bilirubin (μmol/L)	8	6-10
Alkaline Phosphatase (IU)	73	59-90
Alanine Transaminase (IU)	20	15-32
Aspartate Transaminase (IU)	28	22-37
APRI Score	0.33	0.23-0.49
FIB-4 Score	0.98	0.75-1.38

4.1.1.2 Risk Factors for HCV Infection

Risk factors for HCV infection in this group of Kenyan PWID are summarised in Table 4-2. Needle sharing (adjusted odds 2.09, 95% CI 1.29-3.39, p 0.003) and injecting with multiple new members in a group (adjusted odds 2.17, 95% CI 1.31-3.59, p 0.003) were significantly associated with HCV seropositivity. The odds of being HCV seropositive were also higher among those who had achieved, at least, a secondary level of education (adjusted odds 2.94, 95% CI 1.84-4.70, p < 0.0001) and those that were single (adjusted odds 1.85, 95% CI 1.04-3.28, p 0.04).

In this group there was no significant association between tattooing or piercing, receipt of medical injections, blood transfusion, surgical procedures and number of sexual partners (data not shown in table) and HCV infection.

Table 4-2: Risk factors associated with HCV infection

		Total (N=400)	HCV Seropositive (N=143)	Crude OR (N=400)			Adjusted OR (N=386*)		
		N or median	N (%) or median (IQR)	OR	95% CI	p-value	OR	95% CI	p-value
Age (in years)		33	35(30-40)	1.05	1.02-1.08	0.001	1.05	1.02-1.09	0.003
Sex	Male	340	122(35.9)	REF		0.90	REF		0.62
	Female	60	21(35.0)	0.96	0.54-1.71		0.84	0.42-1.67	
Education*(N=386)	Primary school	201.0	50(24.9)	REF		<0.0001	REF		<0.0001
	Secondary school or higher	185	86(46.5)	2.62	1.70-4.04		2.94	1.84-4.70	
Marital status	Married	95	26(27.4)	REF		0.048	REF		0.04
	Single	305	117(38.4)	1.65	1.00-2.74		1.85	1.04-3.28	
Ever shared a needle	No	270	77(28.5)	REF		<0.0001	REF		0.003
	Yes	130	66(50.8)	2.58	1.68-3.99		2.09	1.29-3.39	
Drug user network	Injections alone or in stable group	146	35(24.0)	REF		0.0002	REF		0.003
	Injections in group with new members each month	254	108(42.5)	2.35	1.49-3.69		2.17	1.31-3.59	
Ever had a tattoo or piercing	No	215	49(22.8)	REF		0.32	_		
	Yes	184	42(22.8)	0.81	0.54-1.22				
Surgery	No	212	71(33.5)	REF		0.32	_		
	Yes	188	72(38.3)	1.23	0.82-1.86				

Injected medication	No	66	29(43.9)	REF		0.13	_
	Yes	334	114(34.1)	0.66	0.39-1.13		
Blood transfusion	No	376	131(34.8)	REF		0.14	_
	Yes	24	12(50.0)	1.87	0.82-4.28		

4.1.1.3 Liver Fibrosis Associated with HCV Infection

Liver fibrosis was defined as an APRI score of >1.5 or a FIB-4 score of >3.25 . A higher cut-off was used to identify individuals with advanced fibrosis. The total prevalence of advanced liver fibrosis was 17% (67/399), but among those who were HCV seropositive the prevalence of advanced liver fibrosis was 34% (48/143). Among those with chronic HCV infection, i.e., HCV RNA positive, the prevalence of advanced liver fibrosis was 46% (41/89). Prevalence of advanced liver fibrosis was significantly higher among those who were viraemic with HCV (OR 10.65, 95% CI 5.70-19.93, p <0.0001). These findings are summarised in Table 4-3.

Among HCV seronegative and HCV seropositive, but RNA negative individuals, there was no significant association between the presence of advanced liver fibrosis and other risk factors such as alcohol consumption, use of miraa (local name for khat), herbal medication, HIV co-infection or diabetes.

Table 4-3: Comparing prevalence of advanced liver fibrosis between HCV infected and uninfected PWID

Group	Total (N=399)	Liver fibrosis (N=67)	Crude OR		
	N	%	OR	95%CI	p-value
Never infected by HCV	256	19(7.4)	REF		
HCV infection cleared	54	7(13.0)	1.86	0.74-4.67	
Chronic HCV	89	41(46.1)	10.65	5.70-19.93	<0.0001

There was good concordance between the use of APRI and FIB-4 scores to identify those with advanced liver fibrosis (Table 4-4).

Table 4-4: Concordance between APRI and FIB-4 scores

		APRI score		Total
		No fibrosis	Fibrosis	
FIB-4 score	No fibrosis	375	8	383
	Fibrosis	5	11	16
Total		380	19	399

4.1.2 Kenyan HCV samples

From 89 HCV RNA positive samples, 65 were available for sequencing and 49 whole genomes were assembled following post assembly clean up. This is summarised in Figure 4-1. Kenya HCV genomes were all constructed by reference-based assembly.

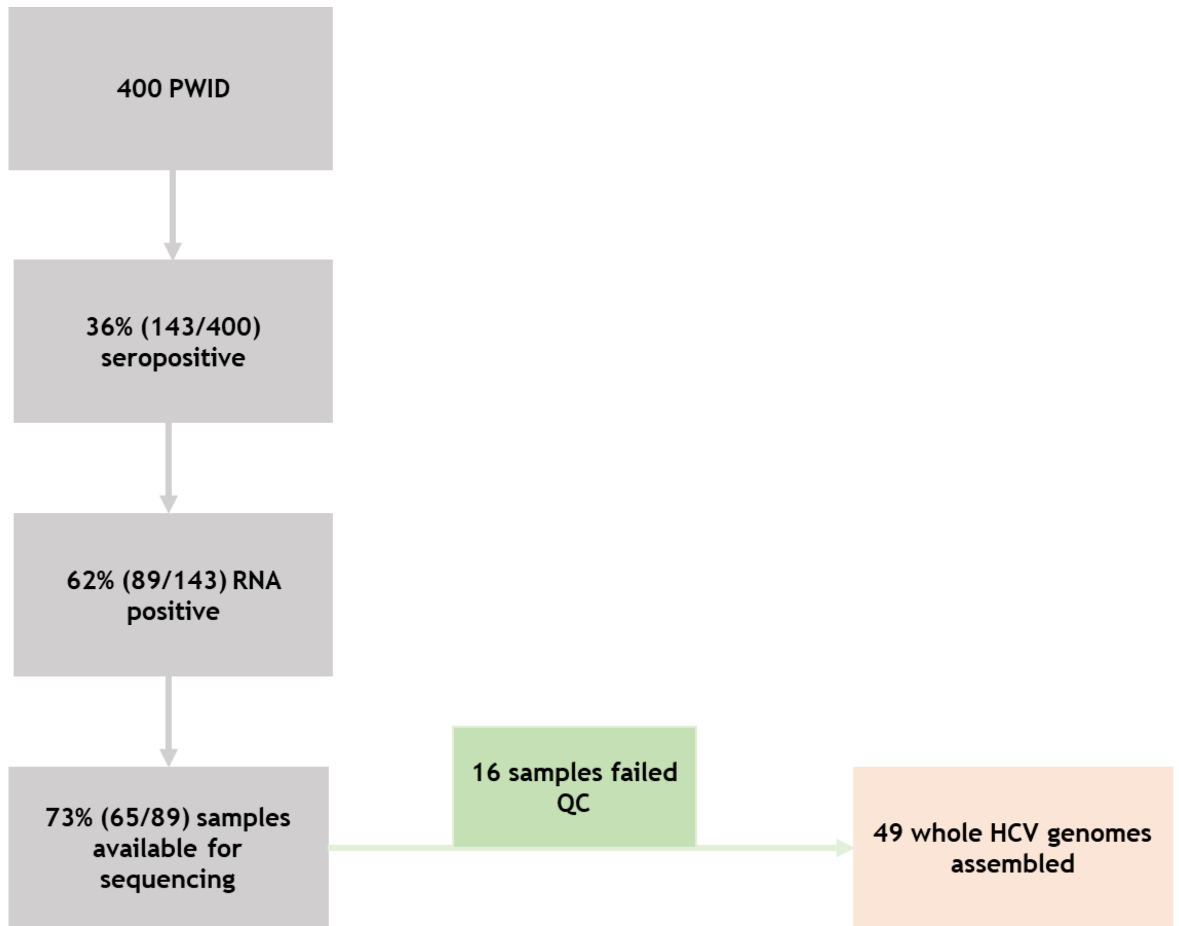


Figure 4-1: Summary of whole Kenyan HCV genomes assembled

There were 5 samples that were discarded due to probable cross-contamination (3.4.4). Details can be found in Figure 4-2

Figure 4-2: Zoom in of ClusterPicker tree cluster 79
Samples that were discarded due to possible cross-contamination are highlighted in a red dashed box. Each sample name is appended with the number of mapped reads. For example, samples KEN001, KEN123, KEN145 and KEN287 were all part of cluster 79 from the ClusterPicker output, and all these samples had mapped reads that were less than 10% of the sample with the highest mapped reads (KEN017) in the same cluster.

4.1.2.1 Resolving Mixed Infections

To look for evidence of mixed infections in individual patient samples, a reference-based assembly pipeline was used to map the raw reads of each sample to multiple different reference HCV genomes. Such an approach is highly sensitive but subject to two potential problems: (1) Mapping of sequences from conserved regions of the genome to more than one reference sequence (2) Bias from low-level cross-contamination from other samples on the same run.

Quality control (QC) analysis was used to estimate the number of mixed infections of multiple subtypes. After mapping, we found that HCV genotypes 1a and 4a were circulating in this group of Kenyan PWID. Before QC analysis, most samples initially mapped to both genotype 1a and 4a reference genomes. Details are provided in Figure 4-3.

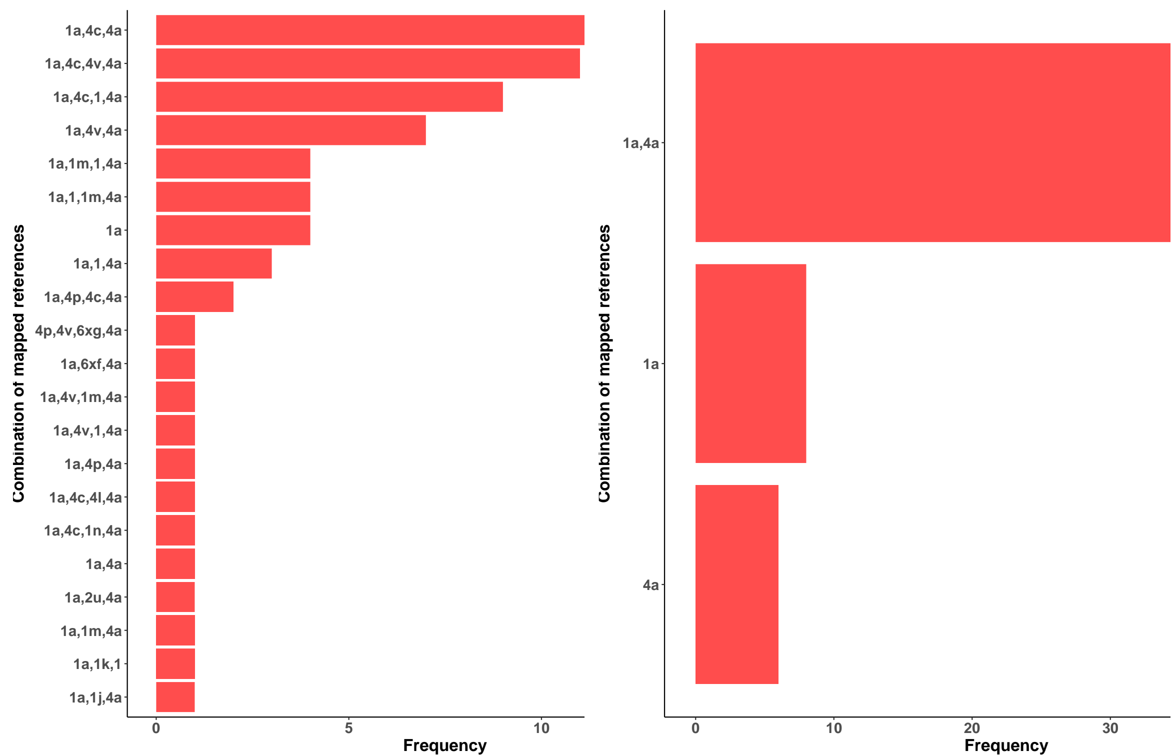


Figure 4-3: Bar chart showing frequency of Kenyan HCV sub-genotype co-infections pre and post cross-contamination analysis
The frequency of HCV sub-genotype co-infections seen in all Kenya samples prior to cross-contamination analysis (left sided panel) and post cross-contamination analysis (right sided panel).

In order to determine if these “mixed infections” were genuine, I looked at the depth of coverage across the whole genome for each sample that was mapped to both HCV 1a and 4a. Coverage statistics were calculated by a python script developed by Joseph Hughes, weeSAM v1.5 (https://github.com/centre-for-virus-research/weeSAM). Each sample was first ranked according to the number of reads mapped to either the 1a or 4a reference genome and then a metric called “Above 0.2 depth” was applied to determine which samples were likely to be genuinely mapping to the selected reference genome. This metric measures the percentage of sites that have a coverage value of the average depth multiplied by 0.2. The lower the value means the more the number of sites that have a low coverage. As shown in Figure 4-4, all samples in the second rank had an above 0.2 depth of less than 50%. There were a few samples that had both the first and second rank genomes with an above 0.2 depth value of less than 50%. Including only samples with an above 0.2 depth of greater than 50% revealed that no samples contained a clear mixed infection of HCV 1a and 4a. For example, sample KEN008 had an above 0.2 depth of 96.12% when mapped to

a 1a reference genome and 38.37% when mapped to a 4a reference genome. This is illustrated as a coverage plot in Figure 4-5 showing evidence of mapping of reads in conserved regions to both references. More examples are provided in Figure 4-6 and Figure 4-7. Sample KEN074 had an above 0.2 depth value of 94.86% when mapped to 4a, but only 37.55% when mapped to 1a. Sample KEN014 was discarded due to above 0.2 depth values of below 50% when mapped to both 1a and 4a.

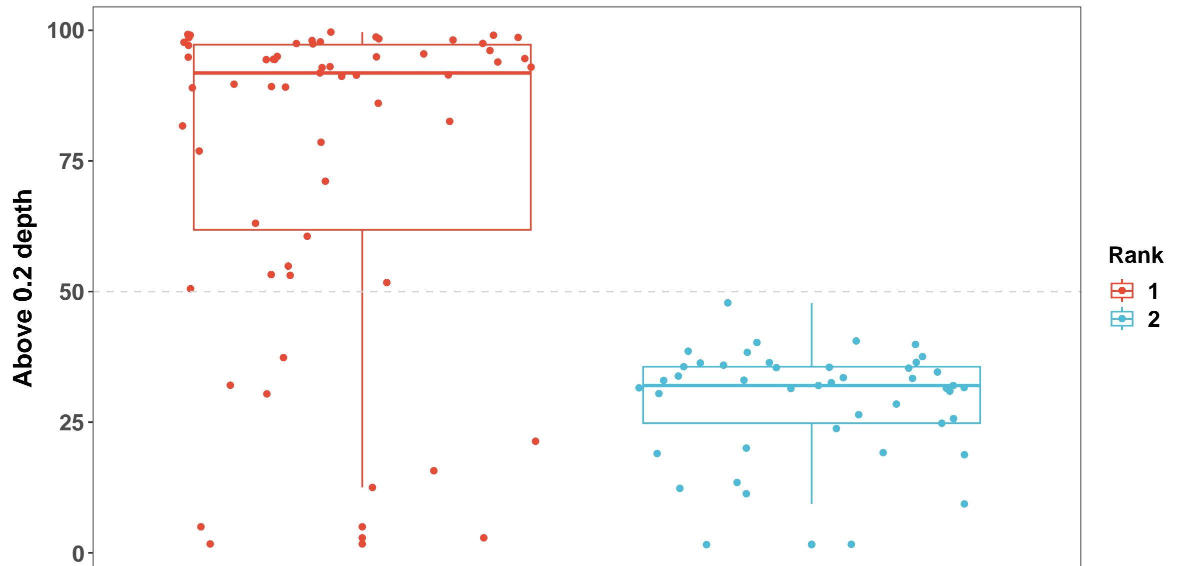


Figure 4-4: Above 0.2 depth for samples that mapped to both HCV 1a and 4a
The metric on the y-axis is expressed as a percentage.

Finally, 1 more sample was discarded as it did not translate to a full-length polyprotein (sample KEN359). In total, 49 whole Kenyan HCV genomes were assembled using a reference-based approach.

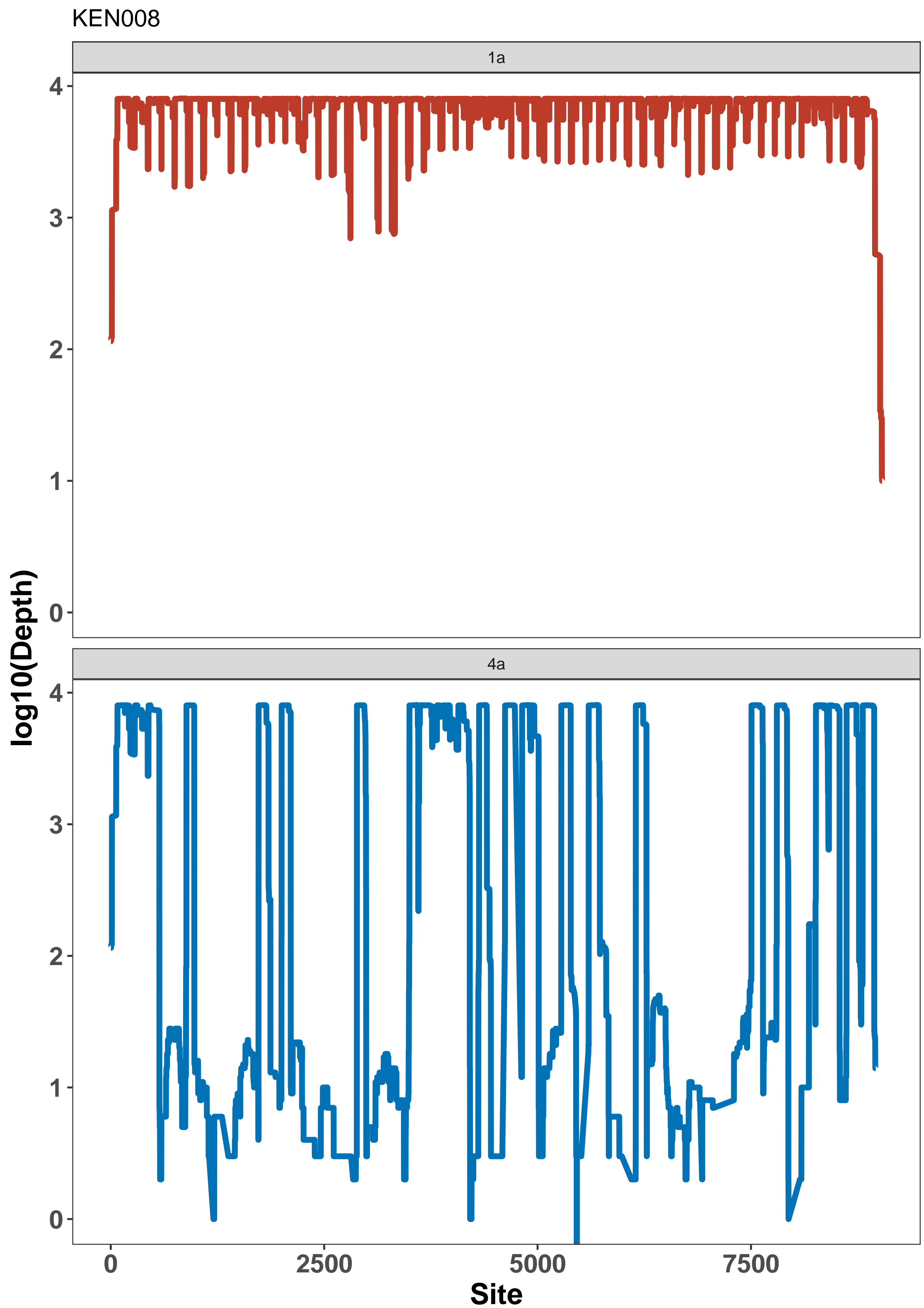


Figure 4-5: Coverage plot for sample KEN008
The accession numbers of the reference genomes used to represent HCV 1a and 4a are AF009606 [280] and Y11604 [281] respectively.

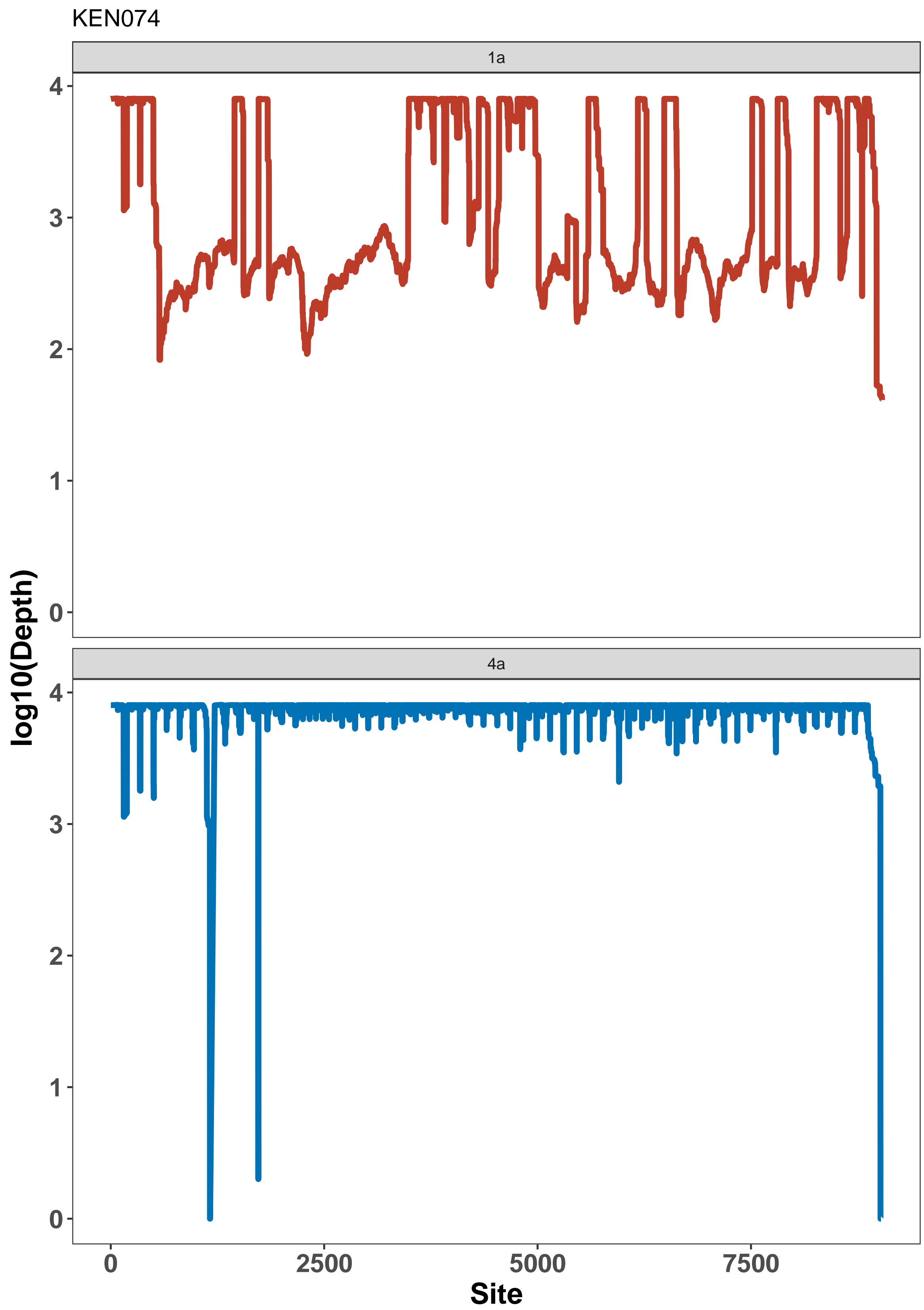


Figure 4-6: Coverage plot for sample KEN074

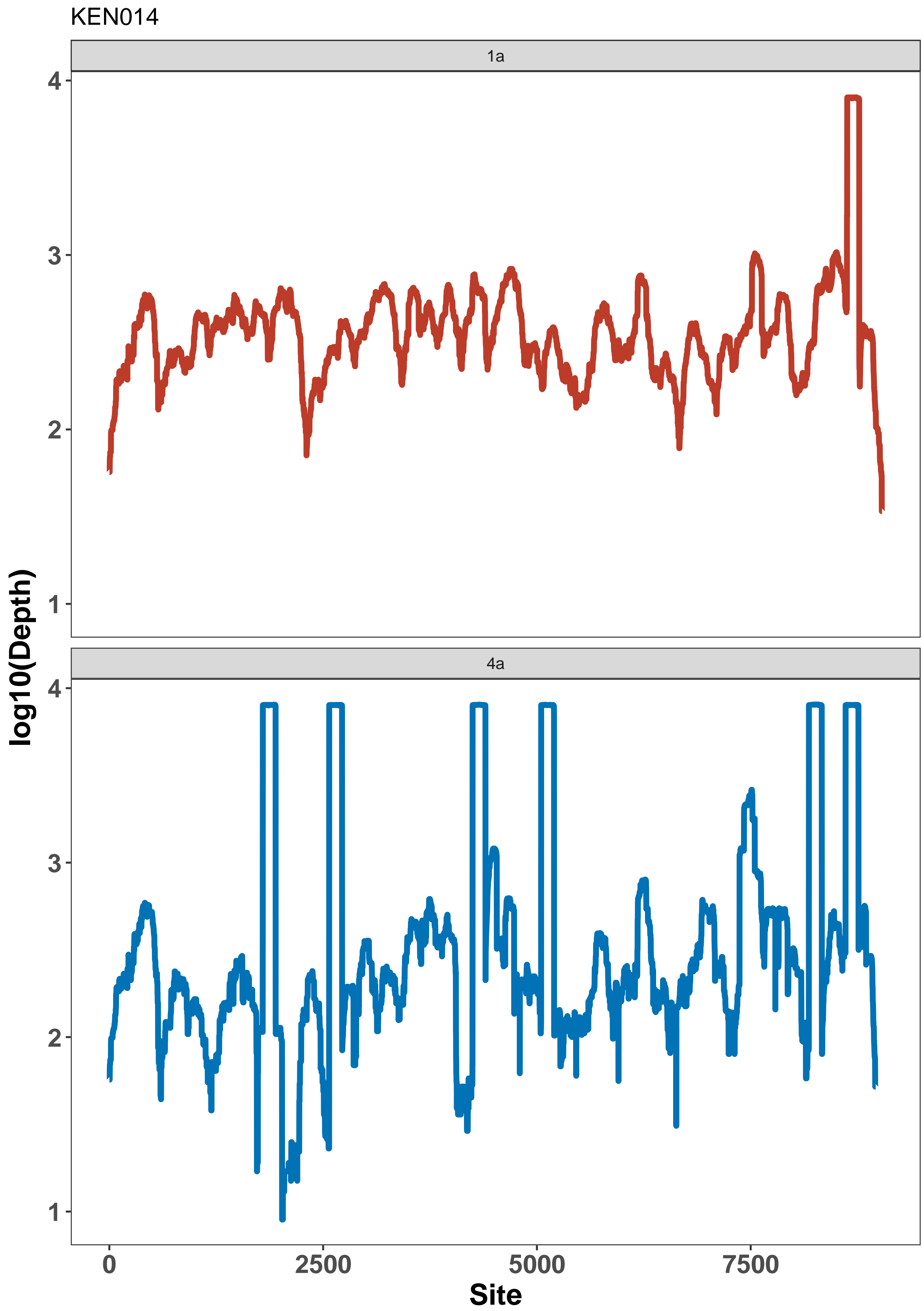


Figure 4-7: Coverage plot for sample KEN014

4.1.2.2 Network Analysis

Phyloscanner [260] was used to determine how closely related the HCV viruses in this group of Kenyan PWID are. The purpose of this analysis was to look at how many connections there were between individuals with chronic HCV infection. Data on known transmitting pairs or groups was not available hence this analysis was not used to confirm direction of transmission. A maximum read threshold of 5000 reads was set(--maxReadsPerHost 5000) to prevent the server being burdened with an over-intensive computational process. The multifurcation threshold was set to the lowest (--multifurcationThreshold 1e-8). This enabled bypassing of the in-built contamination check in Phyloscanner, as I had already checked for cross-contamination in the genomic dataset as explained above. A window threshold of 60% (-swt 0.6) was set. This is based on work by Dr Ben Talbot (publication in preparation), looking at the most appropriate window threshold in a known HCV transmitting couple. Higher window thresholds were explored, but I found that this excluded a lot of genetic data and therefore was likely to have underestimated the density of the network. Finally, a distance threshold of 0.01 (-sdt 0.01) and a minimum read and tip threshold of 1 each (-mr 1 -mt 1) were set.

Tabulated output was formatted and annotated with metadata. The results were fed into Cytoscape (version 3.10.0) to create a visualisable network figure.

4.1.3 Diversity and origin of HCV in Kenyan PWID

Following genome assembly and post assembly processing, 49 whole HCV genomes from Kenya were analysed. 55% (27/49) of the genomes were identified as sub-genotype 1a and 45% (22/49) as sub-genotype 4a (Figure 4-8).

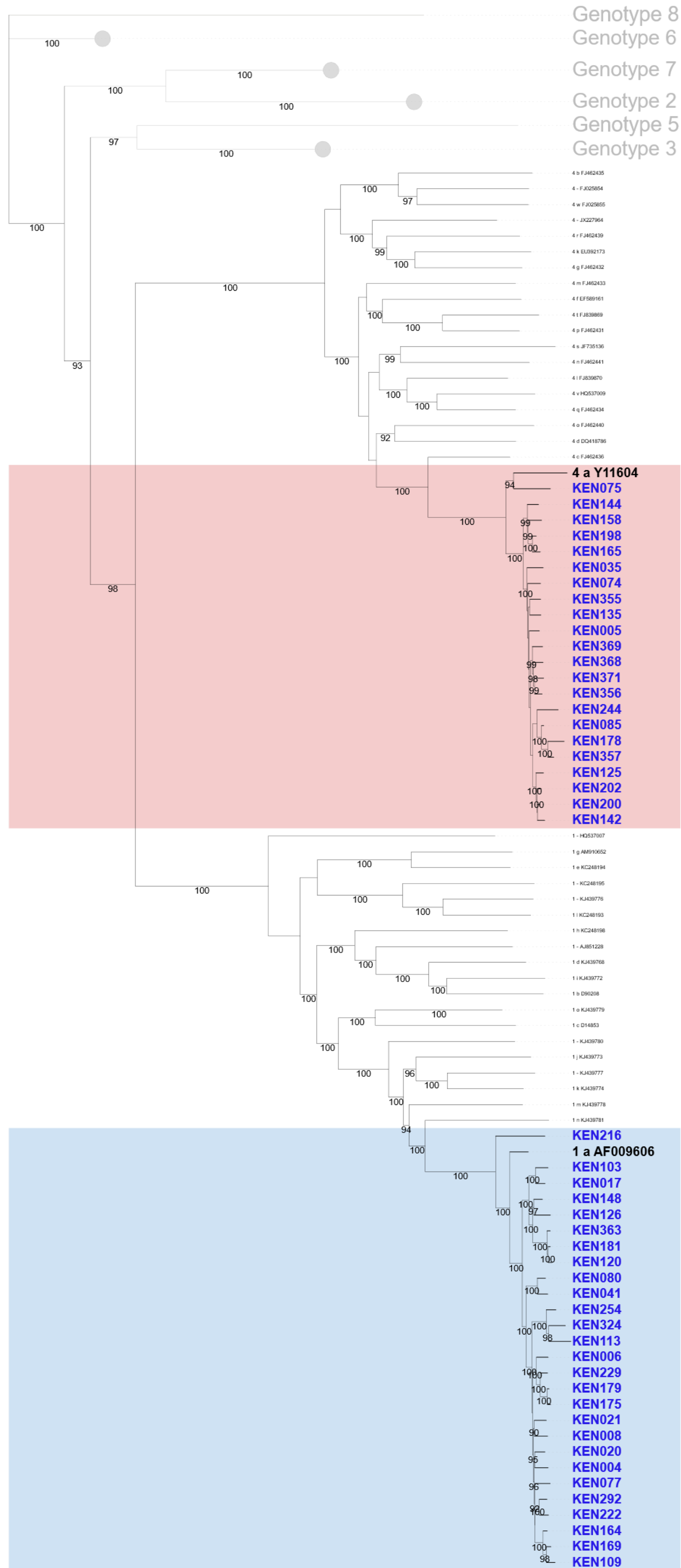

Tree scale: 0.1
Colored ranges
Subtype 4a
Subtype 1a
Genotype 8
Genotype 6
Genotype 7
Genotype 2
Genotype 5
Genotype 3
4 a Y11604
KEN075
KEN144
KEN158
KEN198
KEN165
KEN035
KEN074
KEN355
KEN135
KEN005
KEN369
KEN368
KEN371
KEN356
KEN244
KEN085
KEN178
KEN357
KEN125
KEN202
KEN200
KEN142
KEN216
1 a AF009606
KEN103
KEN017
KEN148
KEN126
KEN363
KEN181
KEN120
KEN080
KEN041
KEN254
KEN324
KEN113
KEN006
KEN229
KEN179
KEN175
KEN021
KEN008
KEN020
KEN004
KEN077
KEN292
KEN222
KEN164
KEN169
KEN109

Figure 4-8: Maximum likelihood phylogenetic tree highlighting HCV genomes from Kenya
A phylogenetic tree of whole HCV genomes including all ICTV reference genomes and genomes sequenced from Kenyan samples. Sub-genotype clades 1a and 4a are highlighted. The remaining tips, in smaller font, within genotype 1 and 4 clades represent non-1a and non-4a genomes, respectively. Clades representing all non-genotype 1 and 4 genomes are collapsed. The tree is rooted on genotype 8. Bootstrap values above 90% are shown.

4.1.3.1 Sub-genotype 1a

A maximum likelihood tree was constructed using all published sub-genotype 1a whole genomes, with the Kenyan sub-genotype 1a genomes sequenced in this study. This is shown in Figure 4-9. The 1a genomes from Kenya form two distinct clusters. The larger cluster is situated within a large cluster of 1a genomes from USA. The second cluster contains a single 1a genome from Kenya (sample KEN216) which is closely related to a 1a genome (EU862836) from an individual in Switzerland and is situated within a larger cluster of closely related 1a genomes from Europe, namely Germany, Switzerland and the United Kingdom. The phylogeny suggests at least 2 separate introductions of genotype 1a to Kenya from USA and Europe (Figure 4-10).

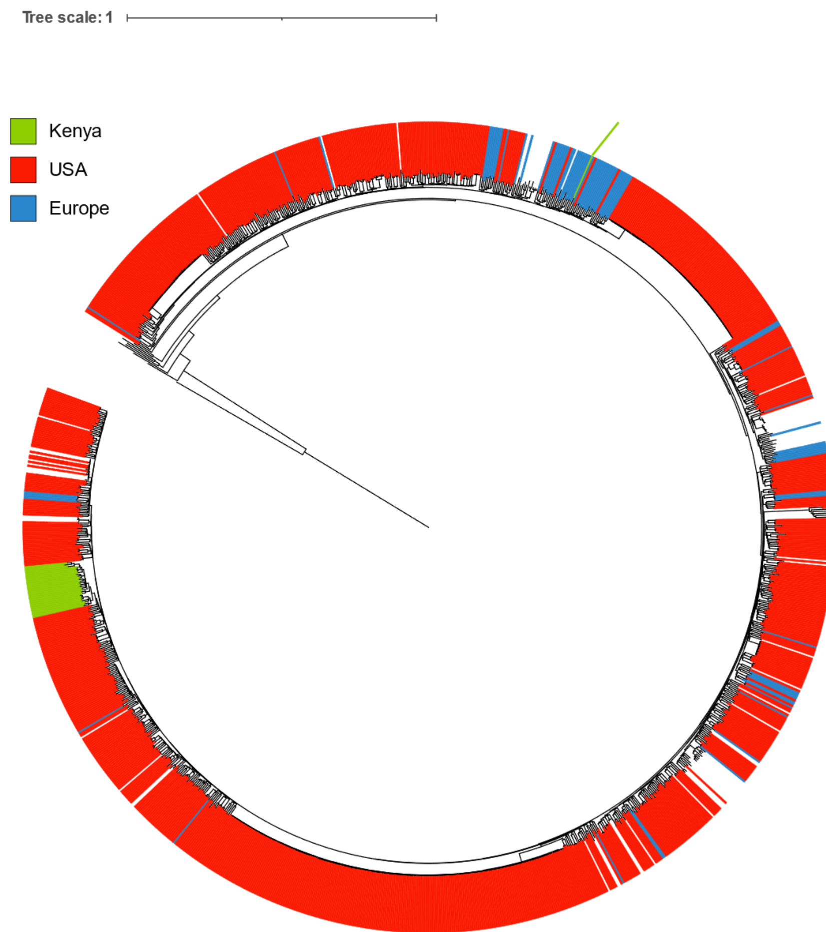
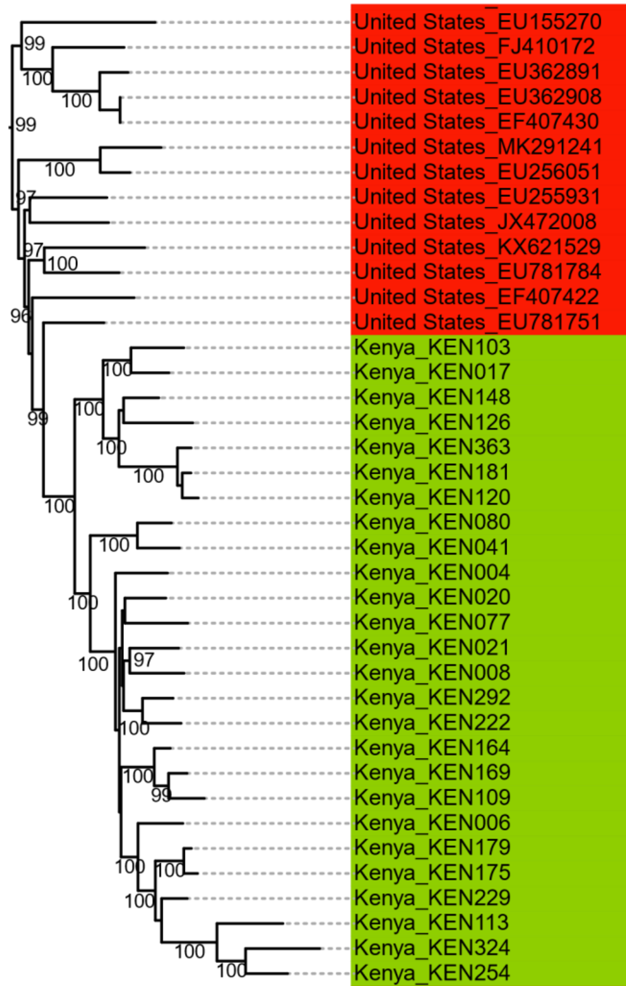


Figure 4-9: A radial phylogenetic tree of all published sub-genotype 1a whole genomes including Kenyan 1a

A maximum likelihood tree, with 1000 bootstraps, of all published sub-genotype 1a genomes as well as the Kenyan 1a genomes. Red and blue coloured branches represent genomes that originated from individuals in USA and Europe, respectively. Green coloured branches represent the Kenyan 1a genomes. An extended green branch is drawn to highlight the single Kenyan 1a genome separate to the main cluster. All published 1a genomes, and associated metadata, were downloaded from HCV GLUE (http://hcv-glue.cvr.gla.ac.uk/#/project/alignment/AL_1). The tree is rooted on the genotype 8 reference as an outgroup (MH590700).


Tree scale: 0.01
United States_EU155270
United States_FJ410172
United States_EU362891
United States_EU362908
United States_EF407430
United States_MK291241
United States_EU256051
United States_EU255931
United States_JX472008
United States_KX621529
United States_EU781784
United States_EF407422
United States_EU781751
Kenya_KEN103
Kenya_KEN017
Kenya_KEN148
Kenya_KEN126
Kenya_KEN363
Kenya_KEN181
Kenya_KEN120
Kenya_KEN080
Kenya_KEN041
Kenya_KEN004
Kenya_KEN020
Kenya_KEN077
Kenya_KEN021
Kenya_KEN008
Kenya_KEN292
Kenya_KEN222
Kenya_KEN164
Kenya_KEN169
Kenya_KEN109
Kenya_KEN006
Kenya_KEN179
Kenya_KEN175
Kenya_KEN229
Kenya_KEN113
Kenya_KEN324
Kenya_KEN254

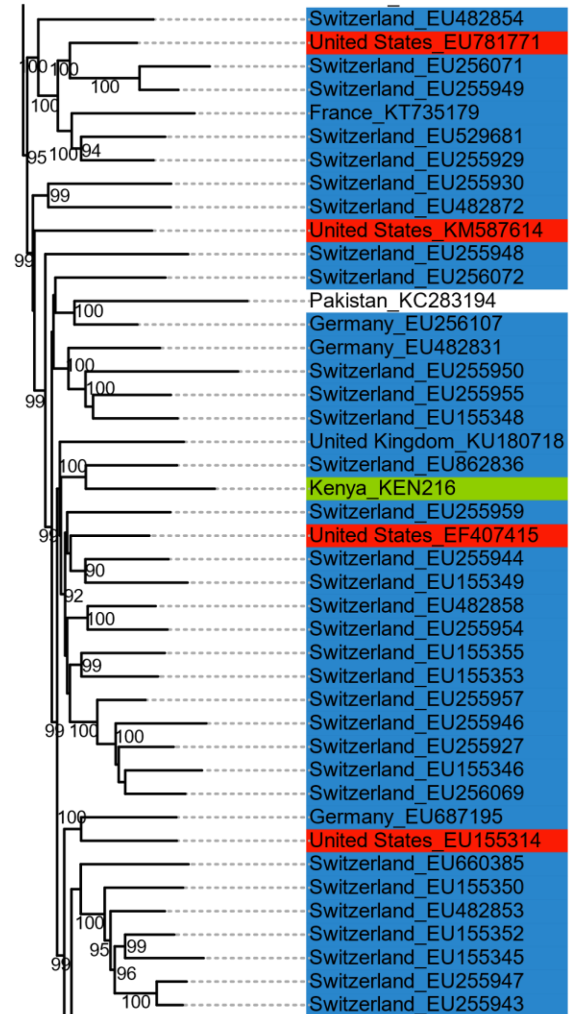
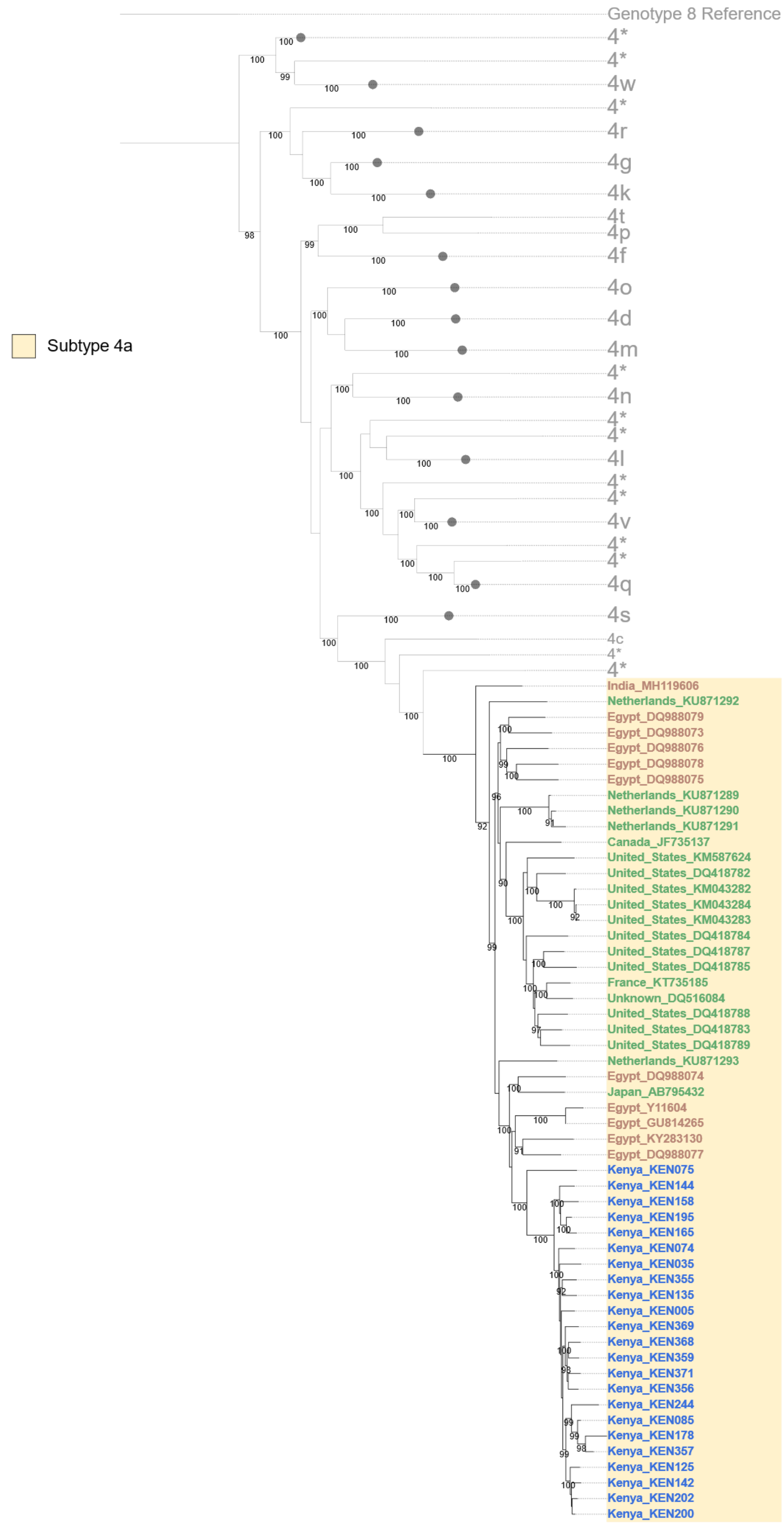

Tree scale: 0.01
Switzerland_EU482854
United States_EU781771
Switzerland_EU256071
Switzerland_EU255949
France_KT735179
Switzerland_EU529681
Switzerland_EU255929
Switzerland_EU255930
Switzerland_EU482872
United States_KM587614
Switzerland_EU255948
Switzerland_EU256072
Pakistan_KC283194
Germany_EU256107
Germany_EU482831
Switzerland_EU255950
Switzerland_EU255955
Switzerland_EU155348
United Kingdom_KU180718
Switzerland_EU862836
Kenya_KEN216
Switzerland_EU255959
United States_EF407415
Switzerland_EU255944
Switzerland_EU155349
Switzerland_EU482858
Switzerland_EU255954
Switzerland_EU155355
Switzerland_EU155353
Switzerland_EU255957
Switzerland_EU255946
Switzerland_EU255927
Switzerland_EU155346
Switzerland_EU256069
Germany_EU687195
United States_EU155314
Switzerland_EU660385
Switzerland_EU155350
Switzerland_EU482853
Switzerland_EU155352
Switzerland_EU155345
Switzerland_EU255947
Switzerland_EU255943

Figure 4-10: Zoom-in to both distinct Kenyan 1a clusters
These subtrees highlight the main cluster of Kenyan 1a genomes and the likely single introduction of HCV 1a into Kenyan PWID, from Figure 4-9. Colour coding follows from the previous figure. Bootstrap values above 90% are shown.

4.1.3.2 Sub-genotype 4a

Similarly, a maximum likelihood tree was constructed using all published genotype 4 whole genomes, with the Kenyan genotype 4a genomes (Figure 4-11). The 4a clade is highlighted and shows a clear Kenyan 4a cluster, thus suggesting a single introduction of sub-genotype 4a to Coastal Kenya. The closest matching genome to the Kenyan 4a cluster is a 4a genome from an individual from Egypt (DQ988077).



Tree scale: 0.1
Genotype 8 Reference
Subtype 4a
4*
4w
4r
4g
4k
4t
4p
4f
4o
4d
4m
4n
4l
4v
4q
4s
4c
India_MH119606
Netherlands_KU871292
Egypt_DQ988079
Egypt_DQ988073
Egypt_DQ988076
Egypt_DQ988078
Egypt_DQ988075
Netherlands_KU871289
Netherlands_KU871290
Netherlands_KU871291
Canada_JF735137
United_States_KM587624
United_States_DQ418782
United_States_KM043282
United_States_KM043284
United_States_KM043283
United_States_DQ418784
United_States_DQ418787
United_States_DQ418785
France_KT735185
Unknown_DQ516084
United_States_DQ418788
United_States_DQ418783
United_States_DQ418789
Netherlands_KU871293
Egypt_DQ988074
Japan_AB795432
Egypt_Y11604
Egypt_GU814265
Egypt_KY283130
Egypt_DQ988077
Kenya_KEN075
Kenya_KEN144
Kenya_KEN158
Kenya_KEN195
Kenya_KEN165
Kenya_KEN074
Kenya_KEN035
Kenya_KEN355
Kenya_KEN135
Kenya_KEN005
Kenya_KEN369
Kenya_KEN368
Kenya_KEN359
Kenya_KEN371
Kenya_KEN356
Kenya_KEN244
Kenya_KEN085
Kenya_KEN178
Kenya_KEN357
Kenya_KEN125
Kenya_KEN142
Kenya_KEN202
Kenya_KEN200

Figure 4-11: Maximum likelihood tree of all published genotype 4 whole genomes and Kenyan 4a genomes
This is a maximum likelihood phylogenetic tree, with 1000 bootstraps, of all published genotype 4 whole genomes and the Kenyan 4a genomes. The whole 4a clade is coloured yellow and tips are coloured by origin from HICs, LICs or Kenya. Non-4a genomes are labelled by sub-genotype in a light grey colour. The tree is rooted on the genotype 8 reference (MH590700) and bootstrap values above 90% are shown.

4.1.3.3 PWID Network Analysis

Network analysis of Kenyan PWID in this study showed 3 networks, 1 large network of HCV genotype 1a and 2 networks of genotype 4a. This is demonstrated in Figure 4-12. While it was clear from the phylogenetic trees shown above (Figure 4-9, Figure 4-10 and Figure 4-11) that circulating HCV 1a and 4a variants among this group of Kenyan PWID are closely related, this network analysis shows how closely interconnected these virus genomes are. Some individuals have multiple connections with others, suggesting that these individuals are focal points of HCV transmission in this group or “superspreaders.” Within the HCV 1a network in Figure 4-12, 6 individuals (KEN020, KEN021, KEN120, KEN164, KEN222 and KEN363) had 10 or more connections to other individuals within the network. Within the larger of the 2 HCV 4a networks, there were 2 individuals (KEN085 and KEN142) with 10 or more connections to other individuals within the network. Geographical distance between recruitment sites did not influence the networks, showing highly interconnected individuals from all 3 sites within two large and one smaller network. There was no statistically significant difference between these networks and age, gender, geographic location, or risk behaviours.

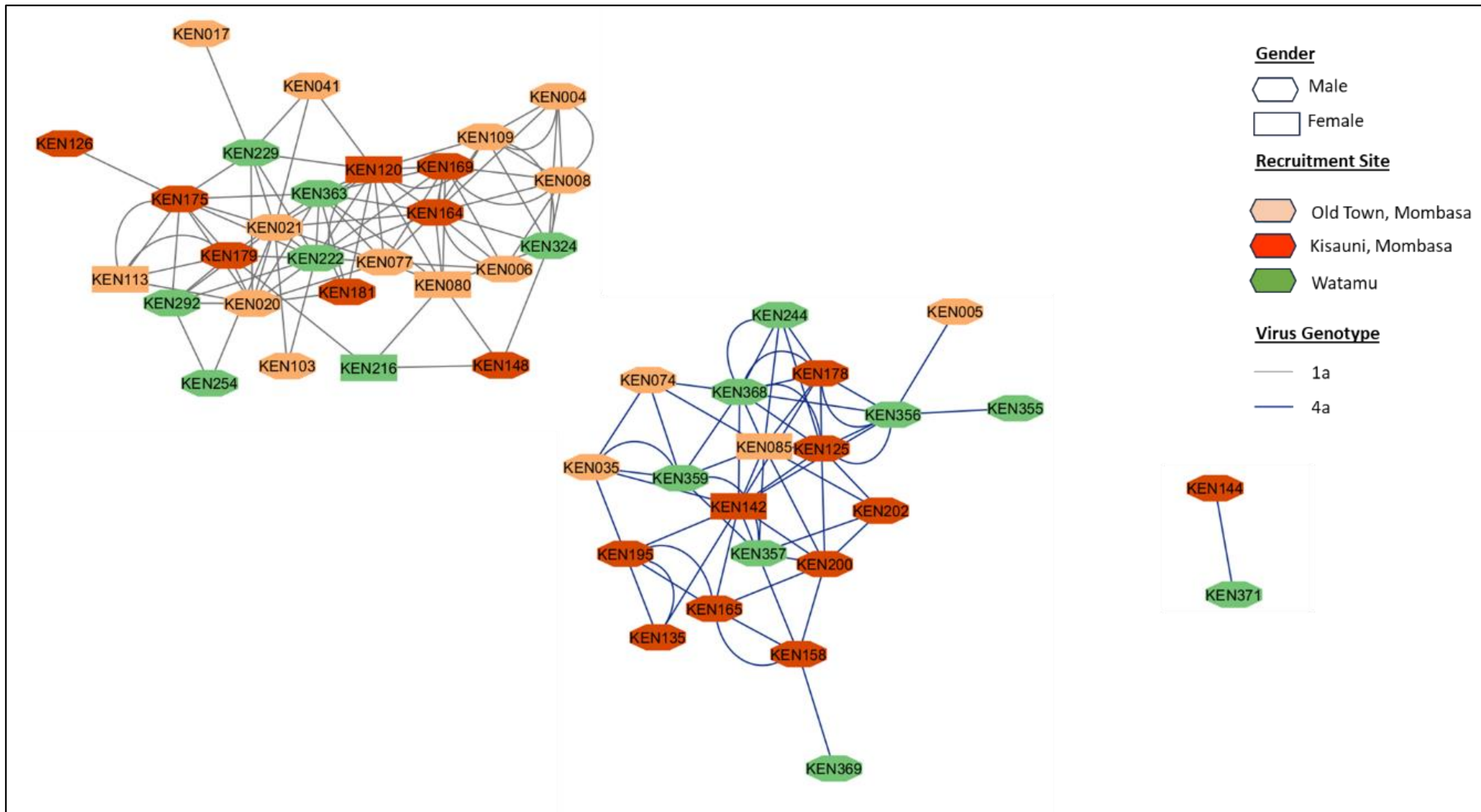


Figure 4-12: Kenyan PWID HCV networks
Figure showing 3 networks of HCV transmission among Kenyan PWID. The nodes represent individuals and are shaped by gender and coloured by recruitment site. Connections between nodes are coloured by HCV genotype.

4.1.3.4 Dating the Introduction of HCV 1a into the PWID Community in Kenya

Table 4-5 shows BEAST parameters for each of the models tested and Figure 4-13 shows the most representative tree, which suggests that HCV genotype 1a was introduced to Kenyan PWID between2001 and 2004, when the Kenyan 1a subcluster diverged from a common ancestor of HCV sequences derived from Switzerland.

Table 4-5: BEAST parameters for each run of the genotype 1a subcluster

Method	No. MCMCs (converged)	Log marginal likelihood	Rate of evolution
Strict	500,000,000	-44565.06	6.79 x10-4
Lognormal	1,000,000,000	-44371.86	2.90 x10-3
Exponential	**500,000,000**	**-44359.59**	**3.16 x10-3**
Gamma	500,000,000	-44363.07	3.03 x10-3

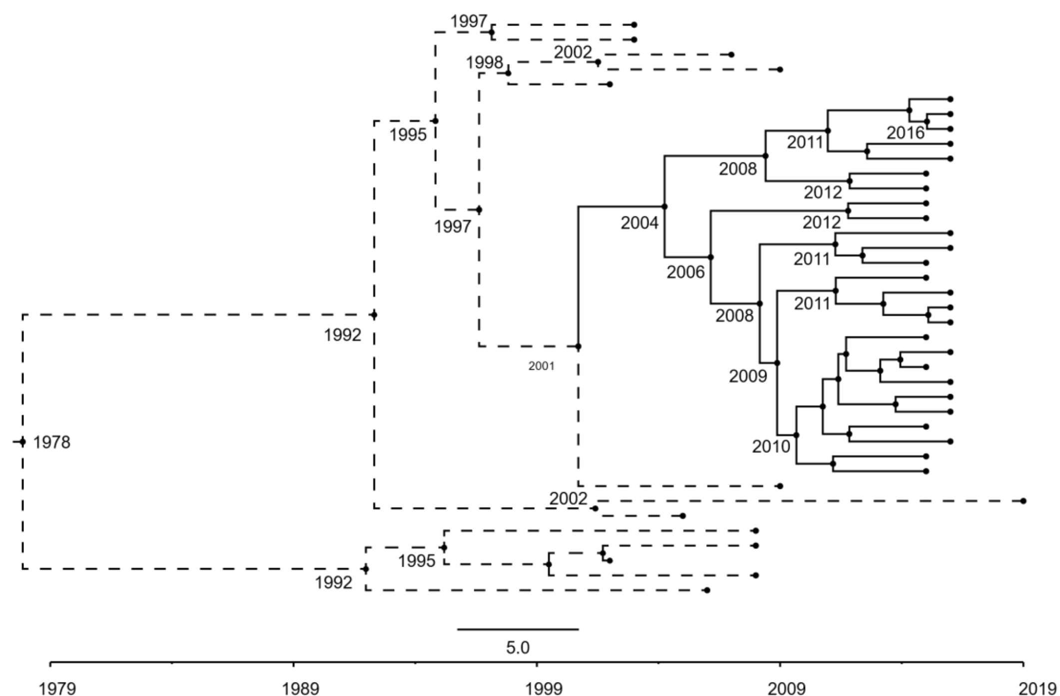


Figure 4-13: BEAST molecular clock analysis of genotype 1a subcluster in Kenyan PWID
The Kenya subcluster (full lines) was nested within a larger cluster of samples originating in Switzerland (dashed lines). The TMRCA of divergence from samples from Switzerland was estimated to be 2001 and 2004.

4.1.3.5 Dating the Introduction of HCV 4a into the PWID Community in Kenya

Table 4-6 shows BEAST parameters for each of the models tested and Figure 4-14 shows the most representative tree, which suggests that HCV genotype 4a was introduced to Kenyan PWID between 1980 and 1998, when the Kenyan 4a subcluster diverged from an HCV sequence from Egypt.

Table 4-6: BEAST parameters for each run of the genotype 4a subcluster

Method	No. MCMCs (converged)	Log marginal likelihood	Rate of evolution
Strict	1,000,000,000	-63562.983	2.55×10^{-5}
Lognormal	1,000,000,000	-63336.708	3.02×10^{-3}
Exponential	1,000,000,000	-63372.983	4.26×10^{-3}
Gamma	**1,000,000,000**	**-63325.099**	$\mathbf{3.79 \times 10^{-3}}$

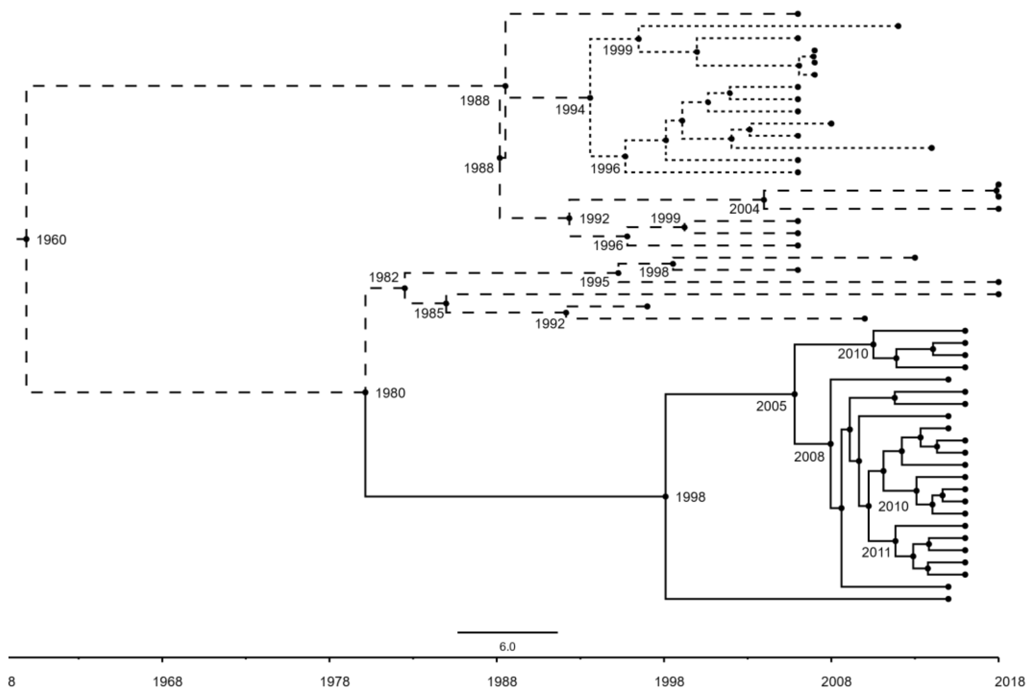


Figure 4-14: BEAST molecular clock analysis of genotype 4a subcluster in Kenyan PWID

The Kenya subcluster (full lines) was nested within a larger cluster of samples originating in Egypt (dashed lines). The TMRCA of divergence from samples from Egypt was estimated to be between 1980 and 1998. A separate cluster associated with exportation from Egypt to the USA and Europe is highlighted with short dotted lines.

4.1.4 RASs in Kenyan HCV genomes

4.1.4.1 RASs in Kenya 1a Genomes

11% (3/27) of the subtype 1a genomes from Kenya had a single known RAS present within the NS5A gene. These three RASs were K24R (KEN216), Q30P (KEN148) and Y93C (KEN120). For KEN216, while R was present at position 24 in NS5A (at least 15% of the reads in that position), the dominant amino acid was still Lysine. The same applies for KEN148. For KEN120, amino acid Cysteine was the only amino acid present in position 93. None of the Kenyan 1a genomes had more than 1 RAS within the NS5A gene and there were no RASs in positions 28 or 31. Figure 4-15 summarises the frequency of RASs in Kenyan HCV 1a genomes.

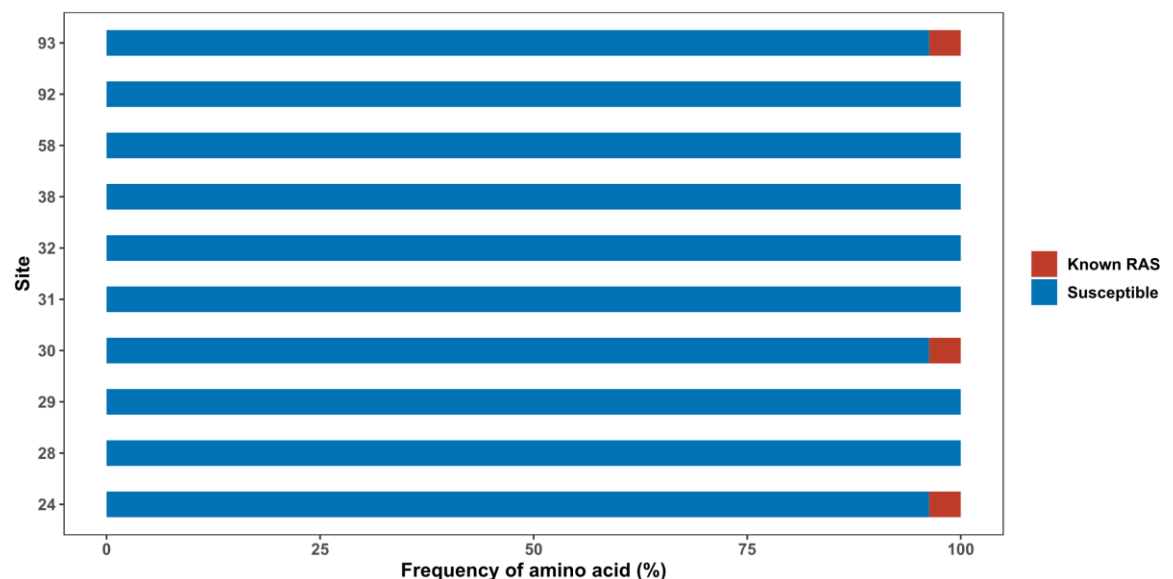


Figure 4-15: Frequency of known RASs seen in NS5A in Kenyan HCV subtype 1a
Susceptible amino acids at positions 24, 28, 29, 30, 31, 32, 38, 58, 92 and 93 are defined as K, M, P, Q, L, P, S, H, A and Y, respectively.

93% (25/27) of the subtype 1a genomes had a known RAS at position 80 in the NS3 gene, i.e. Q80K (Figure 4-16). There were also known RASs in positions 54 (T54S, 7%) and 122 (S122G, 11%). 15% (4/27) of the 1a genomes had 2 RASs present within the NS3 gene. KEN008 and KEN324 had both the RASs T54S and Q80K, while KEN006 and KEN017 had both the RASs S122G and Q80K. KEN004 had the amino acid Alanine in position 158 within the NS3 gene, this amino acid is not known to be associated with resistance to treatment, but is a variant in 1a at this position.

There were no baseline RASs seen in Kenyan 1a genomes in the NS5B gene.

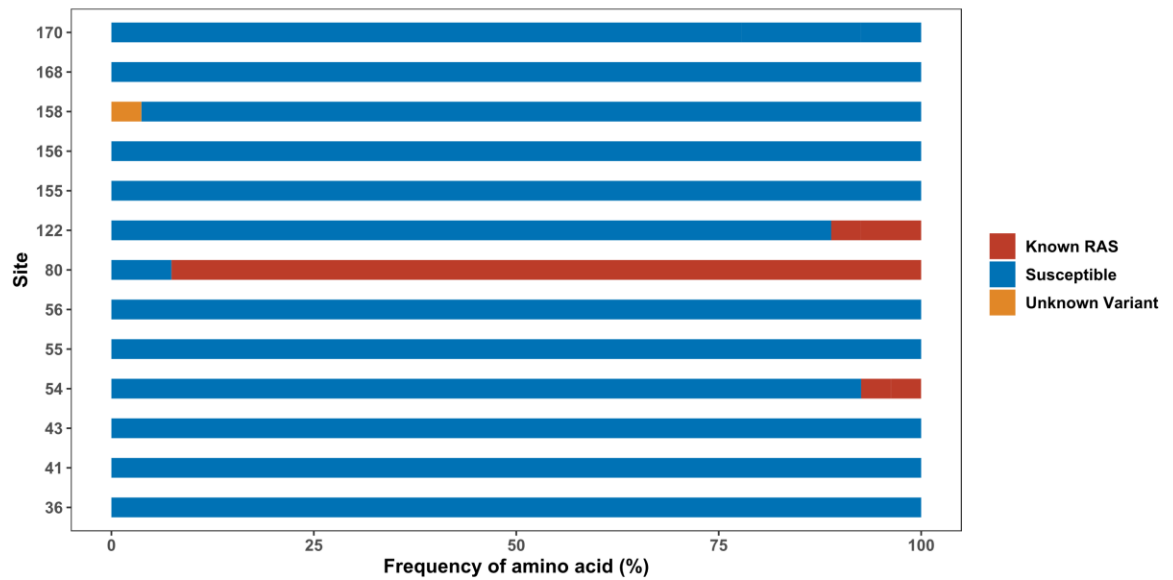


Figure 4-16: Frequency of known RASs seen in NS3 in Kenyan HCV subtype 1a
Susceptible amino acids at positions 36, 41, 43, 54, 55, 56, 80, 122, 155, 156, 158, 168 and 170 are V, Q, F, T, V, Y, Q, S, R, A, V, D and I/V respectively.

4.1.4.2 RASs in Kenya 4a Genomes

No RASs were seen in Kenyan subtype 4a genomes within the NS5A gene. However, 23% (5/22) had previously uncharacterised variants at position 58 (Figure 4-17). These were amino acids Alanine (1/22), Arginine (1/22) and Serine (3/22).

There were no baseline RASs within the NS3 and NS5B genes.

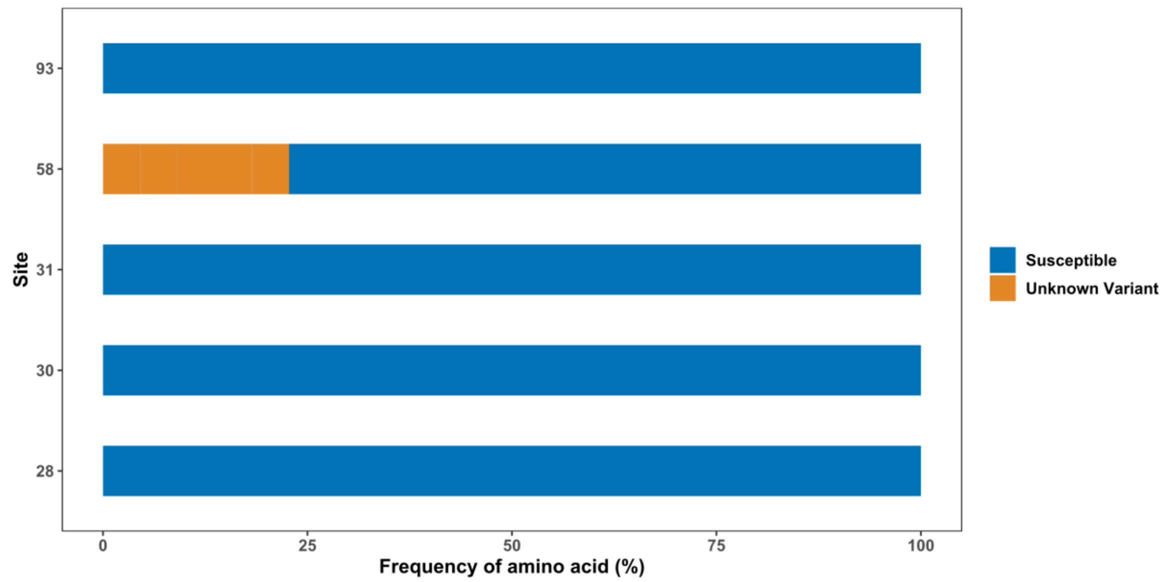


Figure 4-17: Frequency of known RASs seen in NS5A in Kenyan HCV subtype 4a
Susceptible amino acids at positions 28, 30, 31, 58 and 93 are L, L, L, P and Y, respectively.

4.2 HCV in Uganda

This chapter describes the diversity of HCV in Uganda, among a blood donor group and also describes the prevalence of RASs.

4.2.1 Ugandan HCV samples

Out of 81 HCV RNA positive samples, all were available for sequencing. Following post genome assembly clean up, 73 whole HCV genomes were generated (Figure 4-18).

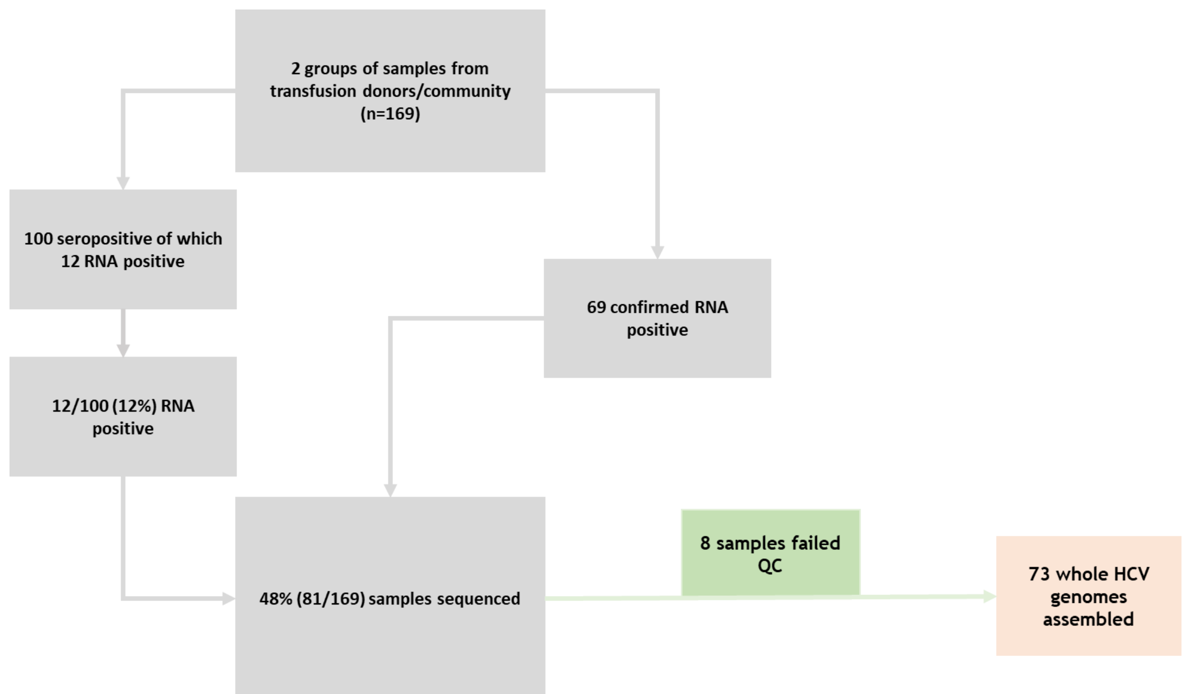


Figure 4-18: Summary of whole Ugandan HCV genomes assembled

4.2.1.1 Resolving Mixed Infections

Ugandan HCV genomes were not as straightforward to assemble using a reference-based approach, as with the Kenyan ones. Following QC analysis, 8 samples were discarded. Initially, multiple samples were found to map to several reference genomes. Details are shown in Figure and Figure 4-20.

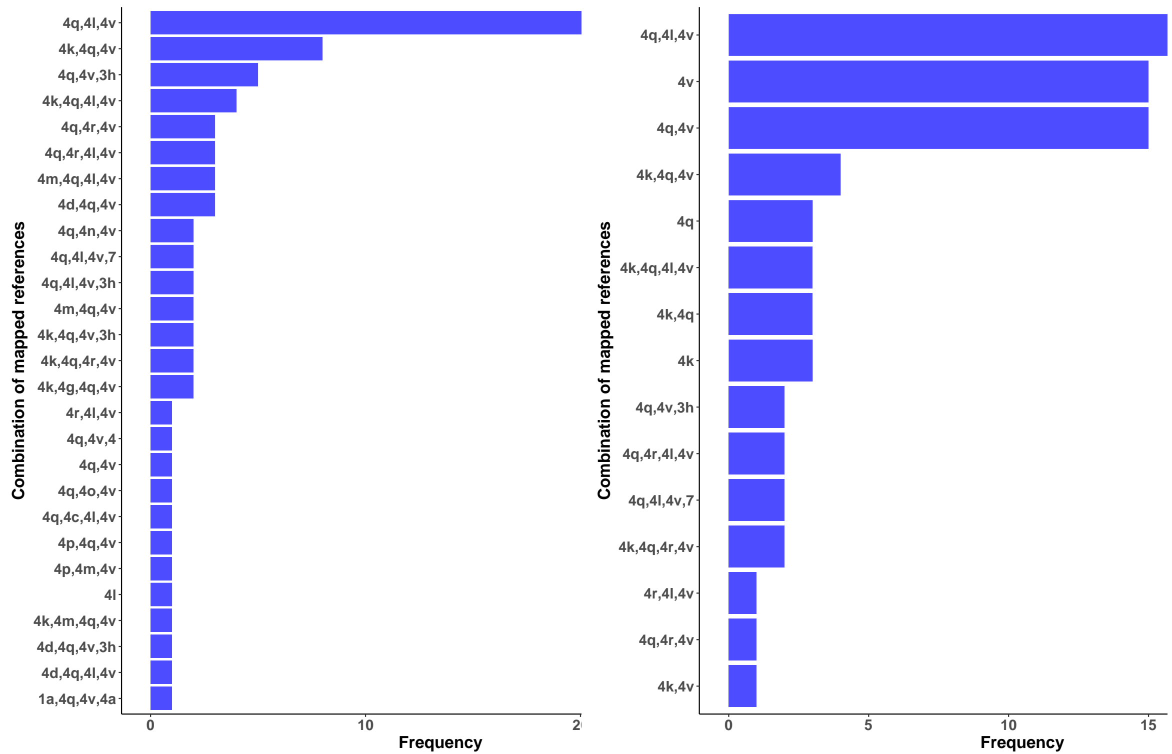


Figure 4-19: Bar chart showing frequency of Ugandan HCV sub-genotype co-infections
The frequency of HCV sub-genotype co-infections seen in all Uganda samples prior to cross-contamination analysis (left sided panel) and post cross-contamination analysis (right sided panel).

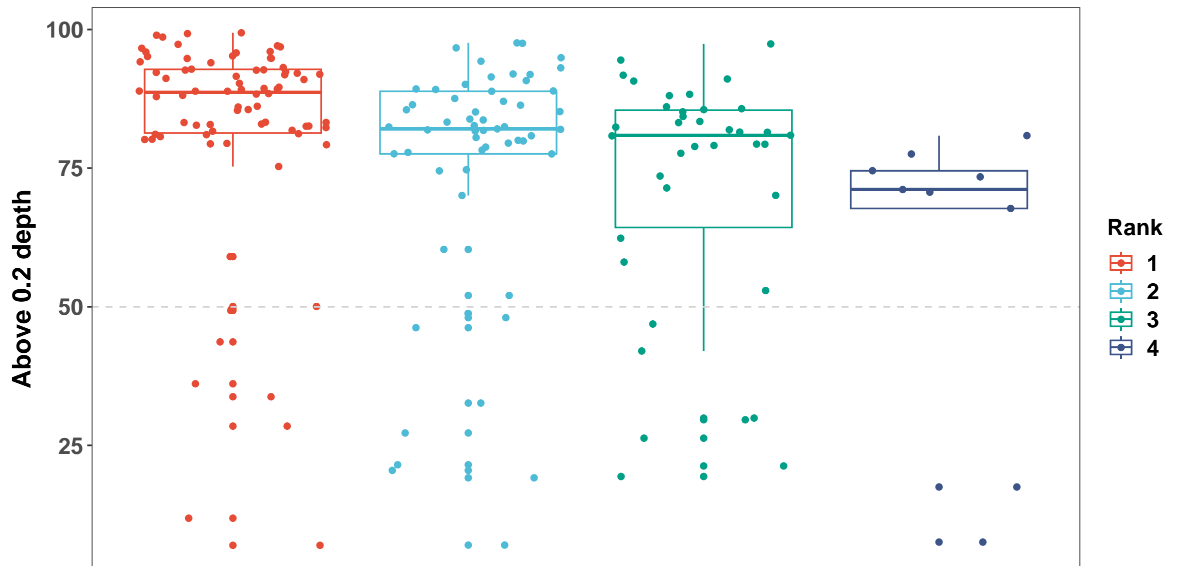


Figure 4-20: Above 0.2 depth for Ugandan HCV samples
HCV reads from Ugandan samples mapped well to several different reference HCV sub-genotypes, occasionally up to 4 different sub-genotypes per sample. Applying the “Above 0.2 depth” metric did not result in a clear-cut distinction between sample genomes that had better depth of coverage across the genome when mapped to different sub-genotypes. As shown in the figure below, a 50% threshold did not work as well as it did for the Kenyan HCV samples to resolve potential mixed infections. Increasing the threshold did not necessarily provide any more clarity.

Examples of coverage plots shown in Figure 4-21 and Figure 4-22demonstrate that the similarity in coverage plots for each sample, when mapped to reference genomes of sub-genotypes 4l, 4q and 4v, suggest a genuine mixed infection, however, subsequent phylogenetic analysis of consensus HCV mapped genomes revealed that the genomes in these samples were very diverse. Therefore, a *de novo* assembly approach was used to generate full length consensus genomes for the Ugandan sequences to avoid mapping bias.

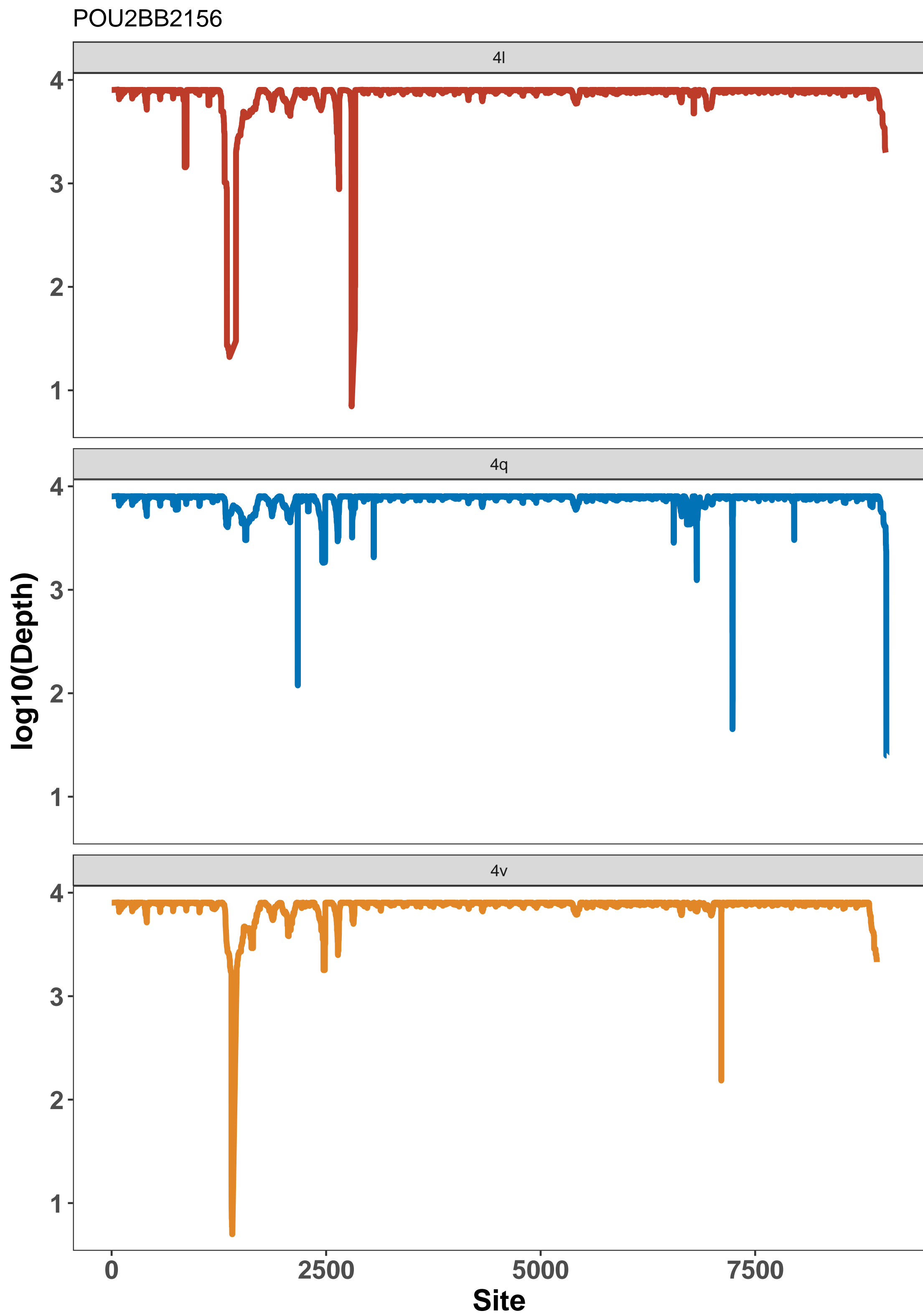


Figure 4-21: Coverage plot for sample POU2BB2156
The accession numbers of the reference genomes used to represent HCV 4l, 4q and 4v are FJ839870 [282], FJ462434 [282] and HQ537009 [283], respectively.

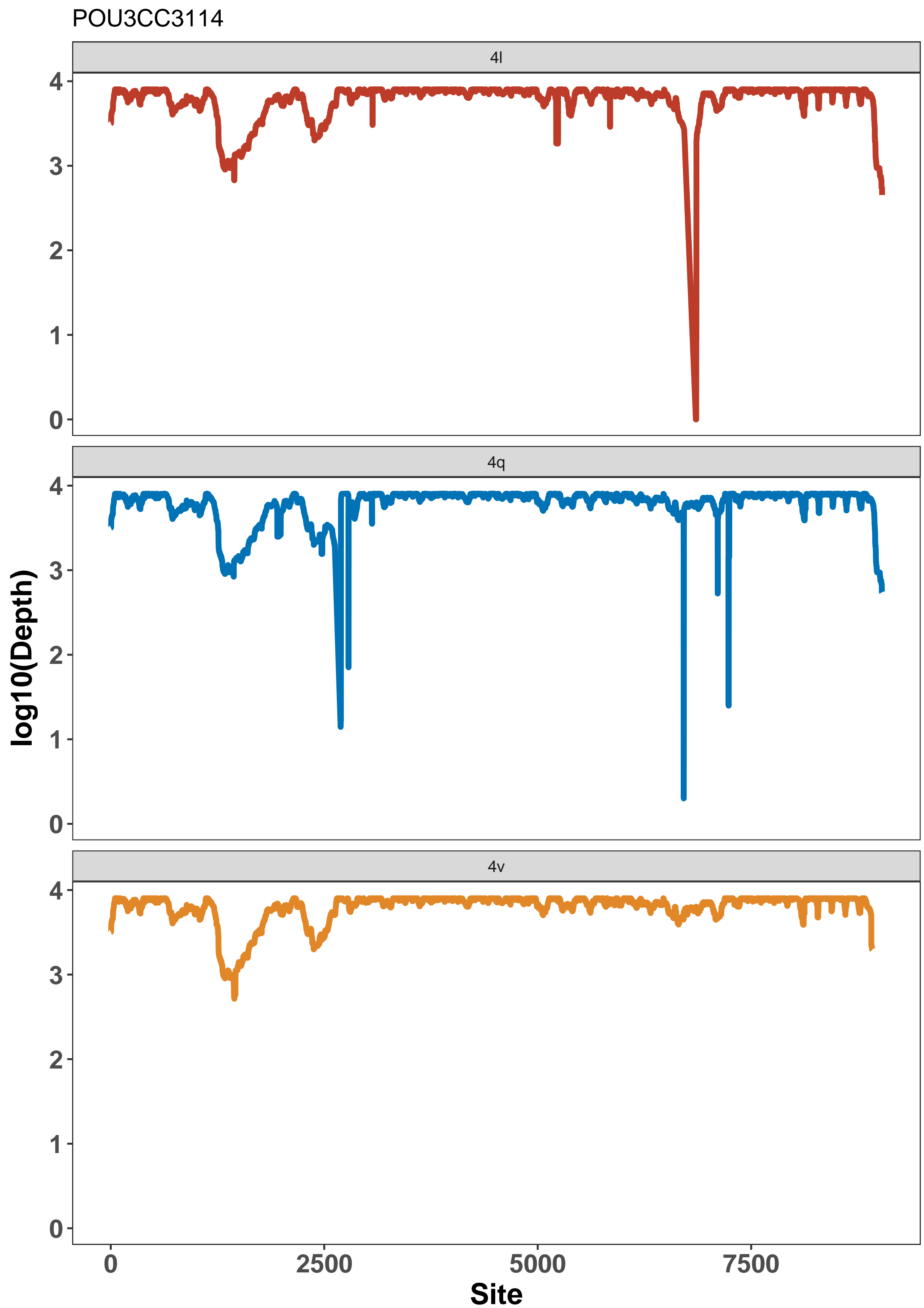


Figure 4-22: Coverage plot for sample POU3CC3114

Where *de novo* assembly failed to generate a whole HCV genome, reference-based assembly, to either a known reference sub-genotype, or a *de novo*

assembled genome, was used. The choice of a *de novo* assembled genome, to be used as a reference for mapping, was determined by phylogenetic analysis. Following assembly, genome statistics (number of mapped reads, coverage and depth) were compared against the corresponding sample genomes that were mapped to known reference sub-genotypes as well as reconstruction of phylogeny for quality control.

Of the 73 Ugandan HCV genomes assembled, 48 were *de novo* assembled and 25 (5 using known sub-genotype genome sequences as the mapping reference and 20 using one of the *de novo* assembled genomes as the mapping reference) were assembled using a reference-based approach.

4.2.1.2 Recombination Analysis

Section 4.2.2.2 details the finding of a genotype 4-genotype 7 mixed infection. This sample was analysed for recombination using Recombination Detection Program version 4 (RDP4). The alignment included whole genomes of all genotype 4 subtypes, and genotype 7, reference genomes. A scanning window size of 200 base pairs was set. Sequences were analysed using RDP, GENECONV, MAXCHI, BOOTSCAN and SISCAN methods.

4.2.2 Diversity of HCV in Uganda

Following genome assembly and post assembly processing, 73 whole HCV genomes from Uganda were analysed (Figure 4-18). 96% (70/73) of the genomes were genotype 4. There were two genotype 7 genomes, including sample POUSEE5157, which was a dual infection with both HCV genotype 4 and 7. This will be described later in this chapter. There was also one genotype 3 (POUSEE5194). A maximum likelihood tree inferred from a general time reversible model with 1000 bootstraps is shown in Figure 4-23.

4.2.2.1 Diversity of HCV Genotype 4 in Uganda

A rich diversity of HCV genotype 4 was found in Uganda as shown in the phylogenetic tree in Figure 4-24, including previously described and novel sub-genotypes. The clade coloured in green includes the reference genomes of 4q and 4v. The clade coloured in blue reveals genotype 4 HCV genomes that are all previously undescribed and includes a genome that was described by Stockdale *et al* in a molecular epidemiological surveillance study of HCV in Malawi [284]. Among the known genotype 4 subtypes, three of the Uganda samples were identified as 4k and four as 4r.

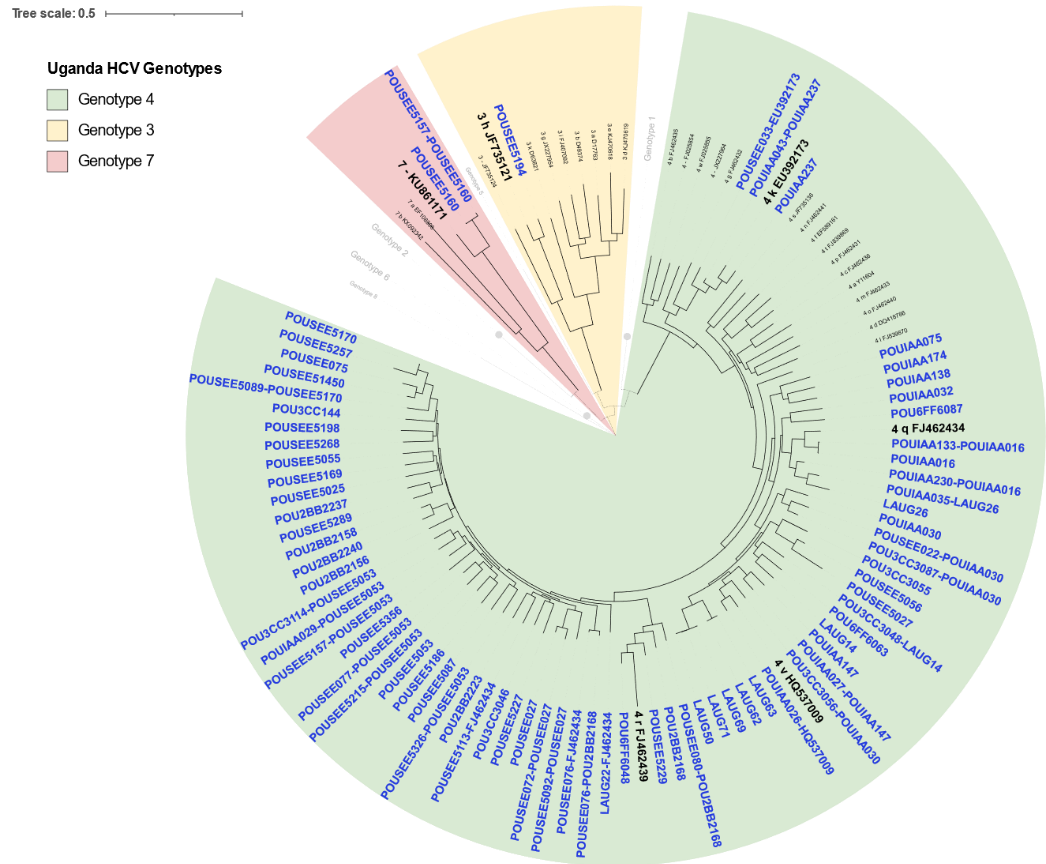


Figure 4-23: Maximum likelihood phylogenetic tree of Uganda HCV whole genomes and selected reference genomes from each genotype.

A maximum likelihood tree inferred from a general time reversible model and a 1000 bootstraps, using IQTREE, on an alignment of whole HCV nucleotide genomes of all known reference HCV sub-genotypes from ICTV (https://ictv.global/sg_wiki/flaviviridae/hepacivirus) and the HCV genomes from Uganda. Tip labels in blue are Uganda HCV genomes and the tip labels in large, bold black font are the closest matching reference sub-genotypes. The remaining reference subtypes in genotype 3, 4 and 7 clades have the tips labelled in a smaller black font. Clades belonging to genotypes 1, 2, 5, 6 and 8 are collapsed and coloured in a lighter grey. Bootstrap values above 90% are shown. Clades representing genotypes 3, 4 and 7 are coloured distinctly.

Figure 4-24: Maximum likelihood phylogenetic tree showing the diversity of HCV genotype 4 in Uganda

A maximum likelihood tree inferred from a general time reversible model and a 1000 bootstraps, using IQTREE, on an alignment of whole HCV nucleotide genomes of all known reference HCV

genotype 4-subtypes from ICTV (https://ictv.global/sg_wiki/flaviviridae/hepacivirus) and the HCV genomes from Uganda as well as additional whole HCV genomes representing 4r, 4v and 4w. Branch labels in red represent the genomes from Uganda. Clades are coloured by subtype. Uncoloured clades represent other genotype 4 subtypes that were not seen in this Ugandan study. Bootstrap values above 90% are shown.

Many of the genomes were genetically similar to 4q and 4v, yet distant enough to be considered a different subtype.

The difference in genetic distance between the 4q (FJ462434) [282] and 4v (HQ537009) [283] reference genomes in the alignment used to construct the phylogenetic tree in Figure 4-24 is 15.3%, but the range of genetic distances between 4q and 4v and the other genotype 4 subtype reference genomes is 18.1%-21.6% and 17.5%-22.1%, respectively. This shows that 4q and 4v share more similarity to each other than the other known genotype 4 subtypes. Some of the Uganda genotype 4 sequences shared a similar genetic distance to 4q and 4v reference genomes as well as other known 4v genomes. For example, samples POUIAA016, POUIAA133-POUIAA016 and POUIAA230-POUIAA016 were 8.7%, 8.8% and 11.2% genetically different to 4q, respectively. Samples POUIAA035-LAUG26 and LAUG26 were 13.8% and 15.1% distant to 4q, which is still less than the 15.3% difference between 4q and 4v. However, sample POUIAA230-POUIAA016 was also 12.8%-13.7% genetically different to the 4v genomes previously identified in Uganda (MH742365, MH742367, MH742369), as described by Davis et al [130]. Samples POUIAA026-HQ537009 and POU3CC3056-POUIAA030 are 7.6% and 12.2% genetically distant, respectively, to the 4v reference genome. As shown in Figure 4-25, there are many samples that are between 11% and 15% distant to previously described 4v genomes from Uganda. Samples LAUG50, LAUG69 and LAUG71 are closely phylogenetically related and are 11.2% distant to samples LAUG62 and LAUG63 (Figure 4-26). This suggests a localised outbreak of HCV, however, epidemiological data was not available to support this finding. Collectively these samples are technically considered as a sub-genotype of 4v as they are 13% distant to the previously described 4v genomes from Uganda, and lie just within the recommended criteria for differentiation. Samples POU6FF6087, POUIAA032, POUIAA075, POUIAA138 and POUIAA174 are more than 15.5% distant to the other genomes in the clade and so represent new subtypes, however, while they form a distinct cluster (Figure 4-24) they are still 14.8%-18.4% distant to each other (Figure 4-26). POUSEE5027 is clearly distinct to all the other genomes in the 4q/4v clade, as evidenced by a long branch on the phylogenetic tree (Figure 4-24).

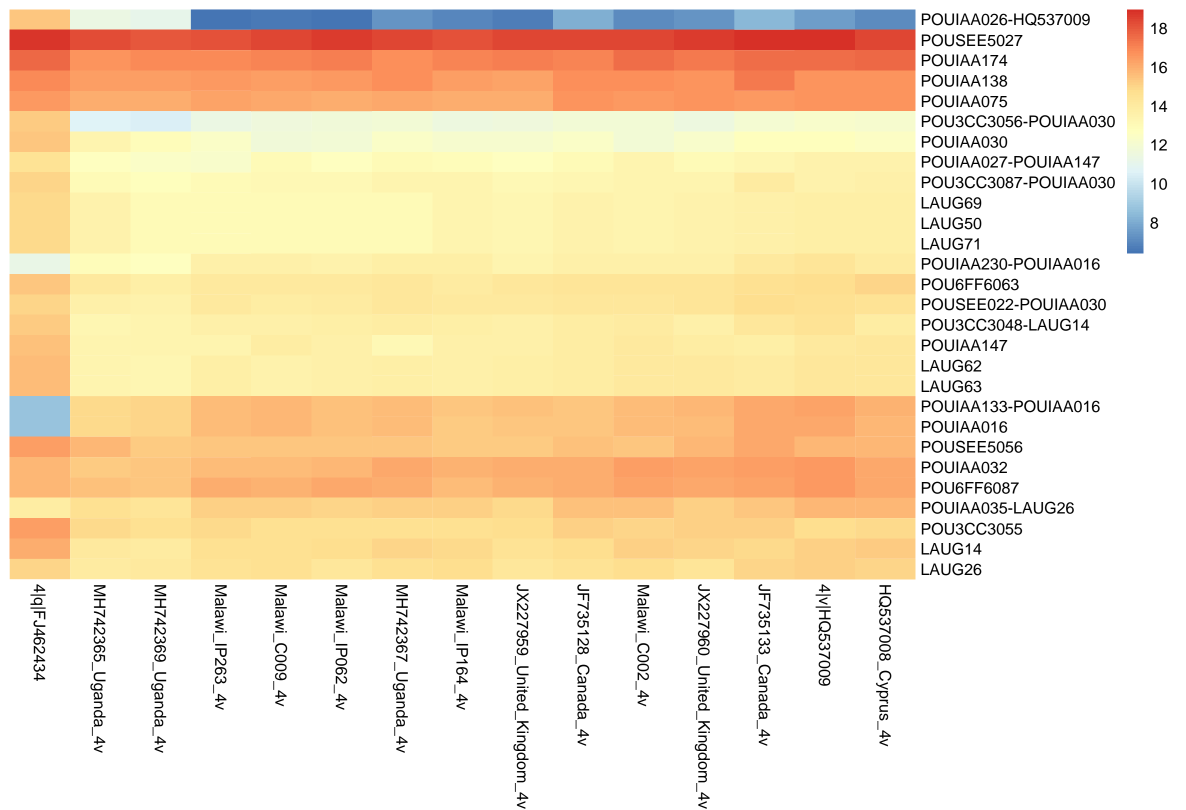


Figure 4-25: Heatmap of genetic distances (%) between selected samples and references within the 4q/4v clade

Genetic distances calculated in Mega X as described in section 3.4.5. The scale shows genetic distances expressed as a percentage. Columns represent selected reference genomes and rows represent the sample genomes within the 4q/4v clade, represented by the green clade in Figure 4-24.

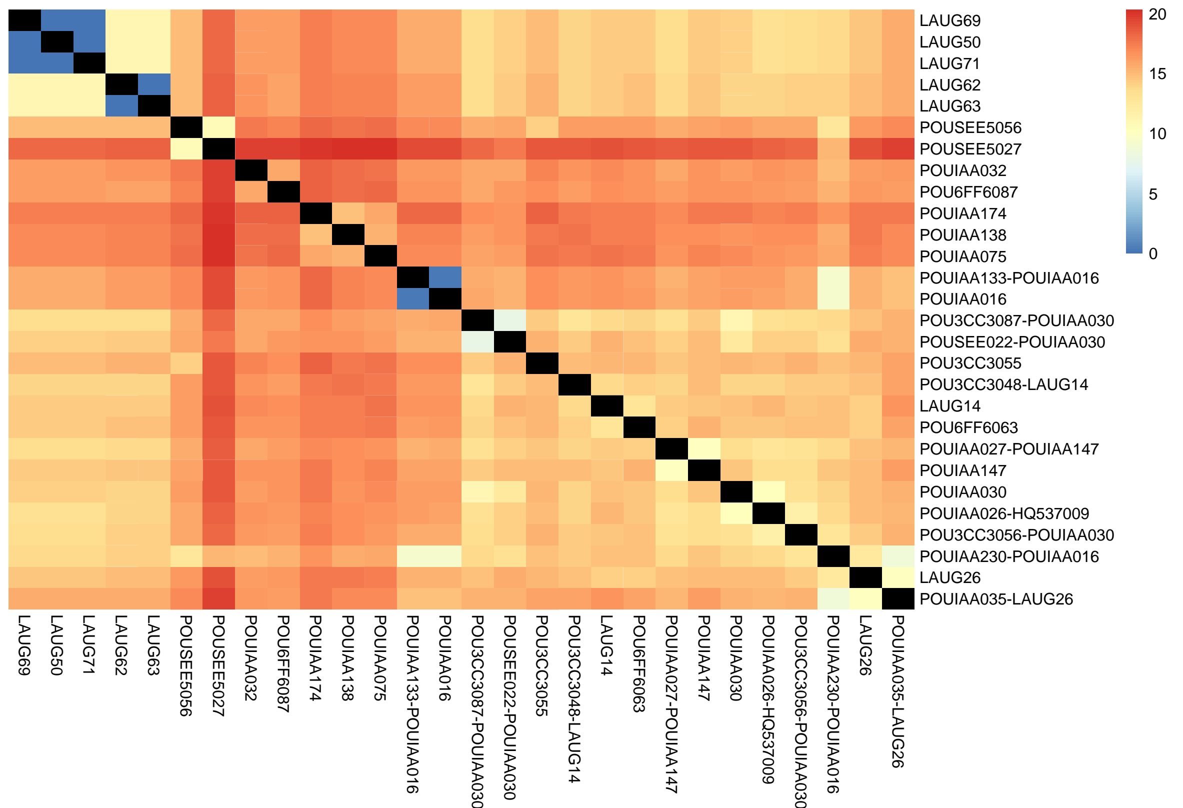


Figure 4-26: Heatmap of genetic distances (%) between samples in the 4q/4v clade

The genomes in the blue coloured clade in Figure 4-24 are very distinct. There is at least a 15% genetic distance between the samples and 4q and 4v genomes, and around a 20% distance between samples and 4r (Figure 4-27). The cluster of samples in the blue coloured clade include a genome from Malawi, which was recently described [284].

The cluster of genomes POUSEE5170, POUSEE5140 and POUSEE075, which are 7.2% to 9.7% genetically distant to each other, fulfil the requirement to be classified as a new subtype, provisionally 4xa. POUSEE5257 is represented by a long branch in this cluster and could also be considered the same subtype because it is only 7.4% different to POUSEE5170, however it is 13.8-14.6% different to POUSEE51450 and POUSEE075, respectively.

While the whole clade could be considered a new subtype 4, there are distinct clusters of genomes even within this clade. In addition to the new subtype 4xa, described above, samples POUSEE027 and POUSEE072-POUSEE027 are 5.3% distant to each other, and samples POU2BB2240 and POU2BB2158 are 6.5%

distant to each other. The genetic distances between these samples and the previously described genotype 4 genome from Malawi is shown in Figure 4-28.

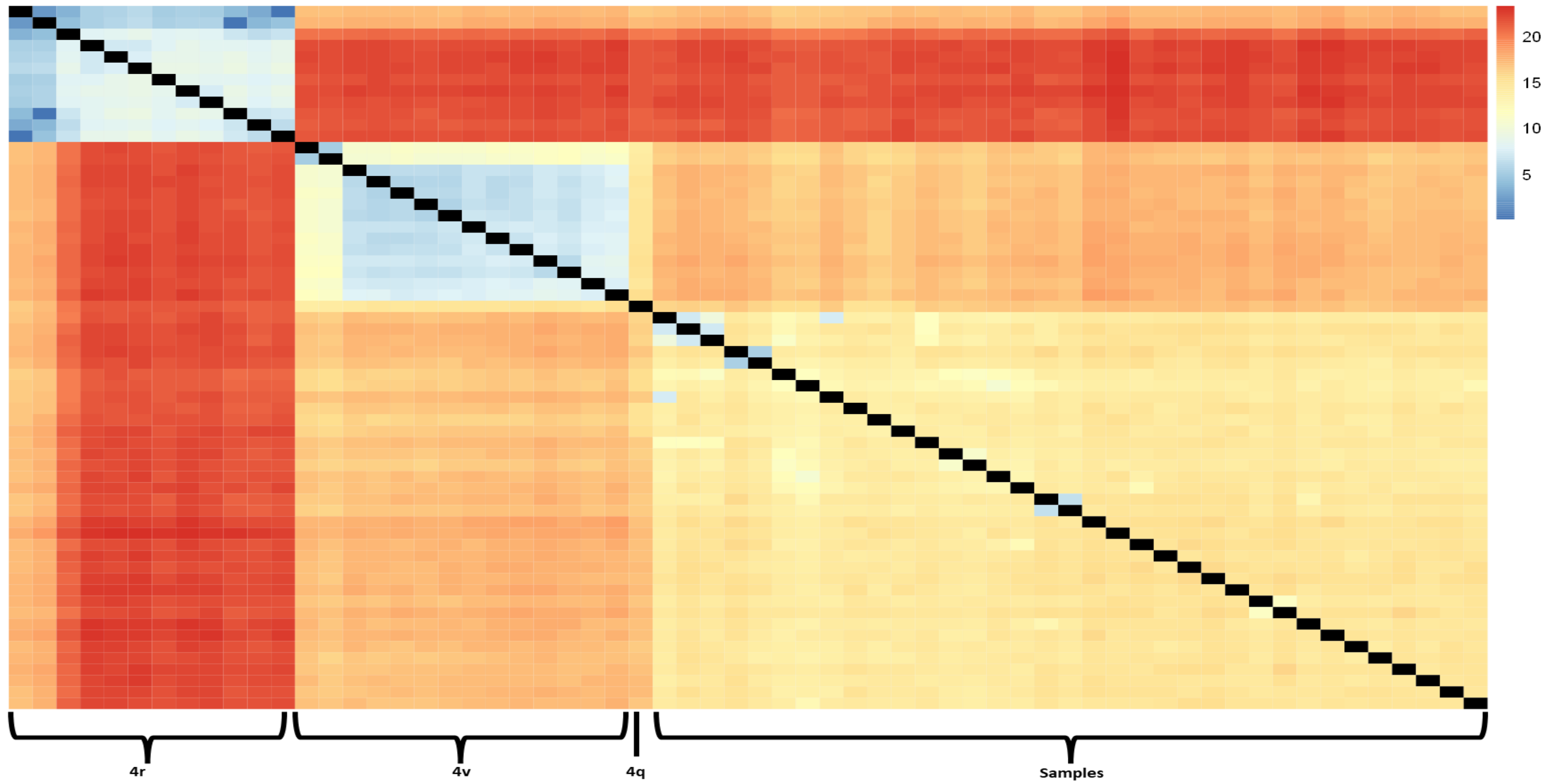


Figure 4-27: Genetic distances (%) between samples and 4q, 4r and 4v genomes
Rows are a mirror of columns.

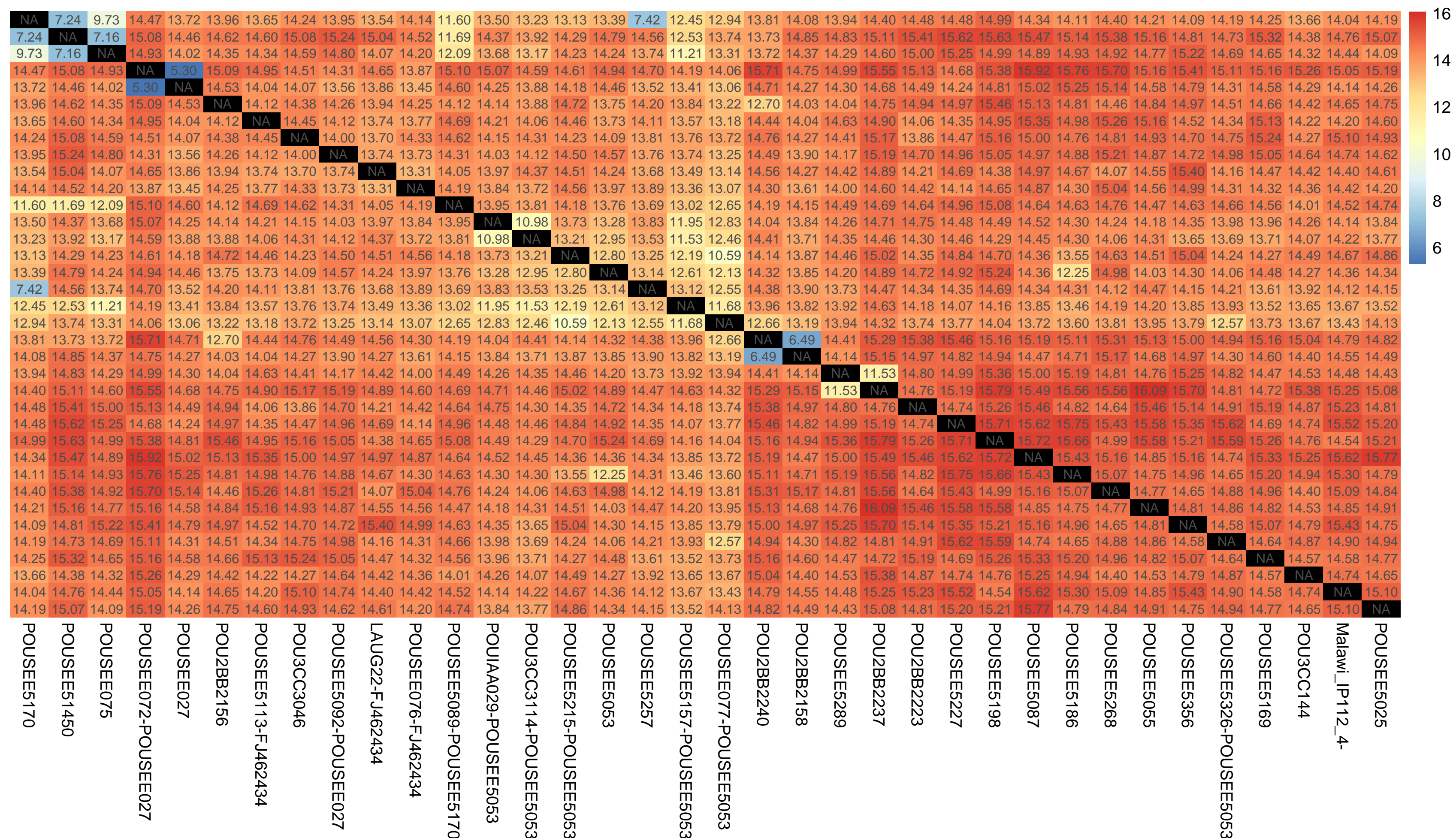


Figure 4-28: Genetic distances (%) within diverse genotype 4 samples from Uganda

In this heatmap the genetic distances, expressed as percentages, are shown within each cell. The previously published genotype 4 genome from Malawi is also shown here, in the second column from the right. Rows are a mirror of columns.

Subtype 4r was also seen in this group of samples from Uganda. This is an important subtype that has been recognised as a more challenging variant to treat with first generation NS5A inhibitors [5]. Samples POUSEE080-POU2BB2168, POU2Bb2168, POUSEE5229 and POU6FF6048 are all less than 15% distant to each other, but not as genetically similar to the 4r reference genomes (Figure 4-29). These samples sit within accepted criteria to be identified as a 4r, but the very high genetic diversity present within this sub-genotype (Figure 4-24) indicates that 4r may have originated in or close to Uganda.

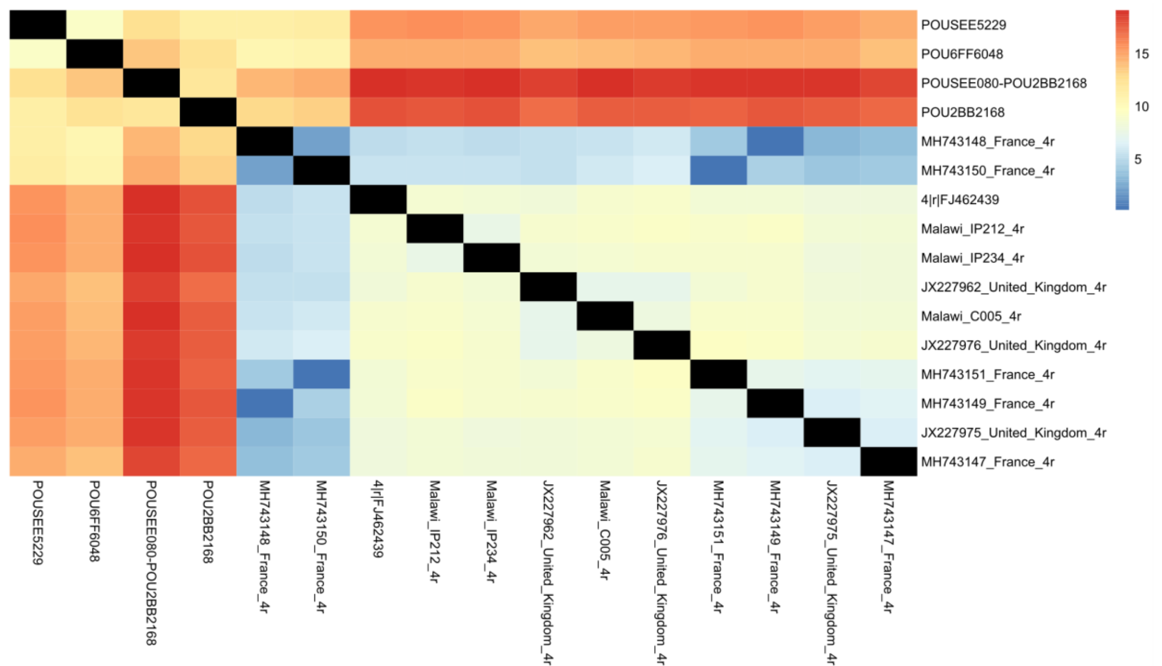


Figure 4-29: Genetic distances (%) between 4r reference genomes and samples

4.2.2.2 Genotype 4 and Genotype 7 Mixed Infection

Two distinct full-length genomes (genotype 4 and genotype 7) were assembled from sample POUSEE5157. To assess if this was a genuine mixed infection, we looked at the frequency of the number of nucleotide differences between the reads and the reference genome. In both the genotype 4 and genotype 7 genomes of sample POUSEE5157, most of the reads had 0 differences to the reference genome. This is illustrated in Figure 4-30.

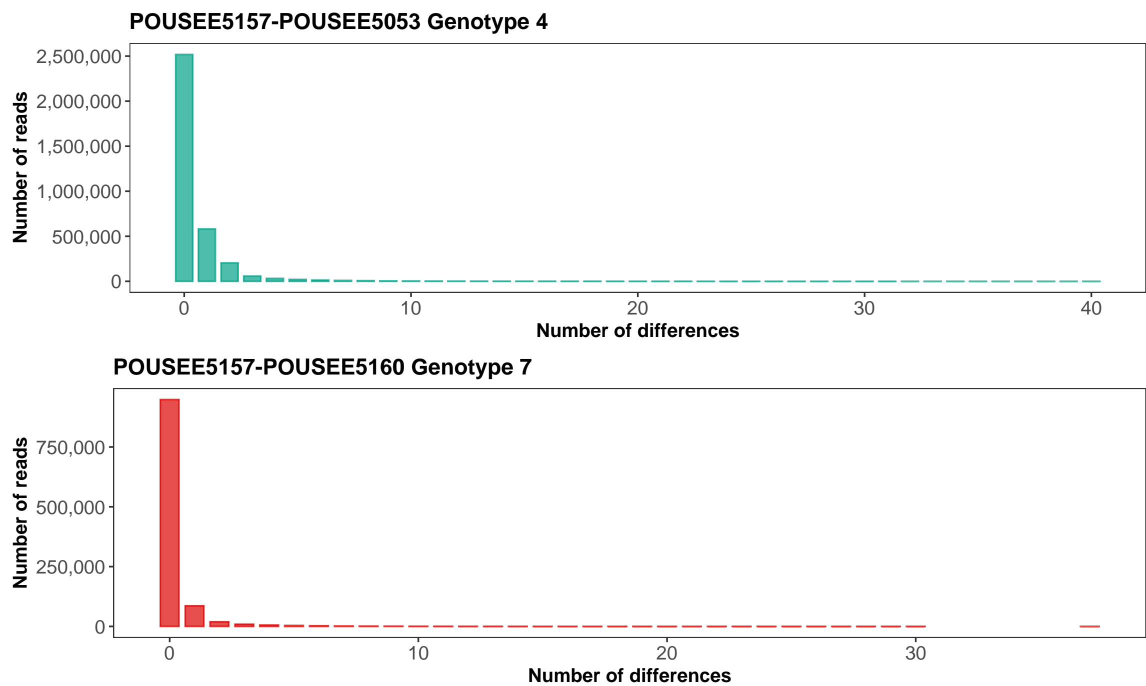


Figure 4-30: Number of nucleotide differences between reads and reference genome for sample POUSEE5157
The number of differences was calculated using a program, SAMDIST, written by Dr Sreenu Vattipally.

Genotype 4 and genotype 7 are at least 30% different to each other, across the whole genome. Using POUSEE5160 as a genotype 7 example and POUSEE5053 as a genotype 4 example (Figure 4-30), a SAM file was created mapping the reads of one to the consensus genome of the other, and vice versa, to plot the frequency of the number of nucleotide differences between reads and the reference genome. This is shown in Figure 4-31 and, as expected, most reads have a higher number of nucleotide differences to the reference genome. We also looked for evidence of recombination between genotype 4 and genotype 7 using Recombination Detection Program (RDP) version 4 [285], but none was found.

Thus, sample POUSEE5157 is likely to be a genuine mixed infection of both genotype 4 and genotype 7.

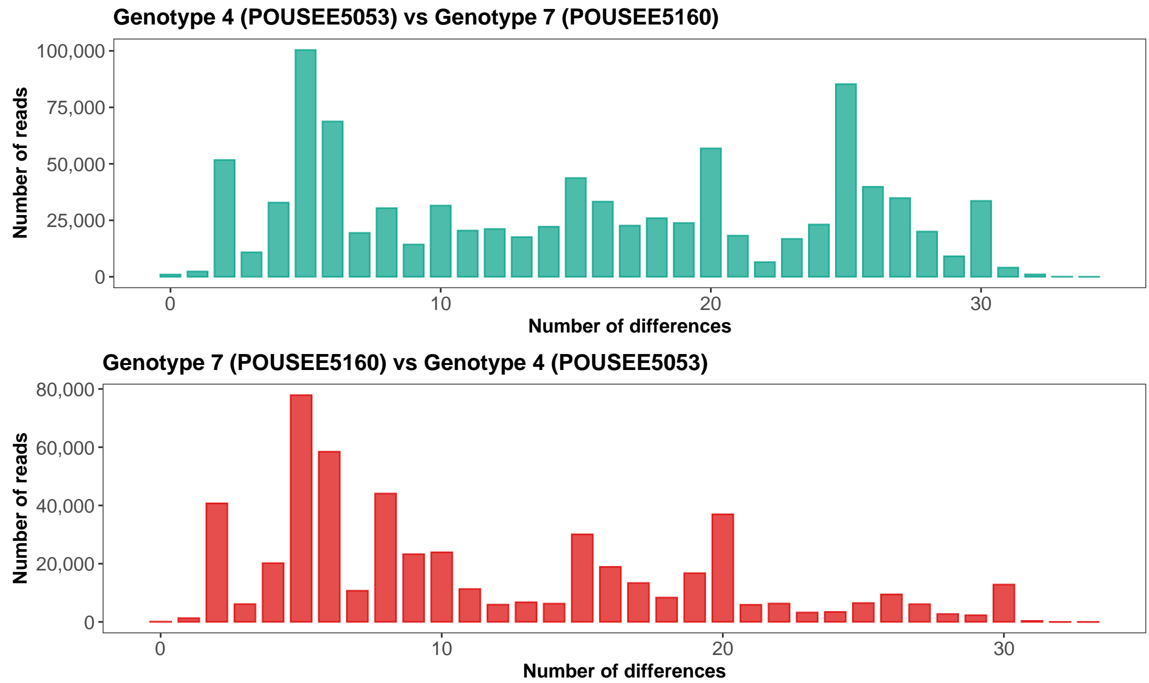


Figure 4-31: Number of nucleotide differences between reads of a genotype 7 sample and reference genome of genotype 4 sample, and vice versa

4.2.3 RASs in Diverse Genotype 4 Ugandan Genomes

100% (69/69) of the diverse genotype 4 genomes from Uganda had the RAS L30R (Figure 4-32). 52% (36/69) had at least 2 RASs, and of these 25% (9/36) had 3 RASs. Other well-described RASs within NS5A were seen at sites 28, 30 and 31 (Figure 4-32). L28M and M31L/V was present in 39% (27/69) and 23% (16/69) of genomes, respectively. Only two genomes had the RAS, Y93H. These were samples POU6FF6048, a subtype 4r, and POUIAA237, a subtype 4k. At position 58, 4% (3/69) of genomes had variant amino acids. Alanine, Arginine or Serine (Table 4-7).

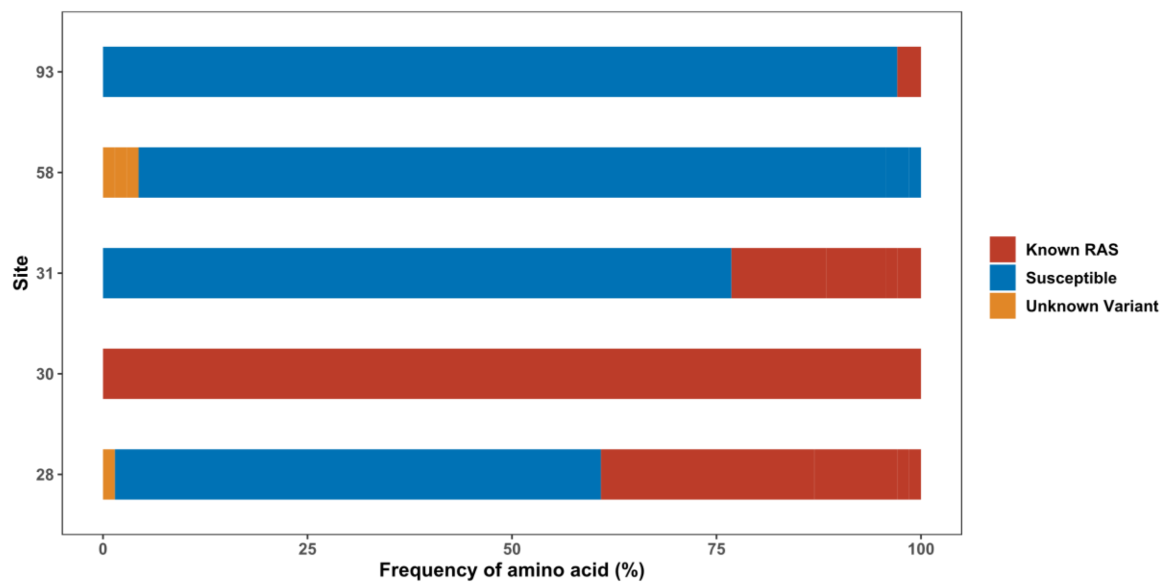


Figure 4-32: Frequency of known RASs seen in NS5A RAS sites among diverse genotype 4 samples from Uganda
Susceptible amino acids at positions 28, 30, 31, 58 and 93 are L, L, M, P/T and Y, respectively. The amino acids at positions 28 and 58, labelled as unknown, are T and A, R and S, respectively.

Table 4-7: NS5A RASs in diverse HCV genotype 4 genomes from Uganda
Amino acids coloured in red denote known RASs. Those coloured in blue denote variants.

Genome ID	NS5A RAS Sites				
	28	30	31	58	93
LAUG14	L	R	M	P	Y
LAUG22-FJ462434	L	R	M	P	Y
LAUG26	L	R	VM	P	Y
LAUG50	L	R	M	P	Y
LAUG62	L	R	M	P	Y
LAUG63	L	R	M	P	Y
LAUG69	L	R	M	P	Y
LAUG71	L	R	M	P	Y
POU2BB2156	M	R	M	P	Y
POU2BB2158	M	R	M	P	Y
POU2BB2223	L	R	M	P	Y
POU2BB2237	M	R	M	P	Y
POU2BB2240	ML	R	ML	P	Y
POU3CC144	M	R	M	P	Y
POU3CC3046	L	R	M	P	Y
POU3CC3048-LAUG14	L	R	M	P	Y
POU3CC3055	L	R	M	P	Y
POU3CC3056-POUIAA030	L	R	M	P	Y
POU3CC3087-POUIAA030	L	R	M	T	Y
POU3CC3114-POUSEE5053	M	R	M	P	Y
POU6FF6048	TM	R	M	P	YH
POU6FF6063	L	R	M	P	Y
POU6FF6087	L	R	M	P	Y
POUIAA016	L	R	M	P	Y
POUIAA026-HQ537009	L	R	M	P	Y
POUIAA027-POUIAA147	M	R	M	P	Y
POUIAA029-POUSEE5053	T	R	L	P	Y
POUIAA030	ML	R	M	P	Y
POUIAA032	L	R	M	P	Y
POUIAA035-LAUG26	L	R	M	P	Y
POUIAA043-POUIAA237	L	R	L	P	Y
POUIAA075	ML	R	M	P	Y
POUIAA133-POUIAA016	L	R	VM	P	Y

POUIAA138	L	R	L	P	Y
POUIAA147	L	R	M	S	Y
POUIAA174	M	R	L	P	Y
POUIAA230-POUIAA016	L	R	M	P	Y
POUIAA237	L	R	VL	P	YH
POUSEE022-POUIAA030	ML	R	M	TP	Y
POUSEE027	L	R	M	P	Y
POUSEE033-EU392173	M	R	M	P	Y
POUSEE072-POUSEE027	L	R	M	P	Y
POUSEE075	M	R	M	P	Y
POUSEE076-FJ462434	L	R	M	P	Y
POUSEE077-POUSEE5053	L	R	M	T	Y
POUSEE5025	L	R	L	P	Y
POUSEE5027	M	R	L	P	Y
POUSEE5053	L	R	M	P	Y
POUSEE5055	L	R	L	A	Y
POUSEE5056	M	R	ML	P	Y
POUSEE5087	L	R	M	P	Y
POUSEE5089-POUIAA237	ML	R	ML	P	Y
POUSEE5089-POUSEE5170	ML	R	M	P	Y
POUSEE5113-POU2BB2168	L	R	M	P	Y
POUSEE5113-FJ462434	L	R	M	P	Y
POUSEE51450	M	R	M	P	Y
POUSEE5157-POUSEE5053	L	R	M	P	Y
POUSEE5169	L	R	M	P	Y
POUSEE5170	ML	R	ML	P	Y
POUSEE5186	L	R	M	P	Y
POUSEE5198	L	R	M	R	Y
POUSEE5215-POUSEE5053	M	R	M	P	Y
POUSEE5227	L	R	M	P	Y
POUSEE5229	TML	R	ML	P	Y
POUSEE5257	M	R	L	P	Y
POUSEE5268	M	R	M	P	Y
POUSEE5289	M	R	M	P	Y
POUSEE5326-POUSEE5053	M	R	M	P	Y
POUSEE5356	M	R	M	P	Y

No RASs were observed in NS5B at positions 282 and 321. A very small proportion of baseline RASs were seen in NS3. 1% (1/69) of genomes had the Y56F substitution and 6% (4/69) of genomes had D168E (Figure 4-33). One of the genomes with D168E also had the NS5A RAS, Y93H (POUIAA237).

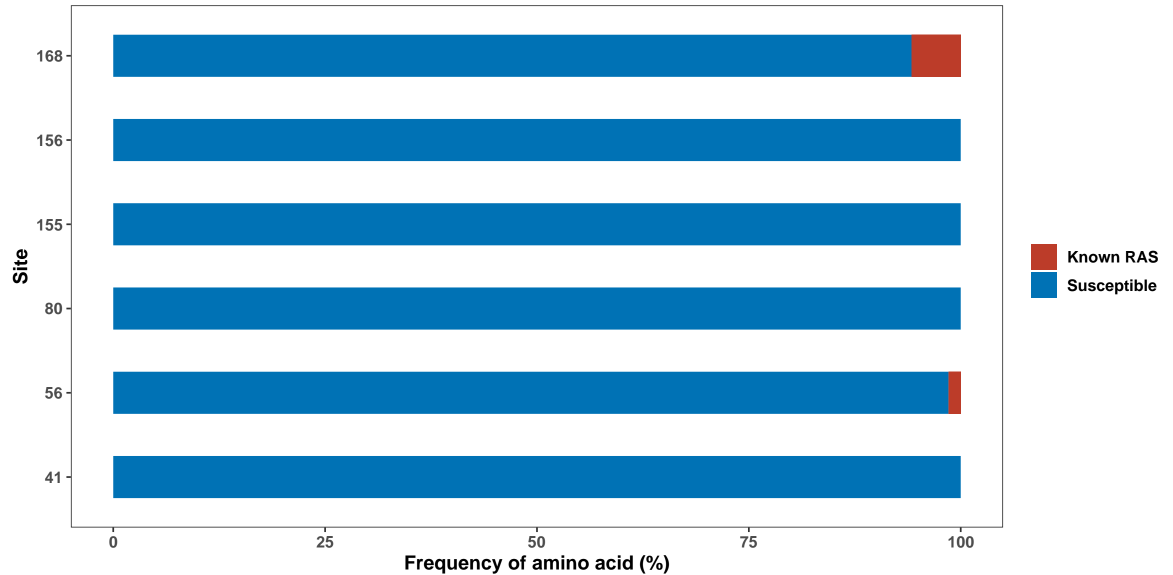


Figure 4-33: Frequency of known RASs seen in NS3 RAS sites among diverse genotype 4 samples from Uganda
Susceptible amino acids at positions 41, 56, 80, 155, 156 and 168 are Q, Y, Q, R, A and D, respectively.

4.3 HCV in Benin

This chapter describes the diversity of HCV in Benin, among HCV infected individuals infected in the hospitals, including health care workers. Data on the prevalence of baseline RASs and the effect of these RASs on DAAs, using an *in vitro* assay is also shown.

4.3.1 Benin HCV samples

The assembly of Benin HCV genomes has been discussed by Lucrece Ahovegbe in the submission of her PhD thesis. This work has been published in The Lancet Microbe. The diversity of HCV in Benin and the presence of baseline RASs is discussed in detail in 4.3.2 and 4.3.2. The effect of DAAs, in particular NS5A inhibitors, on SGR, with NS5A sequences reflective of the diverse HCV genomes in Benin, replication is discussed in detail in section 4.3.4.

4.3.2 Diversity of HCV in Benin

Work presented in this chapter has been accepted for publication by the *Lancet Microbe* (manuscript reference THELANCETMICROBE-D-23-00339R1). Figures presented in this section are also presented in the manuscript. Study design, individual consent and demographic data is presented in the manuscript, however, this section of the thesis will purely focus on the genetic diversity of HCV in Benin. All whole HCV genomes from Benin presented in this work have been published in GenBank. The assigned accession numbers are listed in Table 4-8.

Table 4-8: Table of GenBank accession numbers assigned to whole HCV genomes from Benin

Genome ID	Accession number
Benin_1	OM525854
Benin_10	OM525855
Benin_11	OM525856
Benin_12	OM525857
Benin_14	OM525858
Benin_15	OM525859
Benin_16	OM525860
Benin_17	OM525861
Benin_18	OM525862
Benin_2	OM525863
Benin_22	OM525864
Benin_26	OM525865
Benin_27	OM525866
Benin_3	OM525867
Benin_30	OM525868
Benin_31	OM525869
Benin_33	OM525870
Benin_34	OM525871
Benin_35	OM525872
Benin_36	OM525873
Benin_38	OM525874
Benin_39	OM525875
Benin_4	OM525876
Benin_41	OM525877
Benin_42	OM525878
Benin_43	OM525879
Benin_45	OM525880
Benin_48	OM525881
Benin_49	OM525882
Benin_5	OM525883
Benin_51	OM525884

Benin_56	OM525885
Benin_6	OM525886
Benin_60	OM525887
Benin_64	OM525888
Benin_65	OM525889
Benin_67	OM525890
Benin_68	OM525891
Benin_7	OM525892
Benin_71	OM525893
Benin_73	OM525894
Benin_74	OM525895
Benin_77	OM525896
Benin_78	OM525897
Benin_8	OM525898
Benin_80	OM525899
Benin_81	OM525900
Benin_82	OM525901
Benin_84	OM525902
Benin_85	OM525903
Benin_88	OM525904
Benin_89	OM525905
Benin_9	OM525906
Benin_90	OM525907
Benin_92	OM525908
Benin_93	OM525909
Benin_97	OM525910

72% (57/79) of HCV PCR positive samples from individuals in Benin underwent successful whole HCV genome sequencing (sequencing was not attempted on samples with a Ct value of >30 cycles). 51% (29/57) of these were genotype 1, of which 86% (25/29) were previously unreported subtypes, including 3 new subtypes (1q, 1r and 1s). 14% (4/29) of the genotype 1 genomes were subtype 1b, a known epidemic subtype.

Figure 4-34 shows a phylogenetic tree demonstrating the diverse genotype 1 sequences in Benin. The 3 newly assigned genotype 1 subtypes, 1q, 1r and 1s, were more than 15% different at nucleotide level across the whole genome compared to reference sequences and less than 10% different when compared to each other. This is demonstrated in Figure 4-35.

The remaining 49% (28/57) of HCV genomes were all genotype 2. Of these, 50% (14/28) were previously uncharacterised subtypes, including the newly assigned

subtype 2xa. The remaining 50% of genotype 2 sequences were either 2b (2/28) or 2d. (12/28). The phylogenetic tree shown in

Figure 4-36 demonstrates the diversity of genotype 2 in Benin and Figure 4-37 shows how subtype 2xa was assigned, based on genetic distance to reference genotype 2 sequences, across the whole genome.

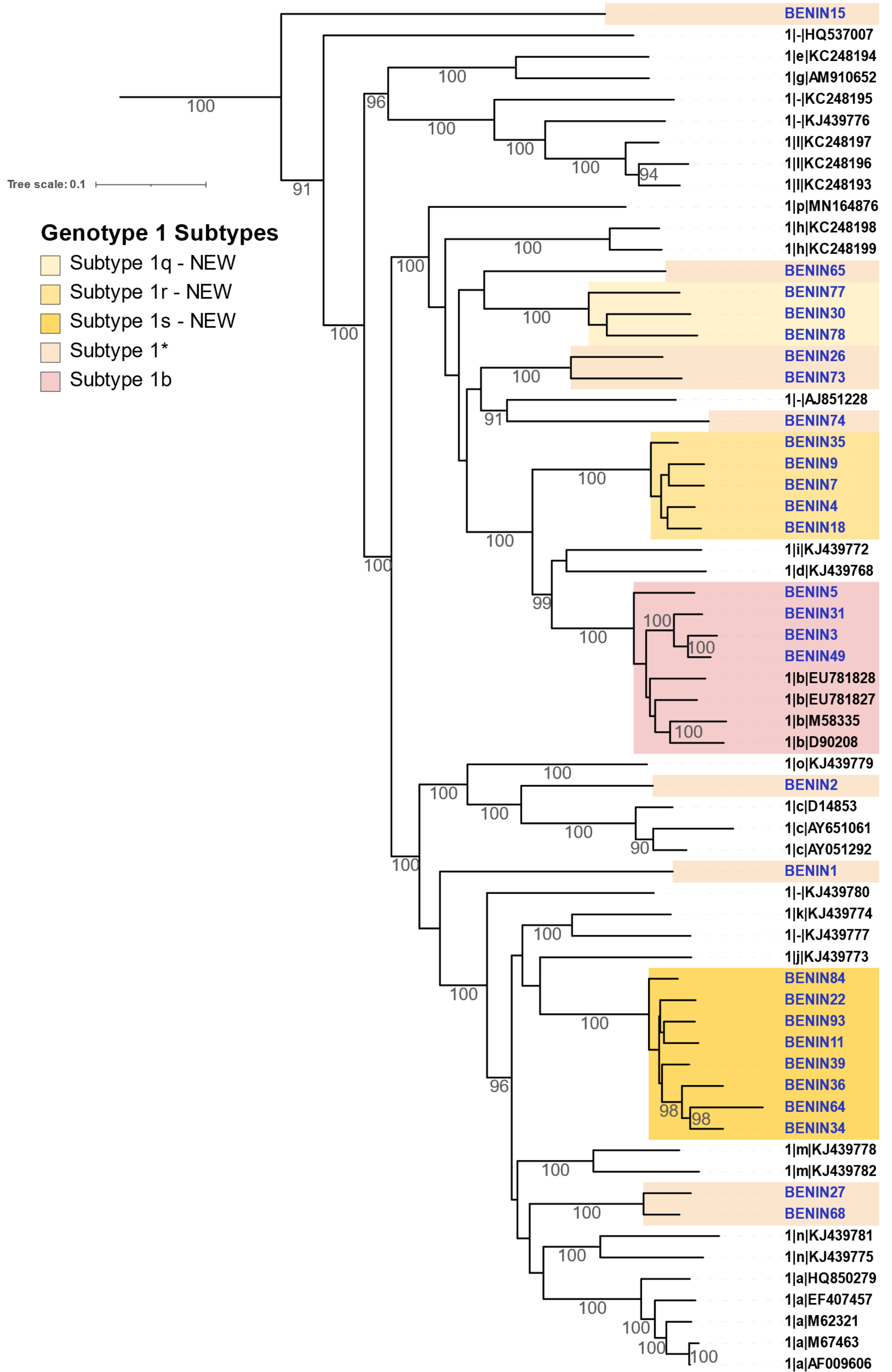
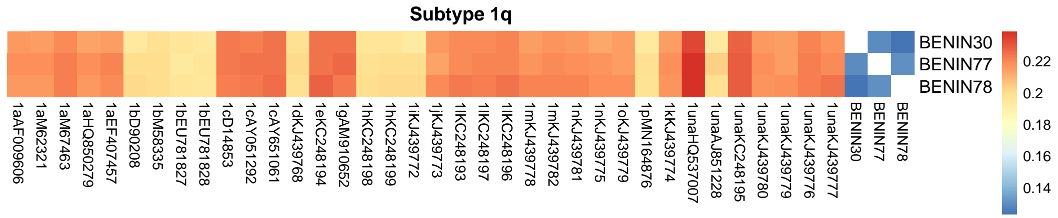
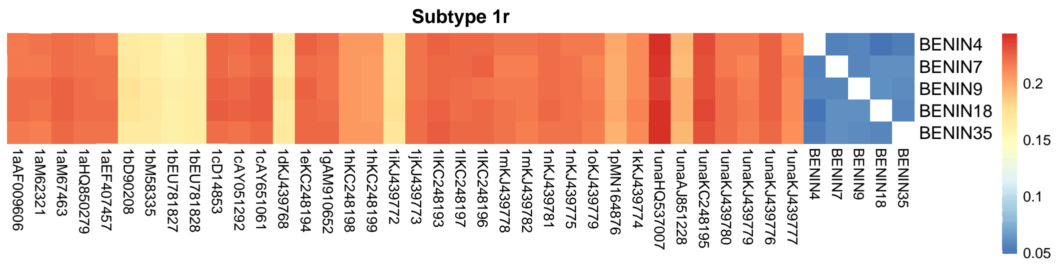

Tree scale: 0.1
Genotype 1 Subtypes
Subtype 1q - NEW
Subtype 1r - NEW
Subtype 1s - NEW
Subtype 1*
Subtype 1b
BENIN15
1|-|HQ537007
1|e|KC248194
1|g|AM910652
1|-|KC248195
1|-|KJ439776
1|l|KC248197
1|l|KC248196
1|l|KC248193
1|p|MN164876
1|h|KC248198
1|h|KC248199
BENIN65
BENIN77
BENIN30
BENIN78
BENIN26
BENIN73
1|-|AJ851228
BENIN74
BENIN35
BENIN9
BENIN7
BENIN4
BENIN18
1|i|KJ439772
1|d|KJ439768
BENIN5
BENIN31
BENIN3
BENIN49
1|b|EU781828
1|b|EU781827
1|b|M58335
1|b|D90208
1|o|KJ439779
BENIN2
1|c|D14853
1|c|AY651061
1|c|AY051292
BENIN1
1|-|KJ439780
1|k|KJ439774
1|-|KJ439777
1|j|KJ439773
BENIN84
BENIN22
BENIN93
BENIN11
BENIN39
BENIN36
BENIN64
BENIN34
1|m|KJ439778
1|m|KJ439782
BENIN27
BENIN68
1|n|KJ439781
1|n|KJ439775
1|a|HQ850279
1|a|EF407457
1|a|M62321
1|a|M67463
1|a|AF009606

Figure 4-34: Maximum likelihood phylogenetic tree of HCV genotype 1 sequences from Benin

HCV genotype 1 near-whole genome reference sequences listed on ICTV website were downloaded from GenBank and aligned with Benin genotype 1 sequences. Tips in blue represent Benin HCV genotype 1 sequences and are coloured by subtype. Subtype 1* sequences represent those that are not yet formally assigned a subtype as fewer than 3 examples of these sequences are published. Tip labels in black represent reference sequences, where "1|-|" denotes reference sequences that have not formally been assigned a subtype. Bootstrap values greater than 90% are shown.





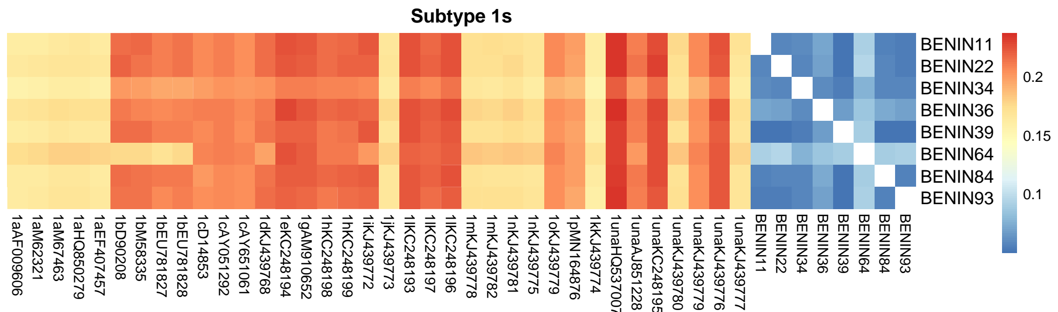
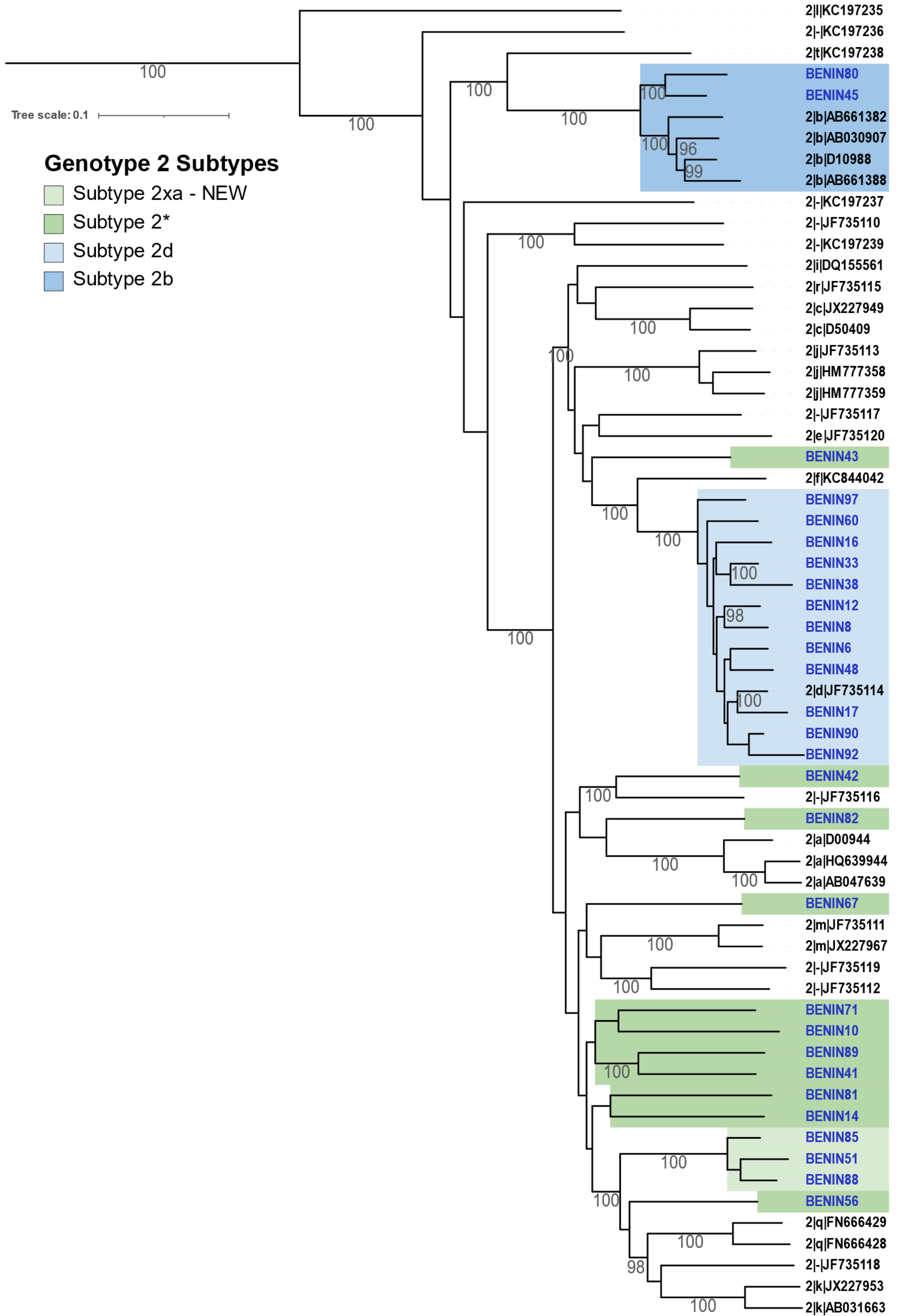


Figure 4-35: Heatmaps comparing genetic distance between HCV genotype 1 reference and Benin sequences

Heatmaps of pairwise distance measurements between genotype 1 reference sequences and Benin genotype 1 sequences representing the newly assigned subtypes 1q, 1r and 1s.



Tree scale: 0.1
Genotype 2 Subtypes
Subtype 2xa - NEW
Subtype 2*
Subtype 2d
Subtype 2b
2|l|KC197235
2|-|KC197236
2|t|KC197238
BENIN80
BENIN45
2|b|AB661382
2|b|AB030907
2|b|D10988
2|b|AB661388
2|-|KC197237
2|-|JF735110
2|-|KC197239
2|i|DQ155561
2|r|JF735115
2|c|JX227949
2|c|D50409
2|j|JF735113
2|j|HM777358
2|j|HM777359
2|-|JF735117
2|e|JF735120
BENIN43
2|f|KC844042
BENIN97
BENIN60
BENIN16
BENIN33
BENIN38
BENIN12
BENIN8
BENIN6
BENIN48
2|d|JF735114
BENIN17
BENIN90
BENIN92
BENIN42
2|-|JF735116
BENIN82
2|a|D00944
2|a|HQ639944
2|a|AB047639
BENIN67
2|m|JF735111
2|m|JX227967
2|-|JF735119
2|-|JF735112
BENIN71
BENIN10
BENIN89
BENIN41
BENIN81
BENIN14
BENIN85
BENIN51
BENIN88
BENIN56
2|q|FN666429
2|q|FN666428
2|-|JF735118
2|k|JX227953
2|k|AB031663

Figure 4-36: 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. Tip labels in blue represent genotype 2 sequences from Benin and they are coloured by subtype, including the newly assigned subtype 2xa. Subtype 2* sequences represent those that are not yet formally assigned a subtype as fewer than 3 examples of these sequences are published. Tip labels in black represent reference sequences, where “2|-|” denotes reference sequences that have not formally been assigned a subtype. Bootstrap values greater than 90% are shown.

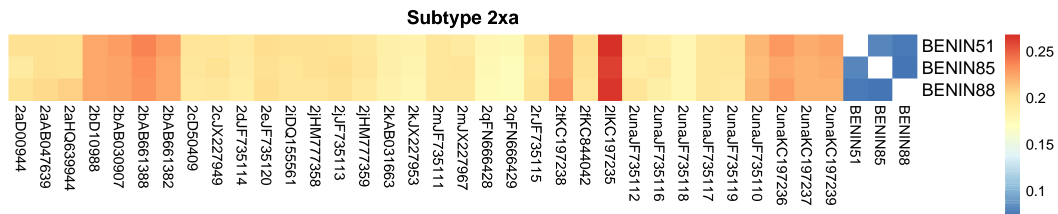


Figure 4-37: Heatmap comparing genetic distance between HCV genotype 2 reference and subtype 2xa

Heatmaps of pairwise distance measurements between genotype 2 reference sequences and subtype 2xa sequences from Benin.

4.3.3 RASs in Benin HCV Genomes

93% (53/57) of the sequenced HCV genomes from Benin had at least 2 RASs within NS5A. In this section I will describe the baseline RASs seen in the diverse genotype 1 and 2 genomes from Benin.

This work is part of a study from which a manuscript has been accepted for publication in the *Lancet Microbe* (in press). Overall, 52 individuals from Benin were treated in this study. The SVR12 rate was 94% (49/52). Genotype 1 infections were treated with Harvoni® (Ledipasvir 90mg/Sofosbuvir 400mg) and genotype 2 infections with Epclusa® (Velpatasvir 100mg/Sofosbuvir 400mg). Pre-treatment genome sequence data were available for 71% (37/52) of treated individuals.

4.3.3.1 RASs in Diverse Benin Genotype 1 Genomes

59% (16/27) of the diverse genotype 1 genomes had at least 2 baseline RASs. From these, 69% (11/16) had 4 RASs and 19% (3/16) had 5 RASs. The observed RASs were K24Q/R, M28L/V, Q30A/K/L/R/S, L31M, H58P/R/S, Y93H/N/S. These are summarised in Figure 4-38 and Table 4-9.

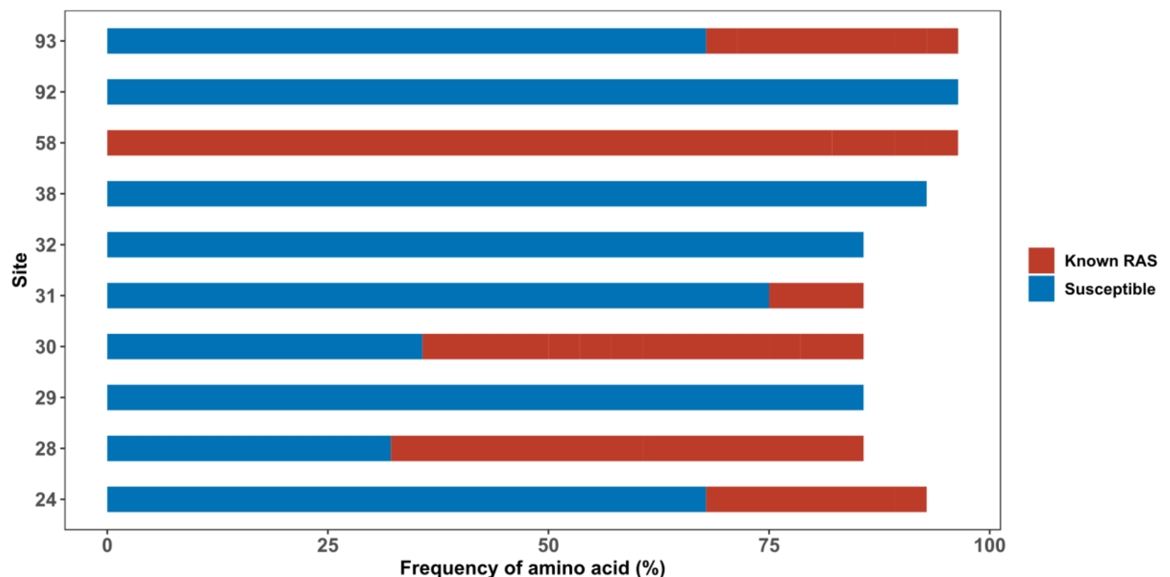


Figure 4-38: Frequency of known RASs seen in NS5A RAS sites among diverse genotype 1 samples from Benin

Susceptible amino acids at positions 24, 28, 29, 30, 31, 32, 38, 58, 92 and 93 are K, M, P, Q, L, P, S, H, A and Y, respectively.

Table 4-9: NS5A RASs in diverse HCV genotype 1 genomes from Benin
Amino acids coloured in red denote known RASs. Empty cells in the table reflect missing sequence data.

Genome ID	NS5A RAS Sites					
	24	28	30	31	58	93
Benin1	K				P	Y
Benin11	K	M	Q	L	P	Y
Benin18	K	V	S	L	P	N
Benin2	K	V	Q	L	P	Y
Benin22	K	M	Q	L	P	Y
Benin26	K	L	S	M	P	Y
Benin27	K	M	Q	L	P	Y
Benin3	Q	L	RQ	L	P	Y
Benin30	Q	L	R	L	R	Y
Benin31	Q	L	R	L	P	YH
Benin34					P	Y
Benin35	K	V	A	L	S	N
Benin36	K	M	Q	L	P	Y
Benin39	K	M	Q	L	P	Y
Benin4	K	V	A	L	P	N
Benin49	Q	L	QK	L	SP	Y
Benin5	Q	L	R	L	P	Y
Benin64	K	M	Q	L	P	Y
Benin65	K				P	H
Benin68	K	M	Q	L	P	Y
Benin7	K	V	A	L	P	N
Benin73	RK	V	R	L	P	S
Benin74	K	L	L	M	P	Y
Benin77						
Benin78	Q	L	K	M	R	Y
Benin84	K	M	Q	L	P	Y
Benin9	K	V	A	L	P	N
Benin93	K	M	Q	L	P	Y

Baseline RASs in NS3 were seen in a small proportion of genomes. 15% (4/27) had V36L/M and 15% (4/27) had Q80K. Of these, genome Benin78 had both RASs. There were also a small proportion of variants seen. V36I was seen in 7% (2/27), Y56F in 4% (1/27) and S122N in 4% (1/27) of genomes (Figure 4-39). No baseline RASs were seen in NS5B.

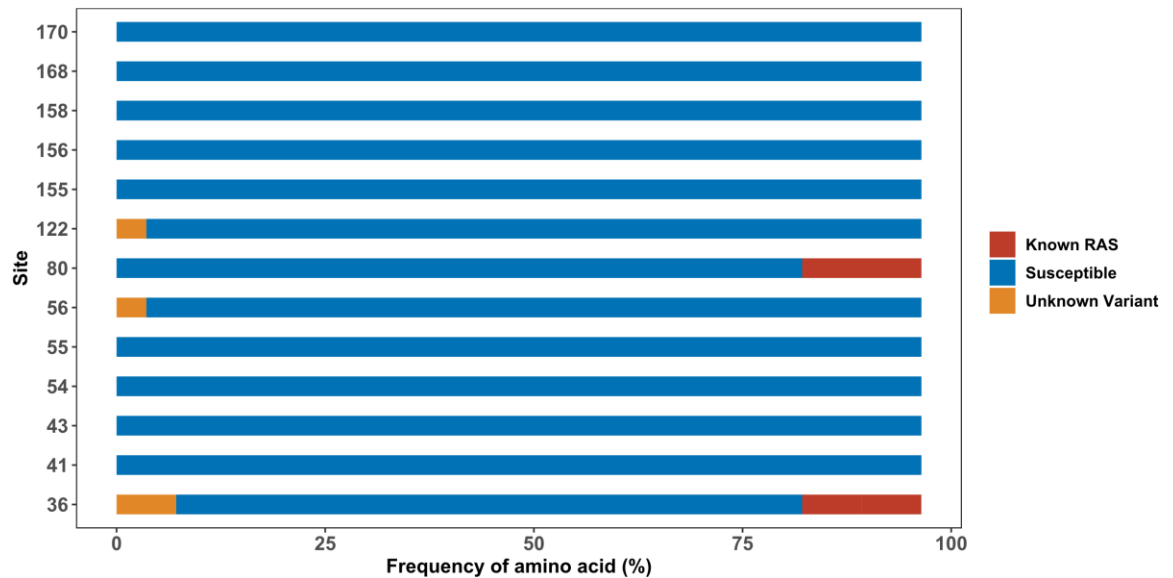


Figure 4-39: Frequency of known RASs seen in NS3 RAS sites among diverse genotype 1 samples from Benin
Susceptible amino acids at positions 36, 41, 43, 54, 55, 56, 80, 122, 155, 156, 158, 168 and 170 are V, Q, F, T, V, Y, Q, S, R, A, V, D and I/V.

4.3.3.2 RASs in Diverse Benin Genotype 2 Genomes

93% (26/28) of the genotype 2 genomes had at least 2 baseline RASs in NS5A (Figure 4-40). Genetic data within the important NS5A RAS sites was missing for 2 genomes. Of the 26 genomes for which genetic data was available, 58% (15/26) had 3 RASs and 4% (1/26) had 4 RASs (Table 4-10). Benin17 (subtype 2d), Benin38 (subtype 2d) and Benin45 (subtype 2b) are viruses from the 3 individuals who failed to achieve SVR12. Both Benin17 and Benin38 had NS5A RASs T24S and L30K. Benin 45 had NS5A RASs T24S, L30K and L31L/M.

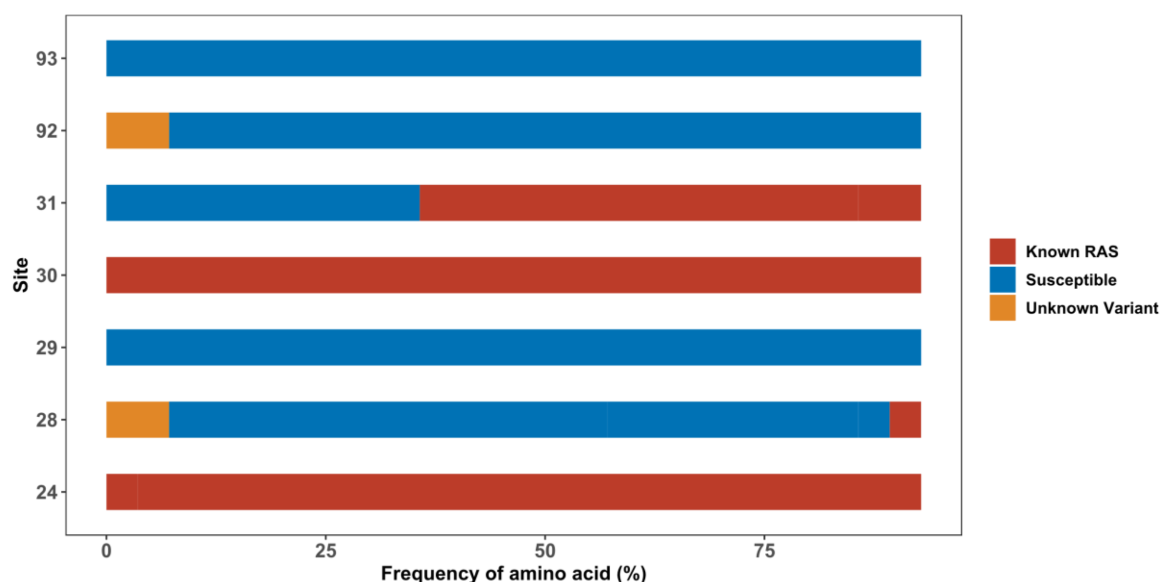


Figure 4-40: Frequency of known RASs seen in NS5A RAS sites among diverse genotype 2 samples from Benin
Susceptible amino acids at positions 24, 28, 29, 30, 31, 92 and 93 are T, L/F, P, L, L, C and Y, respectively.

Table 4-10: NS5A RASs in diverse HCV genotype 2 genomes from Benin
Amino acids coloured in red denote known RASs. Those coloured in blue denote variants of unknown significance at sites associated with resistance. The genome IDs highlighted in green (Benin17, Benin38 and Benin45) represent viruses from individuals that failed to achieve SVR12.

Genome ID	NS5A RAS Sites						
	24	28	29	30	31	92	93
Benin10	S	L	P	K	M	C	Y
Benin12	S	F	P	K	L	C	Y
Benin14	S	F	P	K	M	C	Y
Benin16	S	F	P	K	L	C	Y
Benin17	S	F	P	K	L	C	Y
Benin33	S	F	P	K	L	C	Y
Benin38	S	F	P	K	L	C	Y
Benin41	S	L	P	K	ML	C	Y
Benin42	S	L	P	K	L	C	Y
Benin45	S	LF	P	K	ML	C	Y
Benin48	S	F	P	K	L	C	Y
Benin51	S	L	P	K	M	C	Y
Benin56	S	C	P	K	M	C	Y
Benin6	S	F	P	K	M	C	Y
Benin60	S	F	P	K	M	C	Y
Benin67	S	C	P	K	M	S	Y
Benin71	S	L	P	K	M	C	Y
Benin8	S	F	P	K	L	C	Y
Benin80	S	L	P	K	M	C	Y
Benin81	S	F	P	K	M	C	Y

Benin82	S	L	P	K	L	C	Y
Benin85	S	F	P	K	M	S	Y
Benin88	S	L	P	K	M	C	Y
Benin89	A	V	P	K	M	C	Y
Benin90	S	F	P	K	M	C	Y
Benin97	S	F	P	K	L	C	Y

84% (21/25) of genomes had 1 baseline RAS in NS3, Y56F (Figure 4-41). No baseline RASs were seen in NS5B.

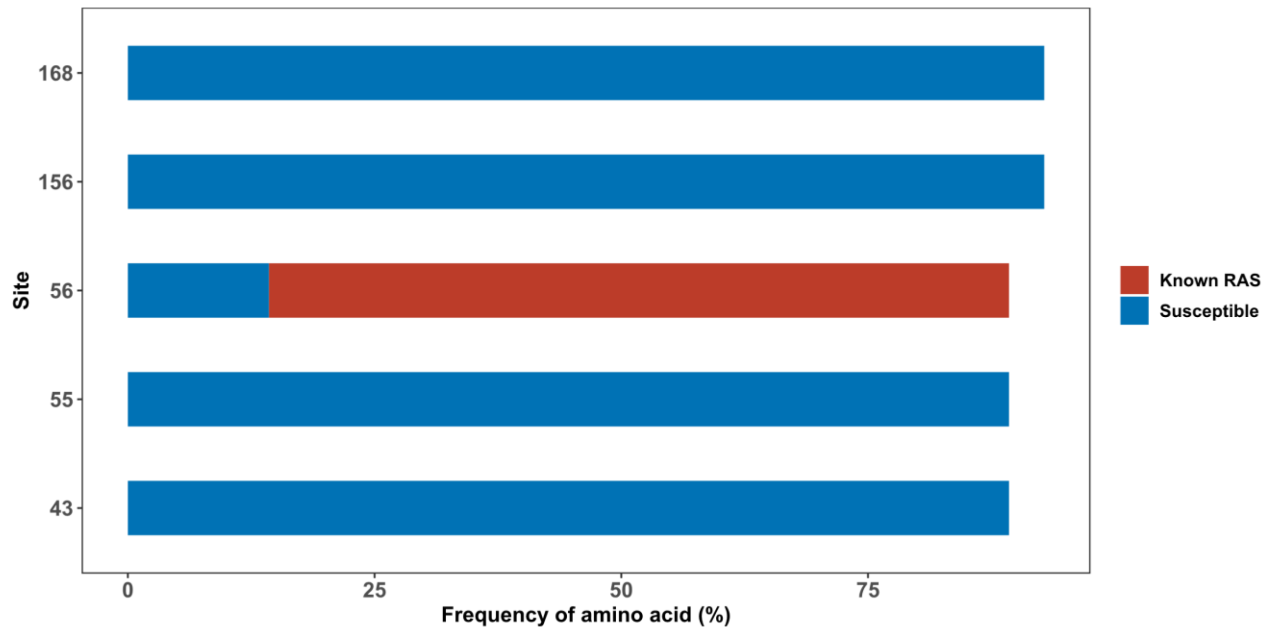


Figure 4-41: Frequency of known RASs seen in NS3 RAS sites among diverse genotype 2 samples from Benin
Susceptible amino acids at positions 43, 55, 56, 156, 158, 168 and 170 are F, V, Y, A, and D.

In the next chapter I will describe how baseline RASs in the NS5A gene may impact the effect of NS5A inhibitors on HCV replication using an *in-vitro* SGR system.

4.3.4 Benin Genotype 2 *in-vitro* Response

NS5A sequences from genotype 2 Benin genomes were selected and SGR constructs were made, replacing the wildtype NS5A sequence from pJFH-1 with the relevant Benin NS5A sequence. The 9 selected sequences included subtype 2d sequences from the 2 individuals that failed to achieve SVR12. The third individual who failed to achieve SVR12 was infected with subtype 2b. Unfortunately, an SGR construct harbouring this NS5A sequence failed to replicate. Other sequences included previously unassigned genotype 2 subtypes as well as those from individuals for whom treatment outcomes were not available. Overall, 9 SGR constructs were taken forward to assess replication capacity and the effectiveness of NS5A inhibitors, Ledipasvir, Pibrentasvir and Velpatasvir. Details of the SGR constructs used in the study are summarised in Table 4-11.

Table 4-11: SGR construct details including representative subtype and treatment outcomes, where applicable

* Wildtype subtype 2a virus used as a control.

**Controls with known resistance-associated substitutions. Two constructs were made; RAS_Control1 contained NS5A polymorphisms F28S+L31I and RAS_Control2 contained NS5A polymorphisms P29S+K30G.

***GND is used as non-replicating control. It is JFH-1 with a single mutation in the NS5B gene that renders it non-replicative.

SGR NS5A Construct	Subtype	Treatment Outcome
Benin6	2d	SVR12
Benin8	2d	SVR12
Benin16	2d	SVR12
Benin17	2d	Failure
Benin38	2d	Failure
Benin43	2*	Untreated
Benin48	2d	SVR12
Benin81	2*	Untreated
JFH-1*	2a	N/A
RAS_Control1**	2a	N/A
RAS_Control2**	2a	N/A
GND***	2a	N/A

The replication capacities of each of the constructs were assessed and compared to that of JFH-1. Three SGR constructs (Benin48, Benin43 and Benin81) were ≥1.5 times more efficient at replicating than JFH-1. Benin17 and Benin38, both

viruses from individuals that failed to achieve SVR12, were 25%-50% less efficient at replicating than JFH-1. These findings are visualised in Figure 4-42.

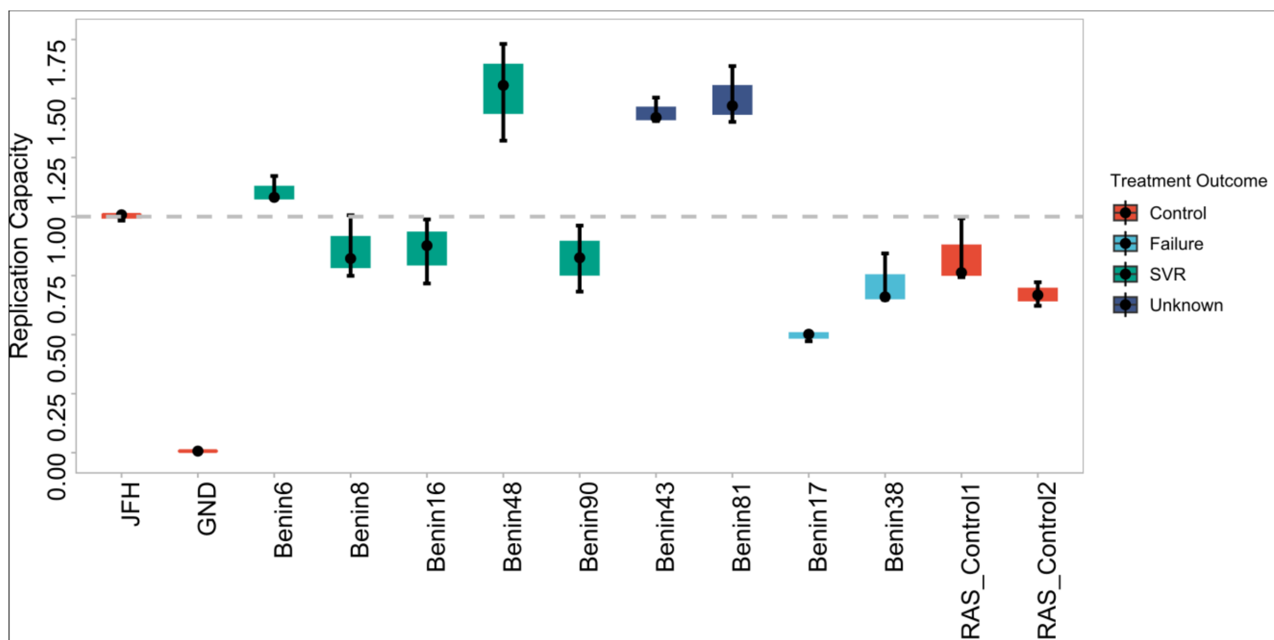


Figure 4-42: Replication capacities of Benin genotype 2 SGR NS5A constructs
Replication capacities of nine Benin SGR NS5A constructs, coloured by treatment outcome. GND is a non-replicating SGR construct. RAS_Control1 contained the NS5A polymorphisms F28S+L31I and RAS_Control2 contained NS5A polymorphisms P29S+K30G.

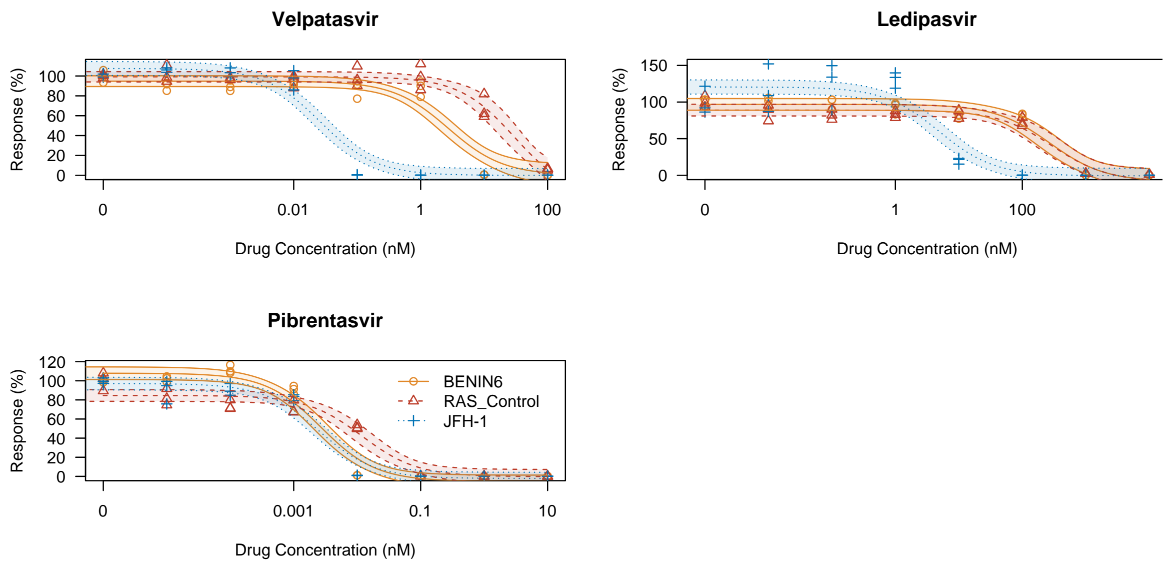
The *in-vitro* efficacy of Ledipasvir, Velpatasvir and Pibrentasvir, was measured against each genotype 2 construct. Drug response curves for each construct, compared to JFH-1 and controls are shown in Figure 4-43. IC_{50} fold-changes for each construct, relative to JFH-1, were calculated and are shown in Figure 4-44. As expected, all Benin SGR constructs viruses showed increased IC_{50} fold changes when challenged with Ledipasvir, which is not recommended for treatment of genotype 2 infections by EASL and WHO [159, 286].

None of the SGR constructs showed increases in IC_{50} fold change comparable to the RAS_Control1 when challenged with Velpatasvir. Although the individual infected with the virus Benin6 did achieve SVR12, this SGR construct showed a 100-fold increase in IC_{50} (IC_{50} = 2.71nM, 95% CI = 1.43nM to 3.99nM) compared to JFH-1 (IC_{50} = 0.03nM, 95% CI = 0.02nM to 0.04nM). Benin43 (IC_{50} = 0.42nM, 95% CI = 0.27nM to 0.58nM) and Benin81 (IC_{50} = 1.46nM, 95% CI = 0.85nM to 2.07nM) also showed increases in IC_{50} of approximately 15-fold and 50-fold respectively, but the individuals infected with these viruses have not yet been treated. The

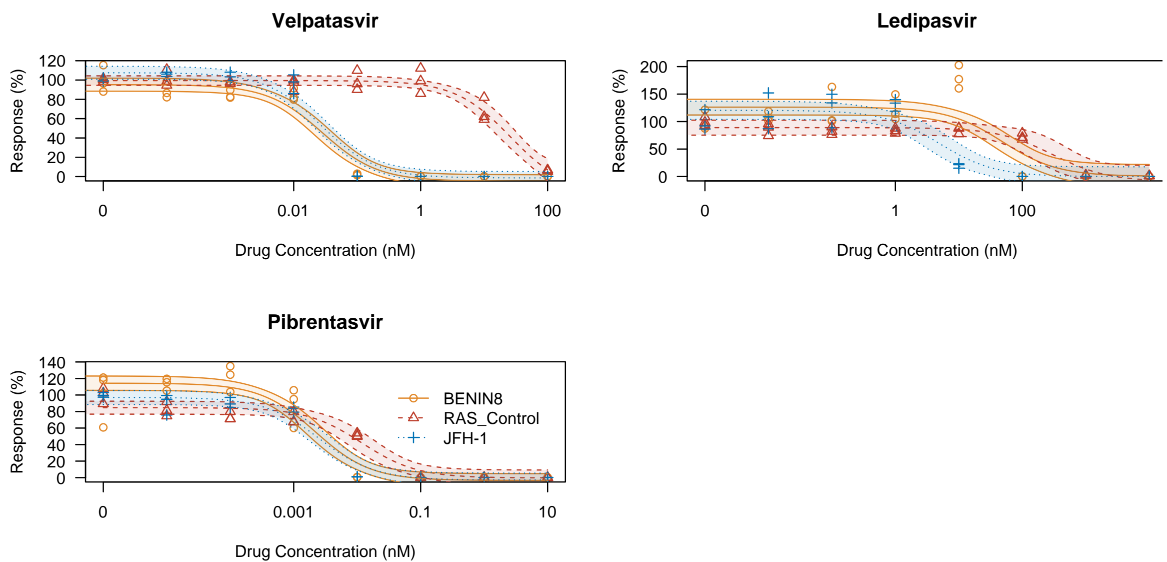
RAS_Control SGR construct (IC_{50} = 29.82nM, 95% CI = 4.26nM to 55.39nM) showed a 1000-fold increase in IC50 compared to JFH-1. Interestingly, Benin17 (IC_{50} = 0.02nM, 95% CI = 0.01nM to 0.03nM) and Benin38 (IC_{50} = 0.02nM, 95% CI = 0.01nM to 0.03nM), the viruses infecting individuals who failed to achieve SVR12, showed comparable responses to Velpatasvir as JFH-1.

Pibrentasvir showed excellent activity against all Benin virus constructs (Figure 4-44).

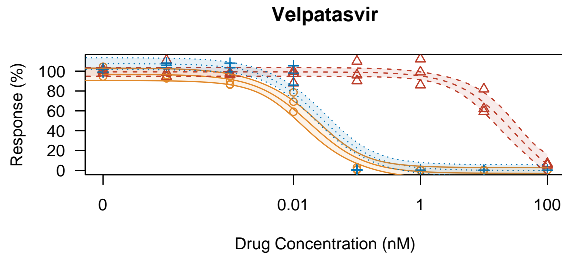
Benin6

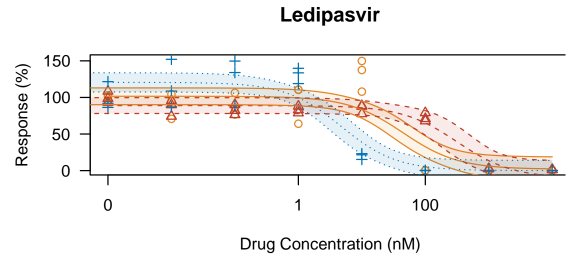


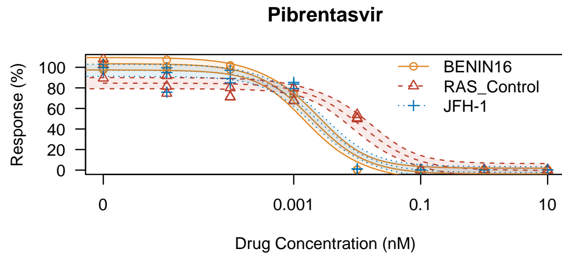
Benin8



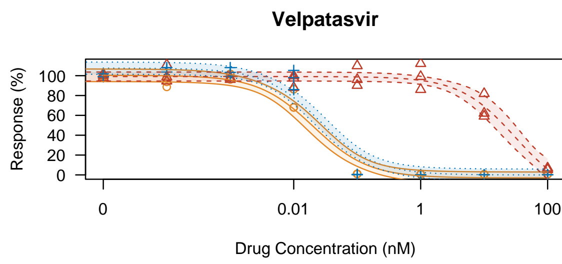
Benin16

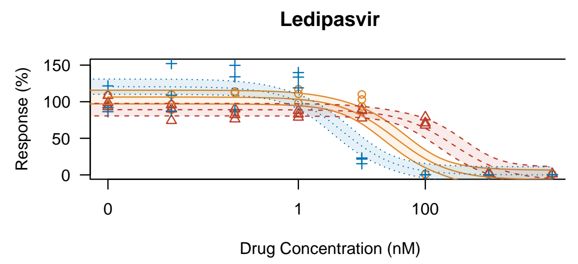


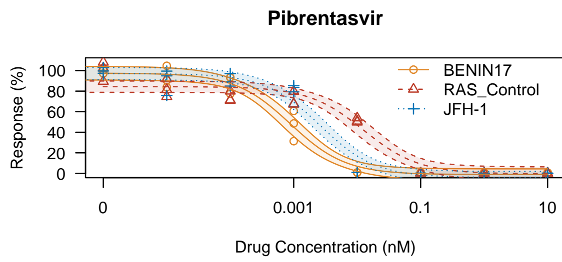




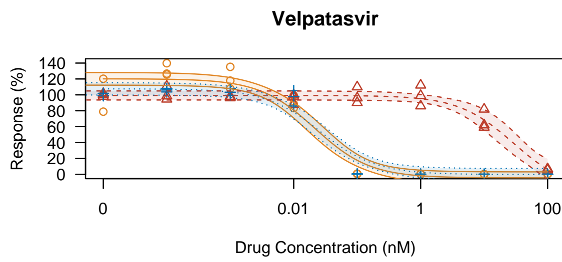
Benin17

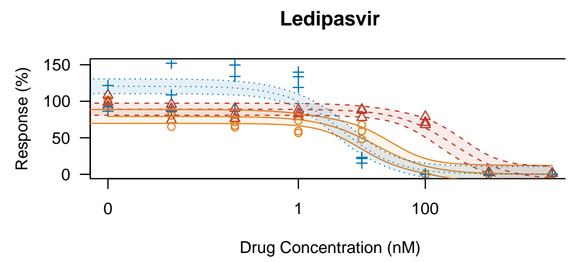


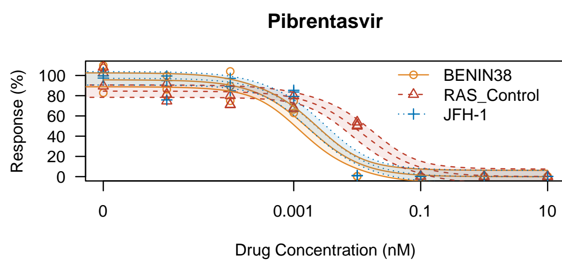




Benin38







Benin43

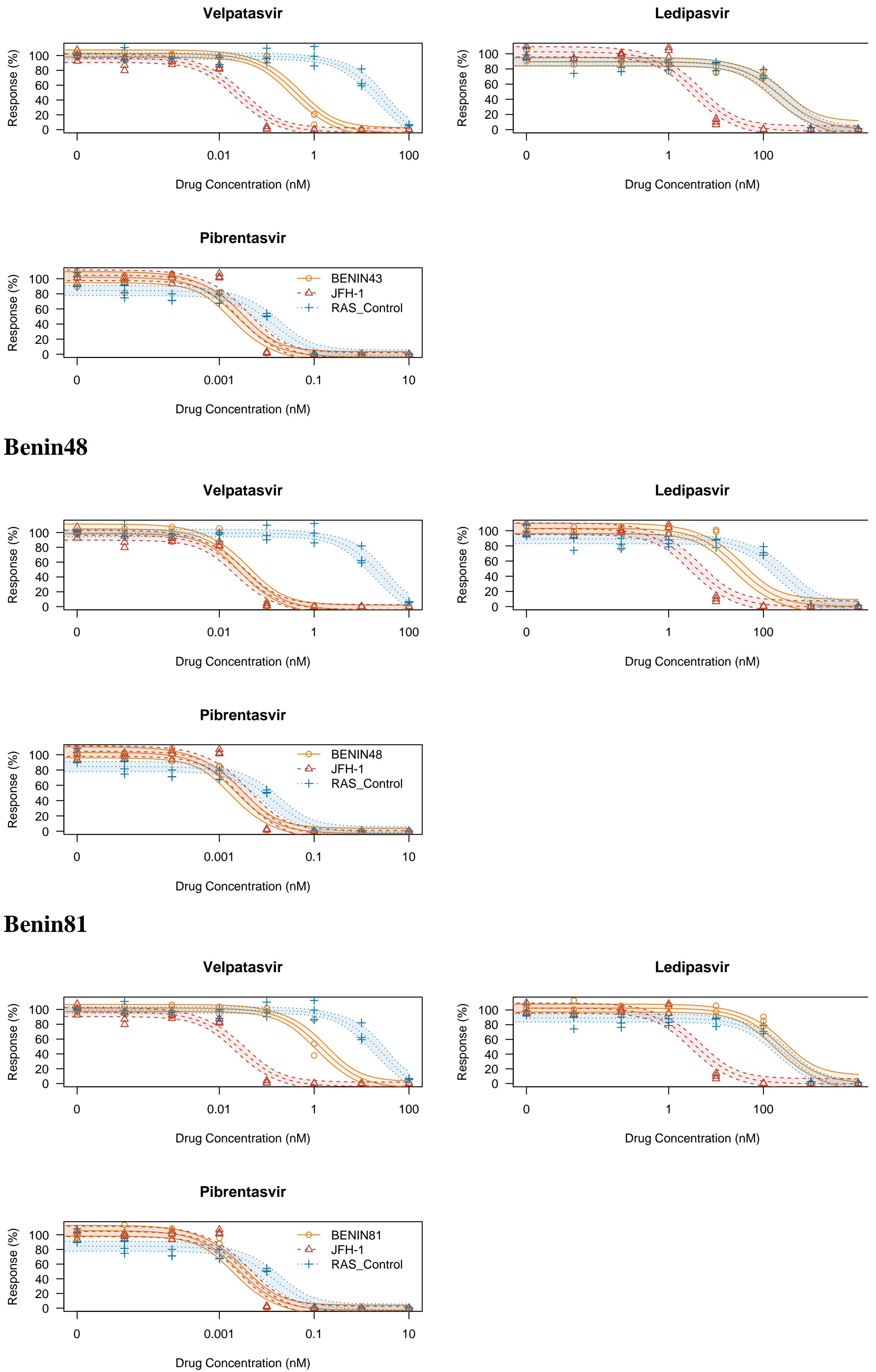


Figure 4-43: Drug response curves for Benin SGR NS5A constructs

Drug response curves are shown for each construct compared to wildtype (JFH-1) and a RAS control construct when challenged with either Velpatasvir, Ledipasvir or Pibrentasvir. RAS_Control1, containing the NS5A polymorphisms F28S+L31I, was used for challenge with Pibrentasvir and RAS_Control2, containing NS5A polymorphisms P29S+K30G, was used for challenge with Ledipasvir and Velpatasvir. Shaded intervals represent 95% confidence intervals.

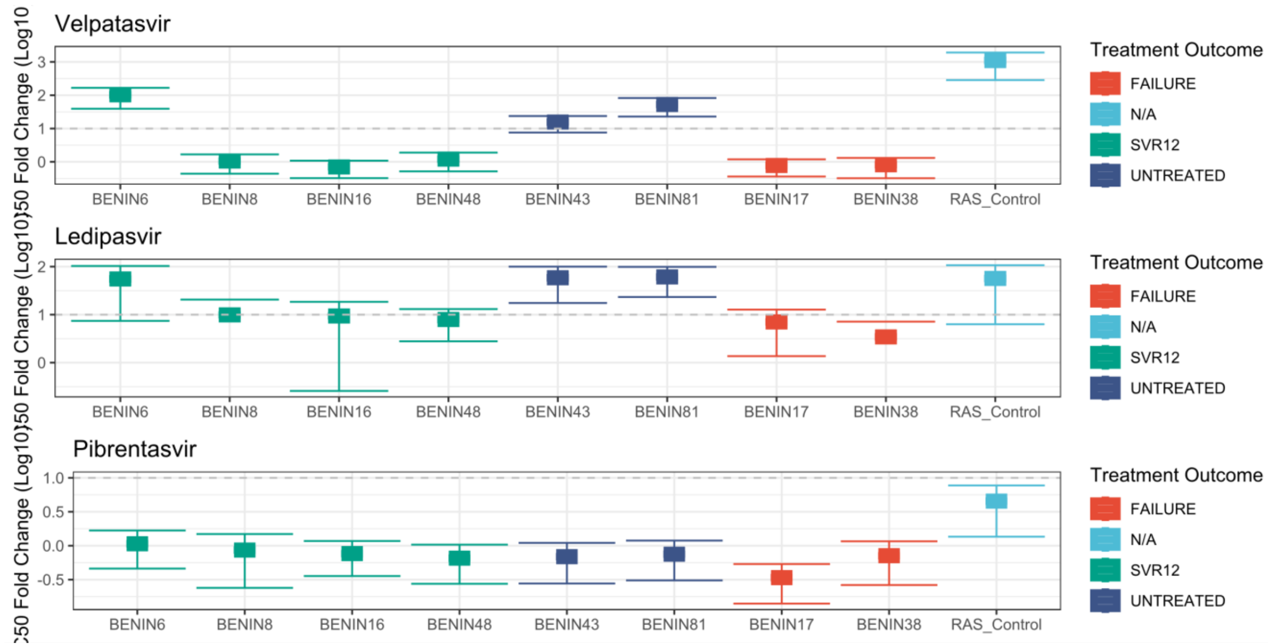


Figure 4-44: IC50 fold changes for each SGR NS5A construct following challenge with Ledipasvir, Velpatasvir and Pibrentasvir
Constructs are coloured by treatment outcome. RAS_Control1, containing the NS5A polymorphisms F28S+L31I, was used for challenge with Pibrentasvir and RAS_Control2, containing NS5A polymorphisms P29S+K30G, was used for challenge with Ledipasvir and Velpatasvir.

4.4 HCV 2a in Glasgow PWID

This chapter discusses a group of HCV 2a infected PWID in Glasgow, some of whom failed treatment with Glecaprevir/Pibrentasvir.

4.4.1 Glasgow Sub-genotype 2a Outbreak in PWID

This study looked at a group of 123 individuals in Glasgow, Scotland, who were all infected with HCV sub-genotype 2a and treated with Maviret® (Glecaprevir 100mg/Pibrentasvir 40mg). 70% of these individuals were PWID. The overall SVR12 rate was 91% (112/123), which is less than what is reported in clinical trials and real-world studies. This work has been published in *Viruses*, 2022 [287]. Whole HCV genomes generated in this study have been published in GenBank and the assigned accession numbers to these genomes can be found in Table 4-12. This section will focus on the *in-vitro* work that formed part of this study.

Table 4-12: Table of GenBank accession numbers assigned to whole HCV genomes from the sub-genotype 2a outbreak in Glasgow, Scotland

Genome ID	Accession number
P1	OP022902
P2	OP022903
P3	OP022904
P4	OP022905
P5	OP022906
P6	OP022907
P7	OP022908
P8	OP022909
P9	OP022910
P10	OP022911
P11	OP022912
P12	OP022913
P13	OP022914
P14	OP022915
P15	OP022916
P16	OP022917
P17	OP022918
P18	OP022919
P19	OP022920
P20	OP022921

A cluster of HCV sub-genotype 2a included 2 individuals, P19 and P20, who both failed to achieve SVR12, as well as individuals P6 and P10 who both achieved SVR12. Phylogeny of this cluster is shown in Figure 4-46and the whole genotype 2 clade is shown in Figure 4-45.

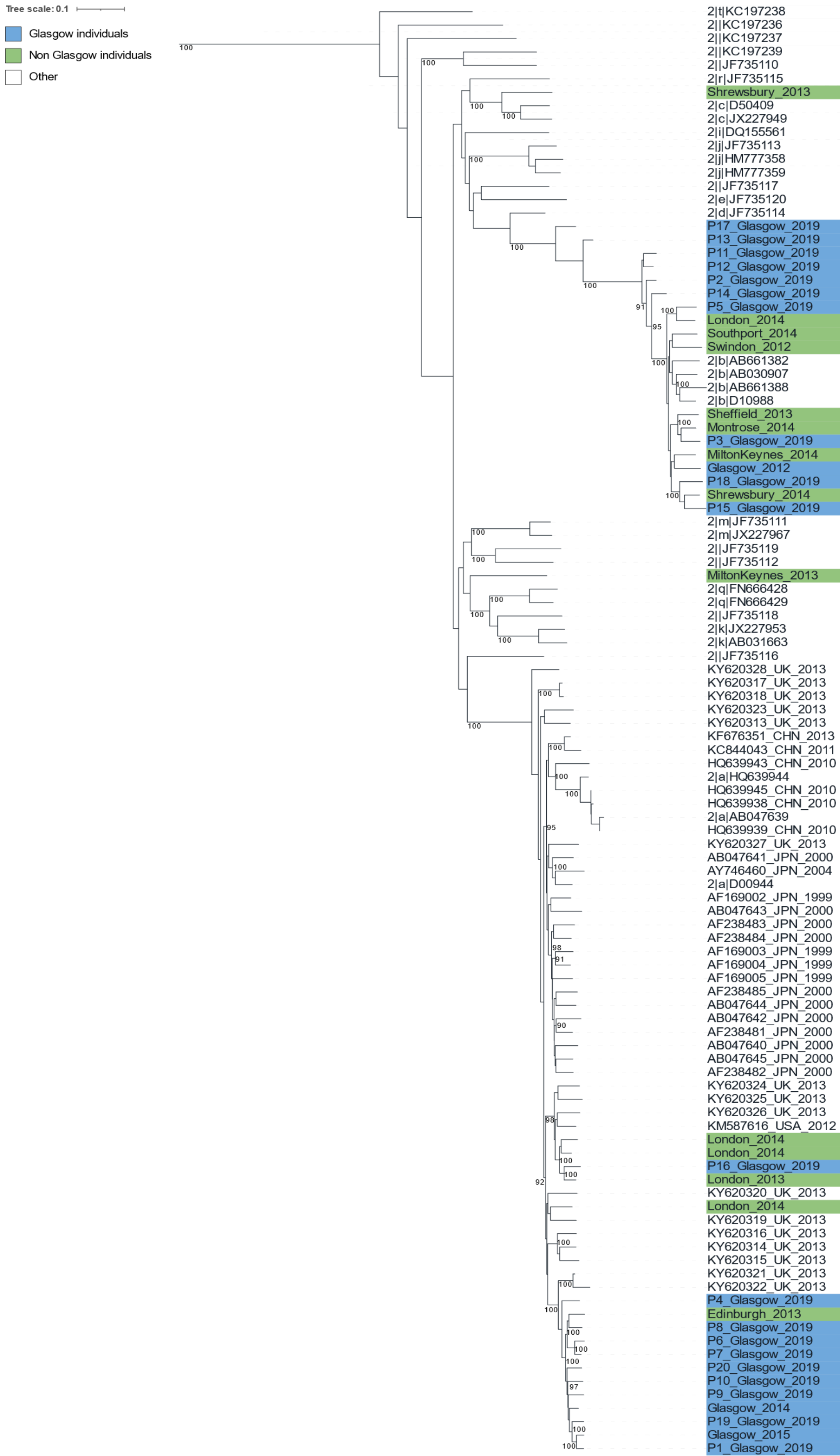


Figure 4-45: Maximum likelihood phylogenetic tree showing the HCV genotype 2 clade and highlighting genomes sequenced as part of this study

Highlighted tip labels distinguish between UK individuals from Glasgow and those outside Glasgow. The sub-genotype 2a cluster, including the individuals who failed to achieve SVR12 is at the bottom of the tree. Non-highlighted tip labels represent reference HCV genotype 2 genomes, that were downloaded from ICTV (https://talk.ictvonline.org/ictv_wikis/flaviviridae/w/sg_flavi/54/hepaciviruses, accessed April 27th 2022), as well as other whole genotype 2 genomes downloaded from GenBank [288]. The phylogenetic tree was constructed with 1000 bootstrap replicates. Bootstrap values greater than 90% are shown.

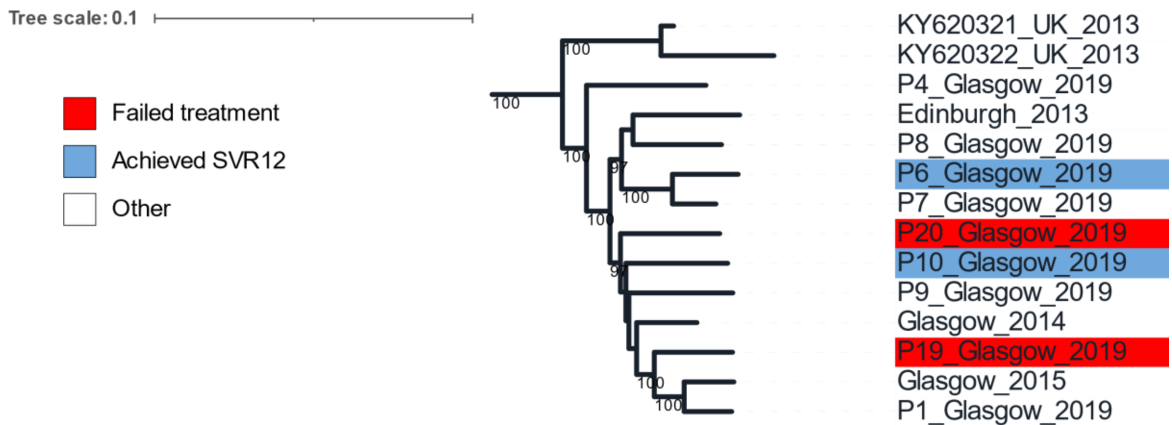


Figure 4-46: Maximum likelihood tree showing the HCV sub-genotype 2a cluster from Glasgow

HCV 2a from individuals that achieved (P6 and P10) and failed to achieve SVR12 (P19 and P20) are highlighted. Non-highlighted tip labels represent other sub-genotype 2a genomes from Scotland, as well as 2 genomes downloaded from GenBank that were also from individuals in UK [289] . The phylogenetic tree was constructed with 1000 bootstrap replicates. Bootstrap values greater than 90% are shown.

None of the known NS3 RASs (A156T/V, D168E/V), associated with resistance to Glecaprevir, were noted in individuals who failed to achieve SVR12. The NS5A RAS L31M was seen in the viruses infecting both individuals who failed to achieve SVR12, but also in those viruses infecting other individuals, within the 2a cluster, who achieved SVR12.

When looking at the overall prevalence of methionine at position 31 in NS5A in all sub-genotype 2a sequences publicly available as well as those sequenced in this study, it was noted that this amino acid was dominant (85%, 125/147), whereas leucine was only prevalent in 15% (22/147) of sub-genotype 2a sequences.

Looking in more detail at the NS5A sequences of HCV 2a infecting P19 and P20 compared to HCV 2a infecting P6 and P10, it was noted that NS5A polymorphisms R353K and P407L were unique to P19 and P20. Looking at the prevalence of these polymorphisms in the remaining sequences in the 2a cluster shown in Figure 4-46, R353K and P407L were present in 90% (9/10) and 70% (7/10), respectively. When looking at all publicly available NS5A sequences from HCV sub-genotype 2a sequences, the prevalence of R353K and P407L was 4% (11/257) and 54% (138/257), respectively.

SGR constructs with NS5A polymorphisms R353K and P407L were synthesised and their replication capacities were compared to pJFH-1, which represented wild-type sub-genotype 2a (see section 3.5.10), and control SGR constructs harbouring known NS5A RASs, i.e. F28S and L31I, as well as P29S and K30G. Replication capacities are shown in Figure 4-47. The control SGR constructs had greater replication capacities than pJFH-1 (median replication capacities of 1.3 for Control_1 and 1.2 for Control_2). The SGR construct harbouring P407L also had an increased replication capacity (median 1.4) compared to pJFH-1. The SGR construct harbouring R353K had a similar replication capacity to pJFH-1.

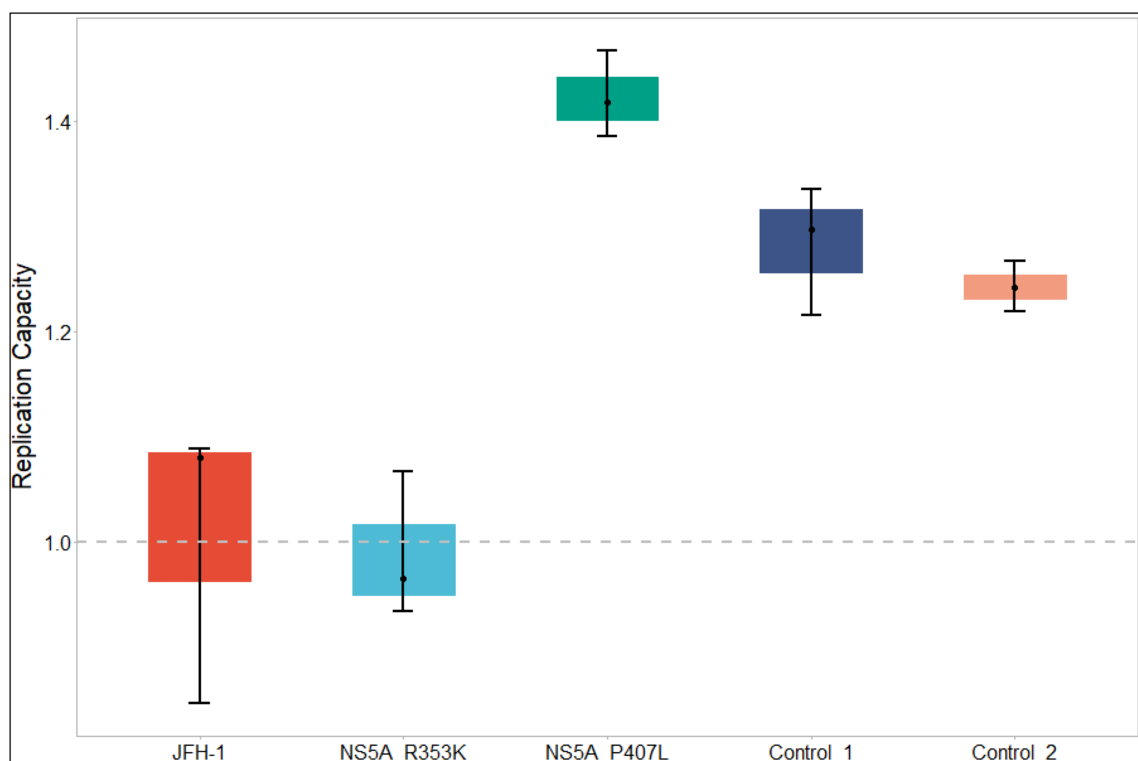
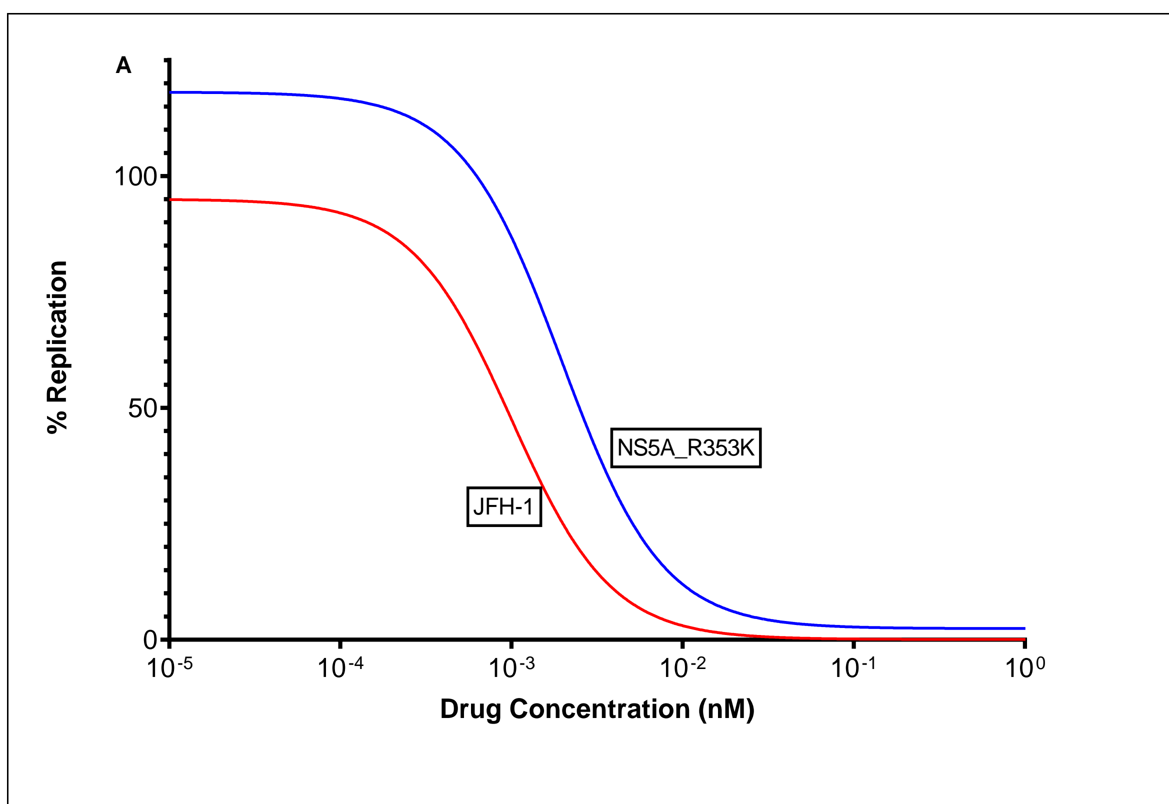


Figure 4-47: Replication capacities of SGR constructs

Replication capacities of pJFH-1, SGR constructs harbouring NS5A polymorphisms R353K and P407L, and controls. Control_1 harbours the known RASs F28S and L31I. Control_2 harbours the known RASs P29S and K30G.

The efficacy of Pibrentasvir was tested on the SGR constructs mentioned above and IC50 drug response curves are shown in Figure 4-48. The IC50 value for SGR construct NS5A_R353 (IC50 = 2.42×10^{-3}, 95% CI = 9.54×10^{-4} to 6.546×10^{-3}, R^2 = 90.5%) was 2-fold higher than the IC_{50} value for JFH-1 (IC50 = 9.24×10^{-4}, 95% CI = 6.68×10^{-4} to 1.28×10^{-3}, R^2 = 99%). The IC_{50} value for P407L was comparable to that of JFH-1.



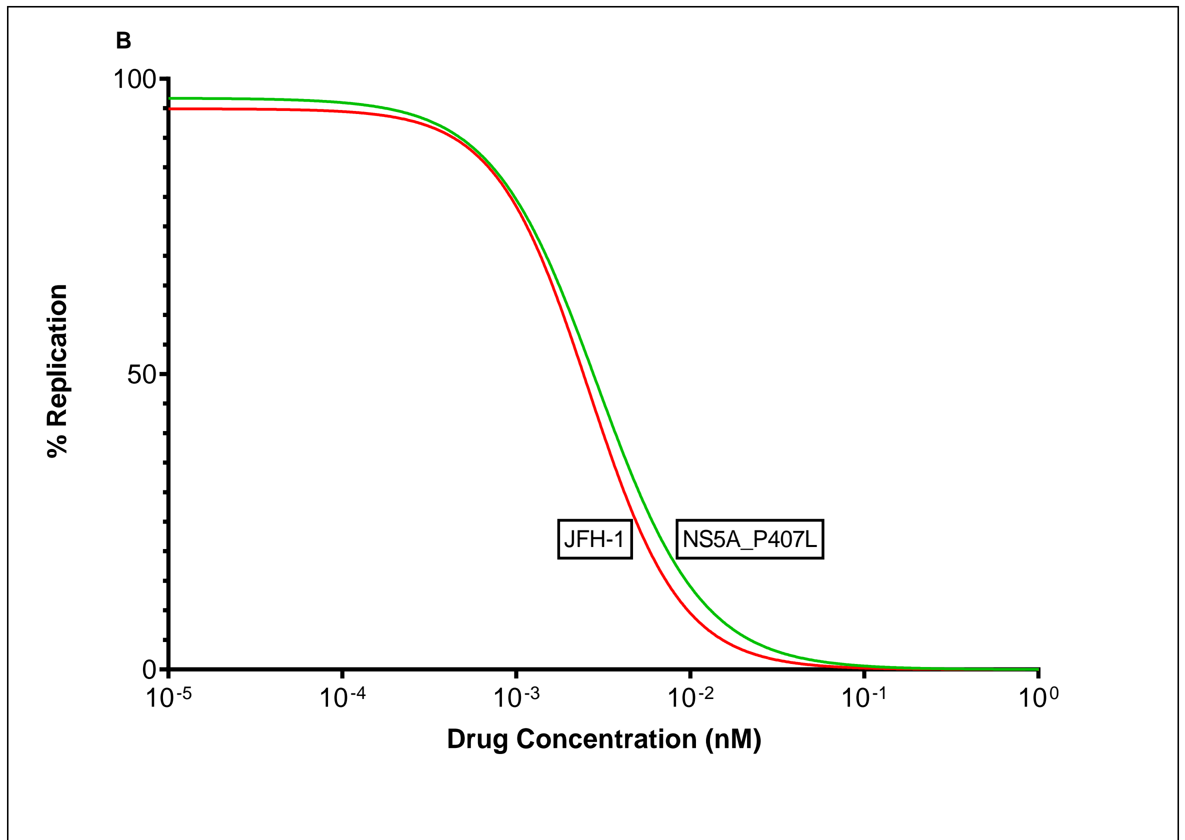


Figure 4-48: IC50 drug (Pibrentasvir) response curves
Drug response curves are shown for SGR constructs harbouring NS5A polymorphisms R353K (panel A) and P407L (panel B).

5 Discussion

5.1 PWID and HCV Associated Liver Disease in Kenya

HCV infection in PWID in SSA is a neglected public health issue. The Kenyan Government has increased efforts to improve health in PWID across the country by providing needle and syringe programmes and opioid substitution, since July 2012, as well as screening for HIV. However, HCV infection screening is lacking and this study has provided evidence as to the scale of the issue, with a 36% (143/400) HCV seroprevalence in recruited PWID. HCV-HIV co-infection was also high (34%) with similar findings of 29% in another Kenyan study [290]. Approximately a third (34%, 135/400) of the study participants were on methadone therapy. Importantly, 92% (369/400) of recruited PWID had never been tested for HCV previously and had not heard about HCV infection and its consequences, reflecting a need to step up public health efforts in the fight against viral hepatitis in this community. Furthermore, 95% (378/400) of study participants had spent time in jail or prison, reflecting Kenyan law, which criminalises any form of drug use. Studies across the globe have shown an association between incarceration and HIV and/or HCV infection among PWID [291].

There was striking geographical variation in HCV seroprevalence in this study, probably explained by seasonal habits. In Mombasa HCV seroprevalence was 59% (118/200) whereas in Watamu, where most participants reported injecting heroin use predominantly during the peak tourist season (July, August and December), it was 13% (25/200). Akiyama et al also found a high seroprevalence of HCV among PWID in their study in Kenya, 13% overall, but 22% in Coastal Kenya [290].

Older age, single status, achieving a secondary level of education, sharing needles and being part of a group of injectors with new members each month (Table 4-2) were all independently associated with HCV seropositivity. Older members were likely to have started injecting before the introduction of needle exchange programs. The higher odds of HCV seropositivity found in those that had achieved a higher level of education has also been reported in Ghana [292]

and we also found that in the Benin study. The reason behind this is unclear, but warrants further investigation.

In our study we also assessed the prevalence of advanced liver fibrosis associated with HCV infection by use of WHO approved methods, APRI and FIB-4 scores [159]. This is the first study to report on HCV associated liver fibrosis in Kenyan PWID. Almost half of the individuals (46%) who were HCV RNA positive had evidence of advanced liver fibrosis (Table 4-3). Unfortunately, it was not possible to confirm these findings with a more accurate method of assessment such as elastography (FibroScan®). There are limitations in the performance of APRI and FIB-4 scores, especially in HIV-HCV co-infection [293, 294]. However, these scores have value in ruling out liver cirrhosis [264] and therefore selecting out individuals who would need further investigation to confirm liver fibrosis of any stage. It is important to highlight that in this study participants were not tested for HBV infection due to lack of availability of POC test kits or serological assays.

5.2 HCV Diversity in Kenya

In this study of Kenyan PWID HCV sub-genotypes 1a and 4a are the dominant lineages. These are well characterised epidemic lineages that are dominant across the globe. Similar findings were reported by Akiyama et al [290] and in an earlier study of HCV in Kenyan PWID [295]. Globally sub-genotype 1a is the most prevalent lineage in PWID, and in Africa, 1a and 4a, are the most frequently observed lineages in PWID [296].

Heroin has been available as a street drug at the Kenyan coast since the 1980s and this has been linked to the tourist boom when Italian investors set up businesses in the region with local partners [297]. Until 1999, the main method of heroin use was inhalation of vapour or smoking. The type of heroin available in the region at the time was locally known as “brown sugar.” During this time supply chains of heroin from Asia changed and a different form of heroin, locally known as “white crest”, began to replace “brown sugar” [298]. This was a type of heroin that could not be inhaled or smoked and thus the shift in practice to injecting heroin took off [299]. While heroin supply to Kenya, and indeed most of the world, is almost entirely from Asia [300, 301], heroin use among locals

was initiated through European and American tourist contacts [297]. This is supported by phylogenetic analysis (Figure 4-9 and Figure 4-10) of HCV sub-genotype 1a, which suggests two introductions of HCV sub-genotype 1a into the Coastal Kenyan community of PWID. Molecular clock analysis of the larger Kenyan 1a subcluster supports a recent introduction (as recent as 2001) from Central Europe (Switzerland) likely through shared injecting drug use with tourists (Figure 4-13).

Phylogenetic analysis of HCV genotype 4a suggests a single introduction to Kenyan PWID from Egypt (Figure 4-11). There are HCV 4a genomes from Netherlands and USA that sit deeper in the 4a clade on the tree, however, the closest related 4a genome to the Kenyan 4a cluster is from Egypt. Egypt has the highest prevalence of HCV in the world and this has been linked to unsafe medical practices through its mass parenteral anti-schistosomal therapy programs, which were only stopped in the 1980s [120]. While genotype 4 is likely to have originated in Central and East Africa given the diversity of endemic genotype 4 subtypes present in the region [302], sub-genotype 4a spread into Egypt and further afield to become the most dominant genotype 4 subtype globally. Indeed, it is found widely throughout the Middle East [303]. The coastal communities in Kenya, mainly the Swahili peoples, have had a long history of trade with the Arab world across the Indian Ocean with many communities from the Middle East integrating and settling into the coastal region of modern day Kenya, at least as early as the eight century C.E [304]. In more recent times there has been an increase in bilateral movement between coastal Kenyan and middle eastern communities and it is estimated that there are up to 120,000 Kenyan working migrants residing in the Middle East [305]. It is therefore possible that HCV sub-genotype 4a was introduced to Kenyan PWID from Egypt via the Middle East. Molecular analysis supports an introduction of 4a into Kenyan PWID around 1980 (Figure 4-14). This may be via iatrogenic spread or via the Middle East, however, sampling of the general population along Coastal Kenya and communities in the Middle East would provide more clarity.

Figure 5-1 shows a diagrammatic suggestion of the spread of HCV genotype 4 from Central and East Africa to Egypt where it diverged into sub-genotype 4a,

through iatrogenic transmission. HCV 4a became a founder virus and was exported globally, including back into East Africa.

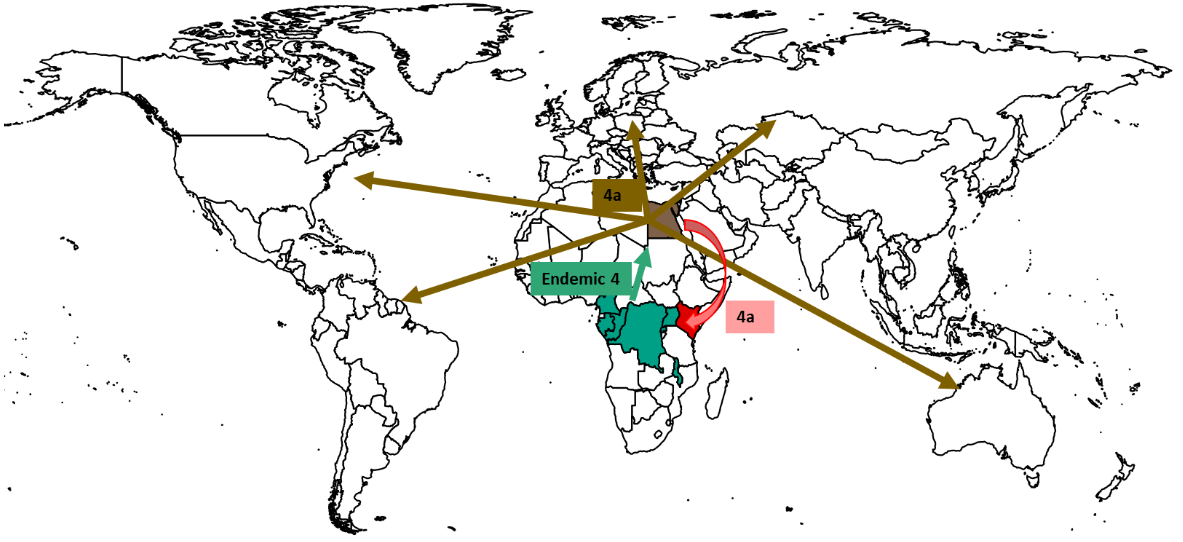


Figure 5-1: The global spread of HCV genotype 4 and introduction of 4a into Kenya

Importantly, as highlighted in the introduction, there is a paucity of HCV genomes from SSA. In Kenya, before this study, there were no published whole HCV genomes. This study has generated 49 whole HCV genomes from Kenya and will be submitted to GenBank in due course.

5.3 HCV Diversity in Benin

In the Benin study a high diversity of HCV genotypes 1 and 2 was seen. Genotype 2 is the most frequently reported HCV genotype in West Africa [306] and both genotypes 1 and 2 are likely to have originated from West Africa [307, 308]. Our data provides further evidence to support this. We discovered 4 new subtypes (1q, 1r, 1s and 2xa) as well as numerous unassigned sub-genotypes (

Figure 4-34 and

Figure 4-36). The most frequent sub-genotype detected in Benin was 2d (43% of all genotype 2 isolates), followed by 1s and 1r. None of the epidemic sub-genotypes 1a, 2a and 2c were detected in this study.

The high diversity of genotype 1 has also been seen in other countries in West Africa, such as Cameroon and Nigeria [4, 308, 309]. Interestingly, we did not detect subtype 1l in Benin. The importance of this subtype will be discussed later.

Sub-genotype 2d was first described in 1994 by Stuyver et al [310] when they detected significant differences in the 5'-UTR region of a genotype 2 isolate from Netherlands compared to 2a, 2b and 2c. While the sample was described as originating from an individual with chronic HCV infection in Netherlands, there was no demographic information on the geographical origins of the individual. The first complete 2d genome was characterised by Li et al in 2012 [116].

Prior to the Benin study there were no published whole HCV genomes from this region. This study has contributed 57 whole HCV genomes to GenBank (Appendix 11).

5.4 HCV Diversity in Uganda

In Uganda we found an extremely high diversity of genotype 4 (Figure 4-24) including a new subtype, which has been provisionally assigned as 4xa. This supports the evidence suggesting that genotype 4 originated in Central and East Africa [302]. We did not detect any of the epidemic sub-genotypes, 4a and 4d. While there were clear clusters of new subtypes in the Benin study, the diversity exhibited within genotype 4 in Uganda was so vast that it was difficult to assign new subtypes using the criteria specified by Smith et al [6]. For example, the blue coloured clade in Figure 4-24 shows numerous genotype 4 genomes that form a distinct clade, which diverges from the same root that gives rise to the 4r clade. However, even within this clade of diverse genomes, there is a wide range of genetic distances of 5.3% to 16.9% (Figure 4-28).

Along with the numerous previously undescribed genotype 4 genomes we found in Uganda we also detected subtypes 4k, 4q, 4v and 4r. These are all subtypes that are endemic to Central and East Africa and have previously been described in Cameroon, Rwanda, Uganda and more recently, in Malawi [5, 130, 283, 284, 311].

There is an argument to call the whole blue coloured clade in Figure 4-24 a new subtype. The data shown in this study is most likely just the 'tip of the iceberg' and almost certainly reflects endemic HCV genotype 4 in the region of its origin.

Genotype 7 was also detected in this study. Little is known about clinical manifestations and treatment response in genotype 7 as it was only recently discovered in individuals who originated from the DRC and Uganda [130, 312].

We also detected a mixed genotype 4-genotype 7 infection in Uganda, which has never been previously reported as far as we are aware. Prevalence of mixed genotype infections varies between regions and by risk group, but overall are not common. For example the prevalence of mixed genotype infections in cross-sectional studies in Egypt, Hong Kong, Italy, Serbia and Saudi Arabia were 5.3% [313], 4% [314], 10.8% [315], 6% [316] and 1.7% [317] respectively. In an Australian study of high-risk prisoners, mixed genotype infection was detected at a high prevalence of 30% [318].however, these studies all used commercial genotyping kits or in-house RT-PCR based methods to genotype their samples. These methods do not have the same sensitivity or specificity as next generation sequencing methods to detect mixed genotype infections, as demonstrated by Fernández-Caso et al [319]. We provide strong evidence of a single genotype 4-genotype 7 mixed infection, as shown in Figure 4-30 and Figure 4-31, in Uganda. No mixed infections were found in the Benin or Kenya studies.

There are 13 published whole HCV genomes from Uganda and this study will contribute a further 73 genomes to GenBank.

5.5 Challenges of Genotyping and Subtyping HCV

In our studies we used a custom probe to enrich our libraries for HCV (3.3.7). Such methods have been shown to provide greater sequence depth and better ability to generate consensus genomes from low viral load samples [320]. We also enriched sequencing reads for HCV *in-silico* and used bespoke pipelines to generate full length HCV genomes as well as examine deep sequence data for presence of RASs in minority virus populations. Despite these methods, we still faced challenges with subtyping HCV, especially with the diverse genomes from Benin and Uganda. This reflects both the incredible diversity of HCV, but also

the dearth of published HCV genomic data, particularly from regions where this diversity is most likely to be seen [108]. Commercial genotyping kits frequently fail to pick up non-epidemic HCV sub-genotypes. For example, in the SHARED study HCV sub-genotype 4r was frequently misclassified as genotype 1 or as a mixed genotype 1 and genotype 4 infection [5, 321]. The first report of HCV genotype 7 was initially mistyped, using the Versant HCV assay (Siemens Healthcare Diagnostics, Germany), as a genotype 2 [312]. Furthermore, the methods we have used are expensive and resource intensive and therefore are not practical for use in resource limited settings.

Finally, the HCV genomic data generated from Benin, Kenya and Uganda in our studies involved shipping of samples to UK from source countries. All library preparation and data analysis were carried out in UK. This approach is not conducive to capacity building within source country [322]. We had originally planned to carry out sequencing of the Kenyan samples in the KEMRI-Wellcome laboratory in Kilifi, Kenya following a planning meeting on site in February 2020, however, the Covid-19 pandemic disrupted these plans. In future studies, we will aim to carry out sequencing on site where possible.

5.6 Summary of HCV Genetic Diversity in SSA

In summary, we found a high diversity of genotype 1 and 2 in Benin and a high diversity of genotype 4 in Uganda as well as the rare genotype 7. This incredible diversity is probably a small window into the true diversity of HCV in SSA, and strengthens the evidence suggesting that HCV genotypes 1, 2, 4 and probably 7 originated in SSA. Conversely, we only detected 1a and 4a, epidemic sub-genotypes that have spread beyond SSA and are established in the human population globally, in Kenyan PWID. This is most likely to represent a recent introduction by European and American tourists through injecting heroin, and subsequent localised outbreak within the PWID community.

5.7 RAS in Diverse HCV Genomes

We noticed clear patterns in the prevalence of baseline NS5A RASs in diverse HCV genomes in Benin and Uganda compared to the epidemic sub-genotypes 1a and 4a seen in Kenyan PWID.

In the genotype 4 genomes from Uganda, all had the RAS L30R (Figure 4-32) and 52% had the presence of, at least, one more RAS (Table 4-7), whereas in the 4a genomes from Kenya no NS5A RASs were detected. In the genotype 1 and 2 genomes from Benin, the prevalence of at least two baseline NS5A RASs was 59% and 93%, respectively. By contrast, in the Kenya 1a genomes only 11% had the presence of a single NS5A RAS and none of the genomes had more than one NS5A RAS.

RASs can be detected at baseline in DAA-naïve individuals. Deep sequencing methods employed to look at baseline RASs in clinical trial patients revealed a prevalence of, at least one RAS in sub-genotype 1a, 13% in North America, 14% in Europe, 7% in Asia and 16% in Oceania [239]. In genotype 1b the prevalence was 16%, 17%, 20% and 19% in the same regions, respectively. This data is from Gilead clinical trials, none of which were conducted in SSA. In our study we see a similar prevalence of baseline RAS in 1a from Kenya (11%), but a much higher prevalence in the 1b genomes from Benin, where all four (Benin3, Benin5, Benin31, Benin49) genomes had at least 4 baseline NS5A RASs (Table 4-9). We also see a higher prevalence of baseline NS5A RASs in the endemic genotype 1 subtypes from Benin.

We also detected baseline NS3 RASs, predominantly in the genotype 1 and 2 genomes. In the Kenyan 1a genomes, 15% had 2 baseline NS3 RASs and 93% had the Q80K RAS (Figure 4-16). By contrast, 30% of the diverse Benin genotype 1 genomes had a single NS3 RAS, either V36l/M or Q80K, and only one of the genomes had 2 NS3 RASs (Figure 4-39). 84% of the genotype 2 genomes from Benin had a single NS3 RAS, Y56F (Figure 4-41). Only a small proportion (6%) of the diverse genotype 4 genomes from Uganda had a single NS3 RAS, D168E (Figure 4-33). No baseline NS3 RASs were detected in Kenyan 4a genomes.

NS3 protease RASs can also be detected at baseline, with the most frequently observed RAS in genotype 1 infections being Q80K, particularly sub-genotype 1a, where 29.5% of the viruses had this RAS. Similar to the NS5A RASs, geographical distribution varied, with a prevalence of Q80K in 48.1% of HCV in North America, 19.4% in Europe and 9.1% in South America [234]. The RAS Y56F has been seen mainly in sub-genotype 1a infected patients failing treatment with Grazoprevir [323], but has not been reported in population sequencing studies of treatment

naïve individuals. In our studies we see a high prevalence of Y56F in genotype 2 genomes from Benin and in a very small proportion (1%) of genotype 4 genomes from Uganda. The NS3 RAS D168E is rarely reported at baseline, with a reported prevalence of 0.5% [234] and 1.7% [324] in genotype 1 infections, but it has been seen with treatment failure to both first and second generation NS3 protease inhibitors [233]. Similarly, in genotype 4 infections D168E has been reported at baseline, but is more frequently seen in individuals failing treatment [325].

We did not find any baseline NS5B RASs in HCV genomes from Kenya, Uganda or Benin. This is an expected finding as NS5B inhibitors have a high genetic barrier to resistance and so mutations that reduce their susceptibility are rarely found at baseline [326, 327].

The difference in the prevalence of baseline RASs, particularly within the NS5A gene, between endemic and epidemic sub-genotypes most likely represents a founder effect. This is demonstrated by the comparison of RASs seen in the Kenyan 1a and 4a genomes to those in the diverse genotype 1 and 2 genomes from Benin and genotype 4 genomes from Uganda. Furthermore, we highlight the dearth of published HCV genomic data from SSA, as evidenced by the lack of population sequencing studies in the region. Our studies provide useful baseline RAS data for the region.

5.8 Implications for Treatment with DAAs

In our studies we provide baseline RAS prevalence data in diverse endemic subtypes of HCV genotypes 1, 2 and 4 in Benin and Uganda. Many of these sub-genotypes have not been previously described and therefore their response to DAA treatment is unknown.

In the Benin study, in which 52 individuals were treated, 94% of treated individuals achieved SVR12, which is reassuring given that most of the infections were caused by diverse, endemic subtypes. All genotype 1 infections were successfully treated, however two (of ten) individuals infected with sub-genotype 2d and one infected with sub-genotype 2b failed treatment. It is not possible to conclude with confidence that sub-genotype 2d is a difficult strain to treat as the numbers in the study were too small, but it warrants concern and

further studies. Our *in-vitro* work supported our reason to be concerned. The SGR construct Benin6 represented the NS5A gene of a 2d sub-genotype that infected an individual who achieved SVR12, however, the IC_{50} value of this construct, when challenged with Velpatasvir showed a 100-fold increase compared to JFH-1 SGR (Figure 4-43 and Figure 4-44). Pibrentasvir showed excellent activity *in-vitro*, and this has also been shown in other *in-vitro* studies testing SGR constructs representing challenging sub-genotypes [328] or constructs with important RASs cloned into the NS5A gene [329].

We know from previous studies that certain sub-genotypes, which are endemic to SSA, are challenging to treat. Childs *et al* showed that 100% of individuals, infected with sub-genotype 1l, 50% infected with sub-genotype 1p and 27% infected with unassigned genotype 1 subtypes, failed treatment with Sofosbuvir/Ledipasvir [4]. These individuals were originally from SSA. Similar findings were reported in a study of the HCV Research UK cohort where 100% of individuals infected with 1l failed treatment with a NS5A/NS5B combination treatment regimen, but those treated with a NS3 based regimen achieved SVR12 [309]. In the Benin study we did not detect any 1l or 1p subtypes and, reassuringly, all individuals infected with unassigned genotype 1 subtypes were successfully treated.

Sub-genotype 4r is also a challenging strain to treat. This was shown in the SHARED study, the first study in SSA to demonstrate successful upscaling of DAA therapy in a resource poor setting, where 56% of individuals infected with sub-genotype 4r failed treatment with Sofosbuvir/Ledipasvir [5]. Similar findings have been reported in real world studies in France and UK [309, 330, 331]. A follow-up study in Rwanda, the SHARED-3 study, analysing the safety and efficacy of Sofosbuvir/Velpatasvir in treating DAA naïve individuals showed an SVR12 rate of 97% [332]. In our Uganda study we detected numerous diverse, endemic genotype 4 strains, including four subtype 4r genomes, although these genomes were still 12-16% genetically distant to the previously published 4r genomes (Figure 4-29). All four genomes had at least 2 baseline NS5A RASs. These individuals were all DAA naïve as far as we are aware.

Genotype 7 is a recently discovered genotype and little is known about its susceptibility to DAAs, however, a case report details successful treatment of a

44-year-old male from Democratic Republic of Congo infected with genotype 7 and treated with 12 weeks of Sofosbuvir/Velpatasvir [333]. Therefore, we would not anticipate any definite problems treating Ugandan individuals infected with genotype 7, although further studies are indicated.

We would also not anticipate any problems treating individuals infected with a mixed genotype infection, provided use of a pan-genotypic DAA regimen. In the era pre-pan-genotypic therapy, there was a suggestion that infection with multiple genotypes was more challenging to treat [334], but this doesn't appear to be an issue with the current pan-genotypic DAAs, such as Sofosbuvir/Velpatasvir or Glecaprevir/Pibrentasvir [335], and Sofosbuvir/Ledipasvir [336].

In the Kenyan study of PWID we found highly connected networks Figure 4-12. These individuals are all treatment naïve, but the prevalence of sub-genotypes 1a and 4a should make for straightforward treatment. Furthermore, the distinction in higher prevalence of HCV in PWID in Kenya compared to the general population, which stands at 0.9% [337], provides an opportunity to focus resources towards micro-elimination of HCV within PWID along the Coast of Kenya. We used a snowball sampling method to recruit PWID into our study, and a similar method could be used to treat HCV infected PWID and gradually reduce the circulating HCV reservoir within the community. This has been suggested in modelling studies [338], and has been shown to be received positively by members within the PWID community [339]. Nonetheless, there would be challenges to ensure a successful micro-elimination program, such as continuous efforts to maintain harm reduction strategies alongside diagnosis and treatment of HCV. Current models of care show high dropout rates at each point of the cascade of HCV care [294] and would need simplifying, as shown in South Africa [340].

Finally, we conducted a survey among members of the HCV SSA network, where only 50% of countries reported having access to Velpatasvir. Most countries had access to Daclatasvir (90%) and Ledipasvir (70%), which may not be adequate for the predominant circulating HCV sub-genotypes in Benin and Uganda, but should be suitable for treating Kenyan PWID. Pibrentasvir and NS3 protease inhibitors were not available among the countries surveyed. Furthermore, our survey

revealed the significant costs of diagnosis and treatment that were borne by individuals [250]. This presents a significant challenge to realistically achieve WHO's elimination goal.

6 Further Work

Follow-up work to the data generated in our studies is planned.

I am planning to date the origin of HCV genotype 4 using the full-length genomes genotype 4 genomes from Uganda. I also plan to investigate the effectiveness of NS5A inhibitors on these genomes, using the same *in vitro* assay that I have described earlier.

Finally, I will be co-ordinating the writing of a WHO paper on HCV drug resistance highlighting current knowledge and identifying research gaps. This work is already underway.

7 Conclusions

In a region with a high burden of HCV infection but a dearth of HCV genomic data, we have published 57 whole HCV genomes from Benin and plan to publish a further 73 whole HCV genomes from Uganda and 49 from Kenya.

In our studies we have described diverse HCV genotypes 1, 2 and 4, including the discovery of previously undescribed subtypes, from Benin and Uganda. This supports and strengthens existing evidence that these genotypes originated in SSA. By contrast we found only epidemic sub-genotypes 1a and 4a circulating among PWID along the Kenyan coast and we show that this is likely to represent a relatively recent introduction from American and European tourists. We also show highly connected PWID networks in coastal Kenya that provide an opportunity to pursue micro-elimination of HCV in this community.

We present useful data on the prevalence of baseline RASs and demonstrate the *in-vitro* effect of diverse HCV genotype 2 on the susceptibility to NS5A inhibitors.

Appendices

The publications listed in section 1.1 are shown here.

ARTICLE IN PRESS

Thematic Miniseries on HCV cure

JOURNAL OF HEPATOLOGY

Non-epidemic HCV genotypes in low- and middle-income countries and the risk of resistance to current direct-acting antiviral regimens

Rajiv Shah[1], Lucrece Ahovegbe[1,2], Marc Niebel[1], James Shepherd[1], Emma C. Thomson[1,3,*]

Summary

The hepatitis C virus (HCV) is an extremely diverse virus, subtypes of which are distributed variably around the world. Viral genotypes may be divided into epidemic subtypes; those that have become prevalent globally, and endemic subtypes that have a more limited distribution, mainly in Africa and Asia. The high variability of endemic strains reflects evolutionary origins in the locations where they are found. This increased genetic diversity raises the possibility of resistance to pan-genotypic direct-acting antiviral regimens. While many endemic subtypes respond well to direct-acting antiviral therapies, others, for example genotypes 1l, 3b and 4r, do not respond as well as predicted. Many genotypes that are rare in high-income countries but common in other parts of the world have not yet been fully assessed in clinical trials. Further sequencing and clinical studies in sub-Saharan Africa and Asia are indicated to monitor response to treatment and to facilitate the World Health Organization's 2030 elimination strategy.

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Keywords: Hepatitis C Virus; Genotypes; Subtypes; Directly Acting Antivirals; SVR; Resistance; Elimination; Sequencing.

Received 9 October 2020; received in revised form 12 March 2021; accepted 21 April 2021; available online xxx

Introduction

The revolution in therapeutic options for the treatment of the hepatitis C virus (HCV) is one of the most important medical advances in a generation and has led the World Health Organisation (WHO) to propose a plan for elimination by 2030. Sustained virological response (SVR) rates of more than 95% have become the norm in high-income countries (HICs), using treatments that have very few side effects and are cheap to manufacture, albeit subject to country-specific pricing differences.[1,2] However, a key weakness of the clinical research underpinning this plan is that almost all of it was carried out in HICs, a weakness repeatedly acknowledged by the international WHO HCV treatment guidelines committee, that focuses on the needs of low- and middle-income countries (LMICs) (Fig. 1).[3] Early indications that SVR rates might not always be as high as expected in some populations emerged from studies on direct-acting antiviral (DAA) treatment in Europe and North America that found diverse genotypes in patients originating from sub-Saharan Africa.[4–6] Subsequently, emerging studies investigating response rates to DAAs in Africa and Asia have confirmed these initial reports on a larger scale.[7]

HCV is one of the most genetically diverse human viruses.[8] This genetic diversity, which varies by geographical region, has not yet been fully characterised, due to under-sampling in many parts of the world, meaning that the likelihood of drug resistance in many viral subtypes has not been assessed. At the time of writing, 8 genotypes and 90 subtypes of HCV have been described with an average pairwise distance of more than 30% between genotypes and an intra-genotype average pairwise distance of 15% between subtypes.[8] These genotypes may be divided into genetically conserved epidemic lineages that have been exported large scale around the world and more localised highly diverse endemic lineages. Several endemic lineages have recently been found to be associated with resistance to some "pan-genotypic" DAA regimens (in particular those containing first-generation NS5A inhibitors) and may represent a threat to the global elimination plans proposed by the WHO.

Response to treatment *in vitro* and in clinical trials in epidemic genotypes prevalent in Europe, the United States, North Africa, parts of Asia and the Indian sub-continent (genotypes 1a,1b, 2a, 3a, 4a, 4d and 6a) has been studied in detail. DAAs with activity against such genotypes have been termed "pan-genotypic" and have enabled the WHO to develop a series of guidelines aimed at elimination of HCV by 2030.[3] These guidelines aim to simplify treatment regimens so that genotyping (which is expensive and can provide a barrier to treatment, particularly in LMICs) is not required at the individual level.

In this review article, we make the case that there is a need for further population-based sequencing and real-world clinical studies to assist policymakers in individual countries to select more nuanced treatment strategies appropriate to

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https://doi.org/10.1016/j.jhep.2021.04.045

Key point

HCV is an extremely diverse RNA virus with 8 known genotypes and 90 known subtypes.

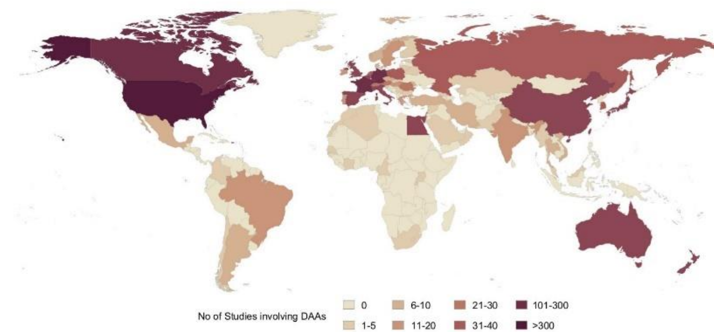

Fig. 1. Map showing locations of DAA-related studies involving HCV-infected individuals. Details of registered clinical trials were downloaded from clinicaltrials.org. DAA, direct-acting antiviral.

the local setting, given emerging evidence that some endemic genotypes present in sub-Saharan Africa and Asia may respond less well than originally anticipated to DAAs, particularly first-generation NS5A inhibitors such as daclatasvir and ledipasvir.

Methods in brief

A comprehensive review was carried out based on searching the literature for all endemic genotypes (excluding the epidemic genotypes 1a,1b, 2a, 3a, 4a, 4d and 6a). The primary source of genetic sequence data and published references was HCV GLUE,[9] which is a comprehensive dataset of HCV genetic sequences with associated metadata from the NCBI. The sequences and metadata are curated and organised into genotypes and subtypes with linked relevant PubMed references. The sequences are linked to a database of polymorphisms known to be associated with DAA treatment failure or reduced DAA efficacy *in vitro*, developed and maintained by an expert resistance group led by Public Health England.

A supplemental literature search was conducted on PubMed to search for every individual endemic sub-genotype, *e.g.* "4r" and "treatment" and any additional studies describing HCV genetic diversity and related geographic distribution as well as reported treatment outcomes using the following search terms: "genotype", "rare", "unusual" or "uncharacterised" and "HCV" or "hepatitis C virus" and "treatment" or "management" or "direct-acting antiviral" or "DAA" or "interferon-free".

To show the number and location of completed and ongoing DAA treatment studies, data were obtained from www.clinicaltrials.gov. Included studies were not limited to randomised controlled trials, provided DAAs were administered to HCV-infected individuals in the country or countries where the study was conducted. Studies involving only healthy volunteers and studies involving use of the first-generation protease inhibitors telaprevir and boceprevir with pegylated-interferon and ribavirin were excluded. A further 2 studies were excluded as no information on the country where the study was conducted could be found.

Data manipulation was carried out in R (version 3.5.3).

Key point

10 epidemic lineages (1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d, 5a, 6a) are well described yet there are many endemic lineages that are poorly characterised clinically, but highly prevalent, particularly in low middle-income countries.

Geographical distribution of genotypes

The distribution of HCV genotypes around the world is highly heterogenous, with the highest levels of diversity in Asia and sub-Saharan Africa; paradoxically, these regions are the least sampled (Fig. 2). The high genetic diversity of HCV in these locations most likely reflects the evolutionary origins of HCV subtypes. In contrast, the more heavily sampled but far less diverse epidemic lineages of HCV (including 1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d and 6a), most likely represent a diaspora of exported founder strains that dispersed rapidly due to medical and recreational use of injections, blood transfusions, and operative medical procedures, peaking during the 20th century. The coincidence of industrialisation alongside changes in medical care and an increase in wealth in some countries has left a legacy of highly sampled relatively conserved epidemic strains in HICs and a highly diverse but largely invisible pool of HCV in LMICs. HCV lineages associated with increased intrinsic resistance to the NS5A inhibitors, with different levels of resistance according to the drug and drug generation, are harboured within this reservoir of endemic strains and could present a barrier to global elimination if transmission is not prevented in affected areas.

Genotype 1a and 1b are the most widespread genotype 1 lineages, distributed around the globe by the founder effect (Fig. 3) through the use of injections in people who inject drugs (PWID) and within healthcare settings,[10] and to a far lesser extent through sexual[11] and vertical

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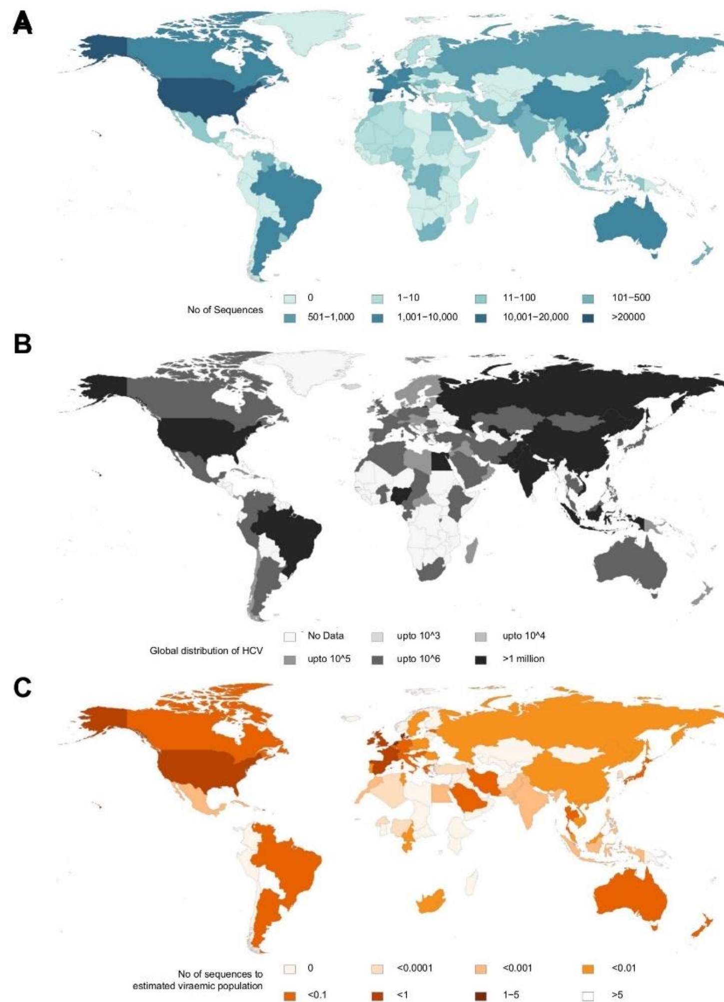

Fig. 2. Global maps illustrating availability of sequence data, distribution of viraemic population and ratio of sequence data to infected population. (A) HCV genetic sequences greater than 500 nucleotides in length uploaded to GenBank and curated using HCV GLUE by country (accessed on 4th August 2020). (B) Global distribution of estimated HCV viraemic population.[50] (C) Ratio of the number of published HCV genetic sequences to the estimated viraemic population per country.

transmission.[12] Genotype 1 is likely to have originated in West Africa where extremely high levels of sub-genotypic diversity are evident.[13]

Genotype 2 is also likely to have originated in West Africa[14] where it is most prevalent and most diverse (Fig. 4). It dispersed widely within West African countries most likely as a result of iatrogenic blood-borne transmission, for example during military medical public health interventions that aimed to treat the whole population of French Cameroon between 1921 and 1957 for African trypanosomiasis, yaws, syphilis and leprosy using injected treatments. Genotype 2 has also spread to other parts of the world. It was exported to the Caribbean by sea as a result of the transatlantic slave trade; sequences sampled in Martinique closely resemble those from the Benin–Ghana area (people in Martinique are known to share ancestry

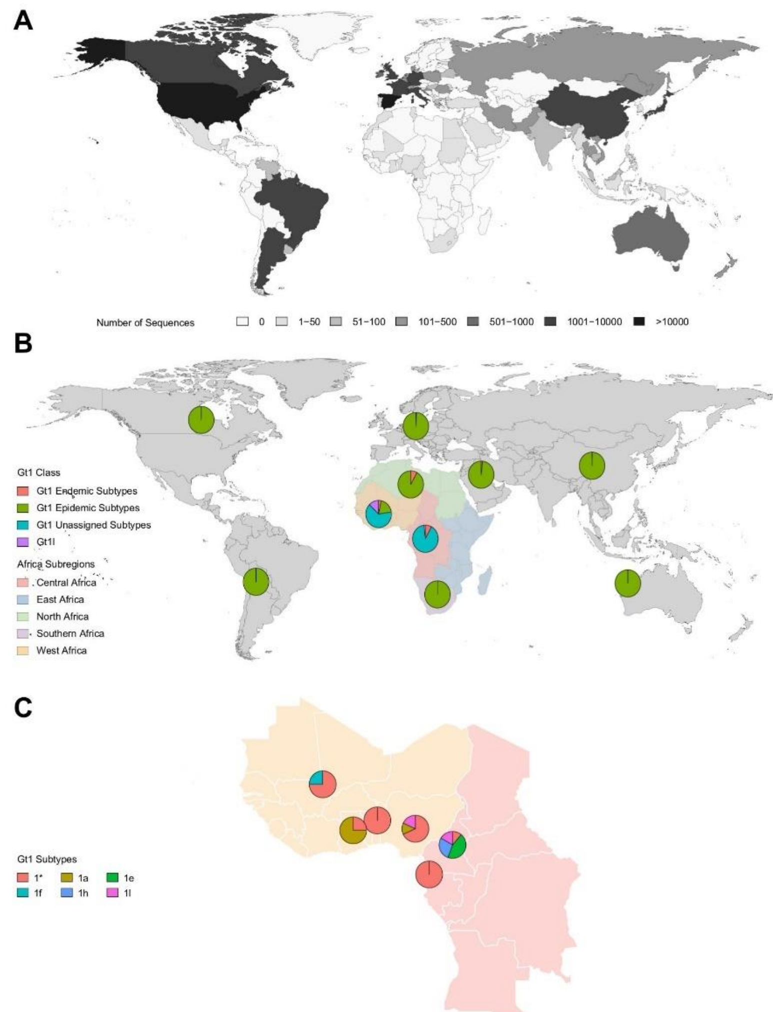

Fig. 3. Genotype 1 genotype and sub-genotype distribution. (A) Global distribution of genotype 1 HCV. (B) Genotype 1 subtype distribution with a focus on the subregions of Africa where genotype 1 is most diverse. Epidemic genotype 1 subtypes include 1a and 1b. Endemic genotype 1 subtypes include 1c, 1d, 1e, 1f, 1g, 1h, 1j, 1k, 1l and 1m. (C) Genotype 1 subtype diversity within West Africa (Benin, Ghana, Mali and Nigeria) and Central Africa (Cameroon and Equatorial Guinea). 1* refers to unassigned Genotype 1 subtypes.

with populations in present-day Ghana, Togo, Benin and Nigeria). Genotype 2 also migrated from South America to Asia, likely at least in part via the slave trade between Indonesia and Suriname. Reflecting this history, genotype 2 strains are also found in the Americas, for example in the USA (2a,2b), Argentina (2a,2c), Venezuela (2b,2c, 2j, 2s), Suriname (2e,2f,2j,2o), Brazil (2a,2b,2c) and Hispaniola (2r) and in Asia – Indonesia (2e), China (2a), Japan (2a,2b), Thailand and Vietnam (2a, 2m).[15]

Genotype 3 is highly prevalent in several regions of the world (Fig. 5), with most heterogeneity in the Indian sub-continent and Southeast Asia.[16] The majority of trials have focused on the

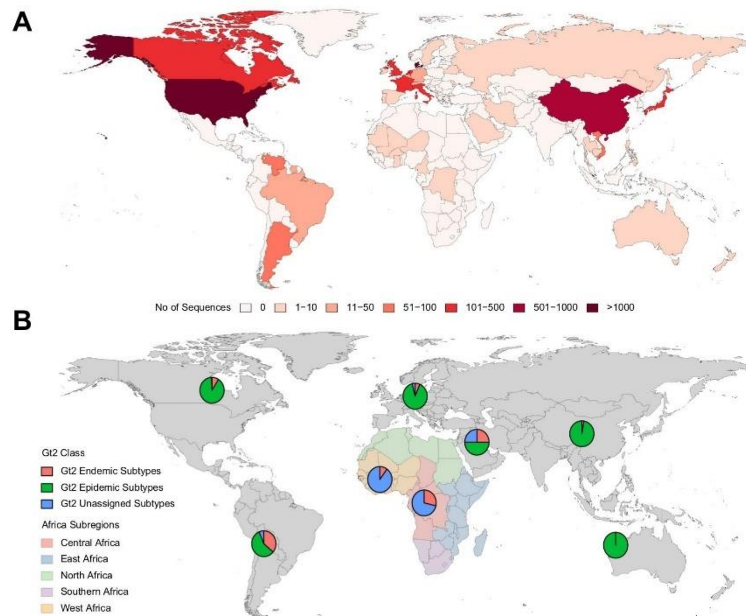

Fig. 4. Genotype 2 genotype and sub-genotype distribution. (A) Global distribution of Genotype 2 HCV. (B) Genotype 2 subtype distribution with a focus on the subregions of Africa where it is most diverse. Epidemic Genotype 2 subtypes include 2a, 2b and 2c. Endemic Genotype 2 subtypes include 2d, 2e, 2f, 2i, 2j, 2k, 2l, 2m, 2n, 2o, 2q, 2r, 2s, 2t and 2u.

epidemic genotype 3a subtype that is prevalent in the Indian sub-continent and has most successfully entered populations in HICs. However, genotype 3b[17] is also distributed widely in Asia, including India, China, Malaysia and Thailand. The less well characterised genotypes 3c (provisional), 3d and 3e have been found in Nepal, 3f, 3g and 3i in India and 3k in Indonesia.[18] Genotype 3 strains are uncommonly detected in South and Central America and in sub-Saharan Africa, but exist in East Africa where genotype 3h has been found in Somalia and in individuals originating from this area.[19]

Genotype 4 HCV (Fig. 6) is likely to have originated in Central and East Africa where multiple diverse endemic strains predominate.[20] Several lineages have spread into North Africa and further afield, of which genotypes 4a and 4d are most dispersed. Genotype 4a is the most prevalent genotype 4 strain around the world and transmission was amplified in North Africa, particularly Egypt as a result of unsafe injection practices in healthcare settings.[21] Clinical trials have focused on this subtype, and to a lesser extent genotype 4d, a strain introduced to Saudi Arabia during the early 20th century,[22] probably from countries in the Horn of Africa including Ethiopia.[23] It has more recently emerged in PWID in Southern Europe and found a sexually-transmitted niche in men-who-have-sex-with-men (MSM) in Northern Europe with cross-over from PWID to MSM likely occurring in urban centres such as Amsterdam, London, Berlin and Paris.[24] While the globally dispersed genotype 4a and 4d lineages are well described, others prevalent in sub-Saharan Africa have only recently been investigated in clinical studies.

Genotype 5 is the most common genotype in South Africa[25] and has been exported to Europe, Asia and North America in rare cases (Fig. 7). There is currently 1 official subtype (5a), however a second highly divergent lineage has been identified in Burkina Faso,[26] suggesting that genotype 5 may be more widespread on the African continent than previously suspected (official sub-genotypes require 3 separate sequences from at least 3 infected individuals to be officially confirmed).[8]

Genotype 6 is the most diverse HCV genotype and is most prevalent in Southeast Asia (Fig. 8) where further geographic heterogeneity of subtypes is evident.[27] While the epidemic strain 6a has been exported widely around the world, other subtypes are far more spatially structured within distinct geographical locations. Genotype 6u is found in Laos[28] and Vietnam[29] while 6v has been detected in China and Thailand.[30,31]

Genotypes 7 and 8 have only recently been identified in individuals originally from the DRC,

Key point

Asia and sub-Saharan Arica host the most diverse HCV lineages but are the least well sampled for genomic data.

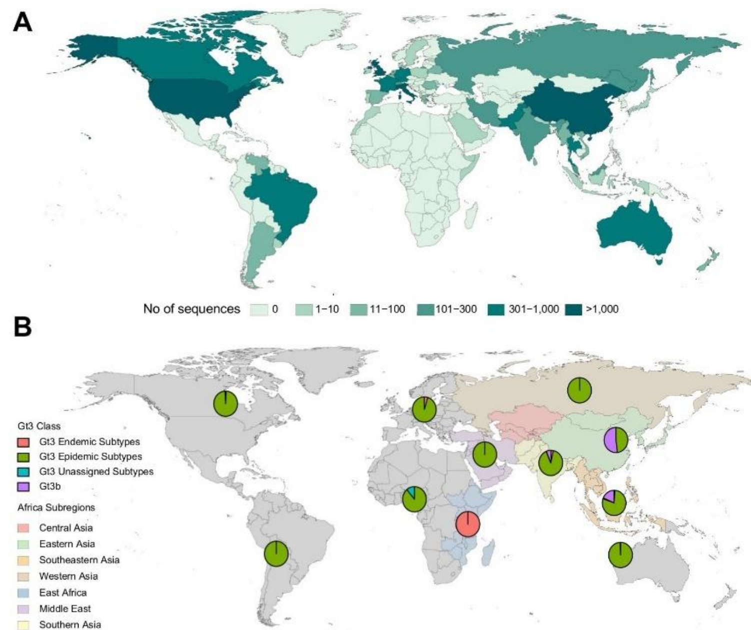

Fig. 5. Genotype 3 genotype and sub-genotype distribution. (A) Global distribution of Genotype 3 HCV. (B) Genotype 3 subtype distribution with a focus on the subregions of Asia and East Africa where it is most diverse. The epidemic Genotype 3 subtype is 3a. Endemic Genotype 3 subtypes include 3d, 3e, 3f, 3g, 3h, 3i and 3k.

Uganda[32] and India.[33] The distribution of these subtypes requires further investigation.

Susceptibility to DAAs in non-epidemic HCV subtypes

Reduced efficacy of DAAs is most often related to polymorphisms within the NS5A gene in epidemic subtypes of HCV and this also appears to be the case with endemic strains.[2,3,34] A list of polymorphisms predicted to confer resistance is included in Table S1. This has been well-documented with the first-generation NS5A inhibitors ledipasvir and daclatasvir but may also affect SVR rates with second-generation inhibitors. Amino acid polymorphisms associated with resistance within NS3 have been reported in endemic strains but have not been tested extensively in clinical trials. Polymorphisms in NS5B are not common – the well described but uncommon and unfit S282T polymorphism is associated with reduced efficacy of sofosbuvir in epidemic lineages. This has occasionally been detected in subtypes 4r and 6l but the *in vivo* impact is unknown.[35,36]

Resistance-associated substitutions (RASs) in epidemic genotypes do not often lead to treatment failure in the absence of other risk factors. Cirrhosis and previous treatment failure are the most common risk factors associated with treatment resistance; however, it is not yet fully established if such risk factors are invariably present in DAA failure with endemic lineages. Further clinical trials are required to investigate this.

In vitro susceptibility to DAAs

The *in vitro* efficacy of second-generation NS5A inhibitors, pibrentasvir and velpatasvir, for infectious clones of genotypes 1a, 1b, 2a, 3a, 4a, 5a, 6a and 7a is high, in keeping with the pan-genotypic activity reported *in vivo* (with the exception of 7a for which a single case report of SVR following sofosbuvir and velpatasvir is available).[2,3,37] The presence of RASs is only occasionally associated with treatment failure in such subtypes, often in patients with cirrhosis.[2]

A recent *in vitro* study investigated the efficacy of first and later generation NS5A inhibitors daclatasvir, elbasvir, ledipasvir, pibrentasvir and velpatasvir against several endemic subtypes (1l, 3b, 3g, 4r, 6u and 6v) using the JFH1 sub-genomic replicon (SGR-JFH1) backbone and the NS5A gene of the respective genotype of interest. In this evaluation, only pibrentasvir had high-level pan-genotypic activity *in vitro*.[34] NS5A sequences of genotypes 1l and 4r typically encode a 28M/30R/

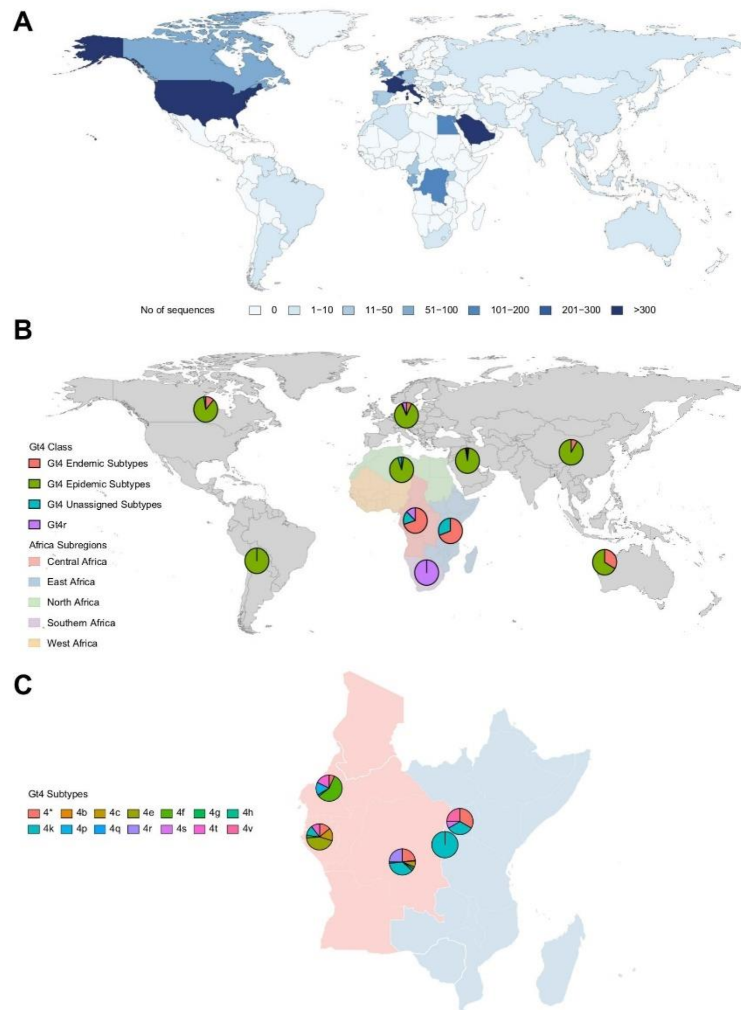

Fig. 6. Genotype 4 genotype and sub-genotype distribution. (A) Global distribution of Genotype 4 HCV. (B) Genotype 4 subtype distribution with a focus on the regions of Africa where it is most diverse. Epidemic Genotype 4 subtypes include 4a and 4d. Endemic Genotype 4 subtypes include 4b, 4c, 4e, 4f, 4g, 4h, 4i, 4k, 4l, 4m, 4n, 4o, 4p, 4q, 4s, 4t, 4v and 4w. (C) Genotype 4 subtype diversity within Central (Cameroon, Democratic Republic of Congo and Gabon) and East Africa (Rwanda and Uganda). 4* refers to unassigned Genotype 4 subtypes.

31M motif at positions that are associated with a resistant phenotype *in vivo*.[5] The NS5A Y93H mutation is also commonly found in genotype 4r-infected patients in whom treatment has failed.[5,6] Subtypes 3b and 3g invariably encode the double NS5A RAS 30K/31M. This double combination has been shown to confer high-level drug resistance when introduced into the 3a subtype, suggesting that subtypes 3b and 3g are more likely to reduce the effectiveness of NS5A inhibitors.[38] The NS5A sequences of subtypes 6u and 6v encode the 28V/30S/93S triple polymorphism in 100% (2/2 and 6/6) of published 6u and 6v sequences. This triple motif is associated with substantially reduced effectiveness

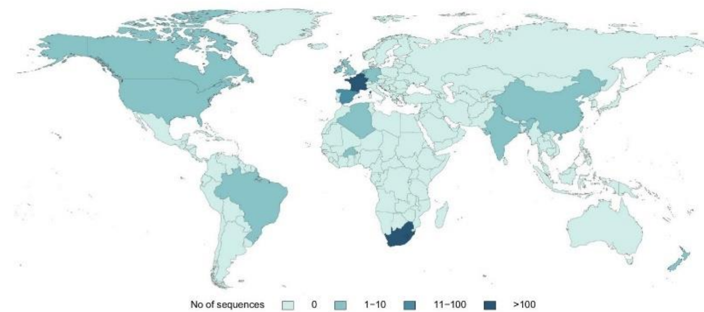

Fig. 7. Genotype 5 genotype and sub-genotype distribution. Global distribution of Genotype 5 HCV. The shading in Asia represents a full length and one partial HCV sequence from China[51] as well as one full length HCV sequence from India (unpublished), reflecting how rare Genotype 5 HCV is in Asia.

of ombitasvir for genotype 6a.[39] In the SGR-JFH1 backbone model (containing genotype-specific NS5A), subtypes 3b and 3g were resistant to daclatasvir, elbasvir, ledipasvir and velpatasvir, while 1l, 4r, 6u and 6v were sensitive to elbasvir and velpatasvir but not to daclatasvir and ledipasvir.[34] Clinical trials are indicated to evaluate the response to treatment of these subtypes in areas where they occur commonly.

Clinical trial and real-world experience with DAAs

Clinical studies have predominantly been conducted in HICs where endemic subtypes occur occasionally, usually in cases of imported infection. Many large clinical trials in HICs have failed to identify subtypes within treatment arms making interpretation challenging. Clinical trials in LMICs have been carried out in a small number of countries. These have been observational and have usually involved only small numbers of patients without the benefit of sequence analysis for the detection of resistance.

Some genotype 1 subtypes prevalent in West Africa have been associated with lower-than-expected SVR rates to current DAA regimens. Two studies in the UK have shown that genotype 1l (prevalent in Nigeria and Cameroon) and several unclassified genotype 1 subtypes are associated with lower-than-expected SVR rates.[4,5] In 1 UK study, carried out in an area with a high number of patients originating from West Africa, only 75% of African patients infected with genotype 1 subtypes rarely found in the UK achieved SVR, whereas a high rate of response was achieved in those infected with genotypes 1a and 1b.[4] Studies in West Africa are awaited.

Evidence confirming the *in vitro* resistance profile of some genotype 3 subtypes has also emerged recently in clinical studies. In a Chinese single-arm phase III trial, 89% of patients without cirrhosis with genotype 3b HCV responded to treatment with sofosbuvir and velpatasvir while only 50% of those with cirrhosis achieved SVR.[17] Trials of patients infected with genotype 3g are awaited.

The SHARED study was a single-arm prospective evaluation of the efficacy and safety of ledipasvir/sofosbuvir in adults diagnosed with chronic HCV genotype 4 infection in Rwanda.[7] Treatment with ledipasvir and sofosbuvir led to high SVR rates of 93% in patients with the highly prevalent genotype 4k variant found in Gabon, the DRC, Uganda and Rwanda; a generic formulation of this treatment has been widely distributed in sub-Saharan Africa. Patients with genotypes 4q and 4v also responded well to treatment, with SVR rates of 90% and 100%, respectively). In contrast, as predicted *in silico*, *in vitro* and in small numbers of patients in European trials, genotype 4r was associated with a far lower-than-expected SVR response to ledipasvir/sofosbuvir of only 56% (27/48 individuals). The SVR12 response rate among all patients in the trial with a non-subtype 4r infection was 93% (234/252 of those treated). Genotype 4r is prevalent in several countries in sub-Saharan Africa, including the DRC, Rwanda, South Africa, Ethiopia and Malawi and has been detected in Saudi Arabia. Genotype 4r NS5A resistance-associated polymorphisms (to daclatasvir, velpatasvir and ledipasvir-based regimens) have also been reported in small numbers of participants in studies in the UK and France.[5,6,40] A phase IV follow-on study is ongoing in Rwanda (SHARED3) to investigate the use of sofosbuvir and velpatasvir as first-line and sofosbuvir/velpatasvir and voxilaprevir as second-line therapy. Genotypes 4b (also found in the DRC), 4c and 4g (found in the

Key point

Clinical trials have involved treatment of HCV-infected individuals in high income countries where epidemic lineages of HCV are the most prevalent circulating virus. Some endemic lineages, for example 1l and 4r, are known to respond less well to direct-acting antiviral treatment than epidemic lineages.

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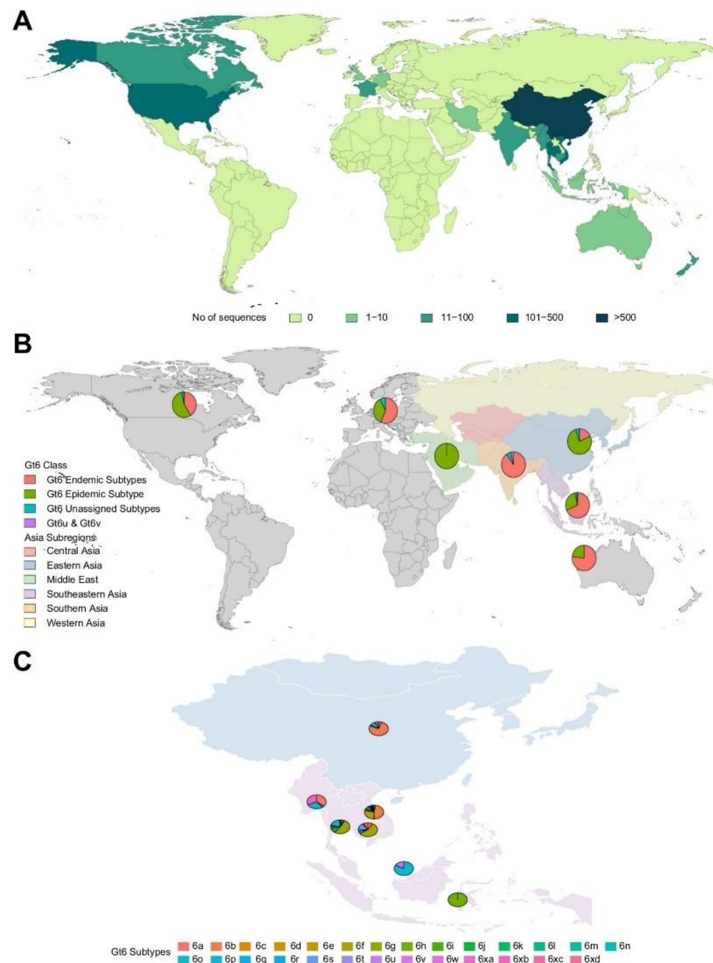

Fig. 8. Genotype 6 genotype and sub-genotype distribution. (A) Global distribution of Genotype 6 HCV. (B) Genotype 6 subtype distribution with a focus on the regions of Asia where they are most diverse. The epidemic Genotype 6 subtype is 6a. Endemic Genotype 6 subtypes include 6b, 6c, 6d, 6e, 6f, 6g, 6h, 6i, 6j, 6k, 6l, 6m, 6n, 6o, 6p, 6q, 6r, 6s, 6t, 6w, 6xa, 6xb, 6xc, 6xd, 6xe. (C) Genotype 6 subtype diversity within Eastern (China) and South-eastern Asia (Cambodia, Thailand, Vietnam, Myanmar, Malaysia and Indonesia). 6* refers to unassigned Genotype 6 subtypes.

DRC and Gabon) and 4l were also detected and treated in the SHARED study but were present in very small numbers. Very little or no SVR data are available for other genotype 4 subtypes not detected in this study, including 4e (Gabon), 4f (Cameroon, Algeria), 4h (the DRC), 4m (Egypt), 4n (Egypt), 4o (Saudi Arabia), 4p (Cameroon), 4s (Uganda) and 4t (Cameroon).[41] Of these, subtype 4b has shown evidence of resistance to ledipasvir *in vitro* and merits further study.[35]

Treatment responses of genotype 6a have also been well documented both *in vitro* and *in vivo* but other genotype 6 subtypes have not.[37,42] Limited data from clinical trials have highlighted high SVR12 rates for genotype 6a.[2,3] It will be important to identify response rates to other genotype 6 subtypes where they are prevalent, particularly in Southeast Asia. Given the presence of a triple RAS motif in subtypes 6u and 6v and the results of *in vitro* assays with first-generation NS5A

Key point

We advocate for further studies to characterise the prevalence and genetic diversity of endemic HCV lineages in low middle-income countries to determine optimal regional treatment strategies and facilitate the World Health Organization's 2030 elimination strategy.

inhibitors daclatasvir and ledipasvir, real-world studies and clinical trials using these and other agents (including the generic NS5A inhibitor ravidasvir) are eagerly anticipated.

Mis-genotyping by commercial diagnostic assays

As well as affecting treatment outcomes, genetic variation may also result in difficulties with genotyping HCV subtypes. While an in-depth discussion and summary of the evidence behind the occurrence of mis-genotyping HCV by commercial assays is beyond the scope of this review, we highlight the importance of this issue, particularly when considering the implications for treatment. For example, in the SHARED study, several subtype 4r samples were mis-typed by an Abbott assay as genotype 1,[7] this assay was also associated with mistyping of genotype 6 subtypes as genotype 1[43] and genotype 7 HCV as genotype 2.[44]

Implications and future planning

Many genotypes that have not been assessed in large randomised controlled clinical trials also appear to respond well to treatment in smaller real-world studies.[2,7,45,46] However, recent *in vitro* experimental and clinical data have shown that some subgenotypes are associated with a reduced susceptibility to current DAA therapies.[2,34] Several genotypes common in parts of sub-Saharan Africa and Asia, including genotypes 1l, 3b, 3g, 4b, 4r, 6u and 6v harbour RASs and have evidence of variable levels of resistance to NS5A inhibitors *in vitro*. Genotypes 1l, 3b and 4r have also been found to respond less well than expected to some DAAs in clinical studies. Genotypes 7 and 8 have not yet been assessed in clinical trials. These genotypes are not rare in affected countries but are most prevalent in LMICs where large scale clinical trials have not taken place due to the absence of highly profitable markets. Future international regulation and/or political pressure on the drug industry may be required to ensure that the WHO HCV elimination target is achieved and policymakers, patients and treating physicians should recommend that pharmaceutical companies based in HICs consider the ethics of access to effective treatment for all in addition to market forces when developing new therapies.

Treating physicians in HICs should consider the possibility of atypical genotypes in patients who may have become infected with HCV in sub-Saharan Africa and Asia – this consideration is now outlined in the most recent EASL guidelines.[2] Those practicing in countries where such genotypes are common in the population should preferentially use WHO-recommended regimens that include NS5A inhibitors with a higher barrier to resistance, such as glecaprevir/pibrentasvir or sofosbuvir/velpatasvir in preference to first-generation inhibitors such as daclatasvir or ledipasvir. Sofosbuvir/daclatasvir remains a recommended first-line "pan-genotypic" regimen in the current WHO treatment guidelines[3] – this combination may be highly effective in some countries but may not be optimal in others. Retreatment options for endemic subtypes have only been partially assessed, typically in very small numbers of patients. Current guidelines and some small studies suggest that glecaprevir/pibrentasvir/sofosbuvir or sofosbuvir/velpatasvir/voxilaprevir with rare genotypes may be a suitable option for retreatment in individuals who have undergone unsuccessful therapy with NS5A inhibitors.[2,5,6,47]

Development of treatment registries in LMICs would be of considerable value in tracking real-world responses to treatment and in quantifying the risk of onward transmission of resistant strains. Sub-genotyping has not always been carried out or reported in many clinical trials. While genotyping is not necessary at the individual level, population-level genotyping studies would help individual countries to set up appropriate local treatment strategies. Full genome sequencing can be carried out at relatively low-cost using technologies such as Illumina and Oxford nanopore. The highly portable Oxford nanopore MinION platform (the size of a mobile phone) has been used to generate full genome sequences in virus outbreaks all around the world, including in low-income countries such as the DRC.[48] Such work has not yet been applied to HCV on a large scale and genetic sequence data is missing from many LMICs. However, it is feasible and should be considered.

Of the 71 million people living with HCV in the world, sub-Saharan Africa,[49] South and Central America and Asia, are home to 10, 3.5 and 31 million people with HCV, respectively, thus sharing a significant proportion of the burden of HCV infection.[50] Careful consideration needs to be given to treatment strategies for HCV in these regions – elimination is within our grasp, but one size does not necessarily fit all.

Abbreviations

DAA, direct-acting antiviral; HCV, hepatitis C virus; HICs, high-income countries; LMICs, low middle-income countries; MSM, men-who-have-sex-with-men; PWID, people who inject drugs; RAS(s), resistance-associated substitutions; SVR, sustained virological response; WHO, World Health Organisation.

Financial support

Medical Research Council (MRC) (MC_UU_12014/1) and Wellcome Trust (102789/Z/13/A).

Conflict of interest

The authors declare that there is no conflict of interest.

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

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Authors' contributions

E.T. is the corresponding author and devised the main conceptual ideas as well as the outline of the review article. E.T. wrote the manuscript supported by R.S. and L.A. R.S. and L.A. gathered and curated the data and analysed the genomic data. M.N, J.S. and R.S. created the data visualisation figures.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2021.04.045.

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Dhiblawe, et al., "Hepatitis C virus in sub-Saharan Africa: a long road to elimination," Lancet Gastroenterol Hepatol, vol. 6, pp. 693-694, Sep 2021 removed due to copyright issues.

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Article

Characterisation of a Hepatitis C Virus Subtype 2a Cluster in Scottish PWID with a Suboptimal Response to Glecaprevir/Pibrentasvir Treatment

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Abstract: Direct-acting antivirals (DAAs) have revolutionised the treatment of Hepatitis C virus (HCV), allowing the World Health Organisation (WHO) to set a target of eliminating HCV by 2030. In this study we aimed to investigate glecaprevir and pibrentasvir (GP) treatment outcomes in a cohort of patients with genotype 2a infection. Methods: Clinical data and plasma samples were collected in NHS Greater Glasgow & Clyde. Next generation whole genome sequencing and replicon assays were carried out at the MRC-University of Glasgow Centre for Virus Research. Results: 132 cases infected with genotype 2a HCV were identified. The SVR rate for this group was 91% (112/123) following treatment with GP. An NS5A polymorphism, L31M, was detected in all cases of g2a infection, and L31M+R353K in individuals that failed treatment. The results showed that R353K was present in 90% of individuals in the Glasgow genotype 2a phylogenetic cluster but in less than 5% of all HCV subtype 2a published sequences. In vitro efficacy of pibrentasvir against sub-genomic replicon constructs containing these mutations showed a 2-fold increase in IC_{50} compared to wildtype. Conclusion: This study describes a cluster of HCV genotype 2a infection associated with a lower-than-expected SVR rate following GP treatment in association with the NS5A mutations L31M+R353K.

Keywords: hepatitis C virus; direct-acting antivirals; glecaprevir; pibrentasvir; sustained virological response; resistance associated substitutions; phylogenenetics; subgenomic replicons

Citation: Shah, R.; Barclay, S.T.; Peters, E.S.; Fox, R.; Gunson, R.; Bradley-Stewart, A.; Shepherd, S.J.; MacLean, A.; Tong, L.; van Vliet, V.J.E.; 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. https://doi.org/10.3390/v14081678

Academic Editor: Philippe Gallay

Received: 24 June 2022
Accepted: 27 July 2022
Published: 29 July 2022

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1. Introduction

An estimated 58 million people in the world have chronic Hepatitis C virus (HCV) infection, of whom one third will develop liver cirrhosis with associated risks of decompensated liver disease and hepatocellular carcinoma (HCC). In 2015, the WHO estimated that there were 1.75 million new infections, predominantly due to unsafe health-care procedures and injecting drug use [1]. The development of interferon-free, all oral regimens of direct-acting antivirals (DAAs) has revolutionised HCV treatment. Indeed, the global elimination of HCV has generated much interest and appears to be a realistic goal, as evidenced by the World Health Organisation (WHO) 2030 target [2].

While the rates of sustained virological response 12 weeks after treatment (SVR12) are as high as 95% [3], treatment failure occurs in a small number of individuals. Genetic surveillance of cases of treatment failure is useful, to characterise the emergence of viral drug resistance [4], a risk that occurs due to the extensive natural genetic diversity of HCV.

Under drug pressure, mutations within the targeted viral proteins NS3, NS5A and NS5B may emerge, giving rise to treatment failure [5]. The NS3-inhibitors, including glecaprevir, bind to the NS3 protease catalytic site, preventing posttranslational processing of the viral polyprotein and the release of proteins that are essential to produce viral particles. The NS5A inhibitors such as pibrentasvir and NS5B RNA polymerase inhibitors impair viral replication and the production of viral particles. Treatment guidelines recommend combination DAA therapy [6–8] to reduce the risk of such resistance developing. However, 5–10% of individuals may fail to achieve an SVR12, with the highest risk in individuals who are cirrhotic, require retreatment, are non-adherent or are infected with diverse HCV subtypes such as 1l, 4r and 6b, usually acquired in Asia or Sub-Saharan Africa [9].

Here, we describe a cluster of individuals infected with genotype 2a with a suboptimal response to treatment with glecaprevir/pibrentasvir (G/P), a regimen previously reported to have a high genetic barrier to resistance. We also investigate a subset of individuals from this group who failed treatment. Resistance was associated with the combination of L31M and R353K mutations within NS5A.

2. Methods

2.1. Clinical Cohort and Samples

Demographic and clinical data on individuals with HCV genotype 2 infection, managed within NHS Greater Glasgow and Clyde (GGC), and treated with G/P, were collected between 2013 and 2019. Plasma samples from fifteen of these individuals, including two that failed treatment, were available for whole HCV genome sequencing. Liver cirrhosis was defined by a transient elastography score of greater than 12 kPa. Fisher's exact tests were conducted for categorical variables and Wilcoxon Rank Sum test for continuous variables. Ethical approval was granted by the National Research Ethics Service Committee, East Midlands (11/EM/0323) and from the West of Scotland Research Ethics Committee (12/WS/0002).

2.2. HCV RNA Quantification

HCV viral load testing was carried out at the West of Scotland Specialist Virology Centre, Glasgow Royal Infirmary. Viral loads were determined by the Abbott Alinity m in vitro realtime reverse transcriptase test (Abbott Molecular, IL, USA). It has a limit of detection (LOD) of 12 International Units per millilitre (IU/mL) and a quantitation range of 12 to 2×10^6 IU/mL. Validation shows all HCV genotypes are detected and quantitated equally.

2.3. Library Preparation and Next Generation Sequencing

Extraction of viral RNA from the plasma samples (200 µL) was performed with the Agencourt RNAdvance blood kit (Beckman Coulter, Brea, CA, USA). The RNA was then eluted in nuclease free water (11 µL) and reverse transcribed with Superscript III (Invitrogen, MA, USA), using random hexamers. NEB Second Strand Synthesis kit (New England Biolabs, MA, USA) and KAPA Library Prep kit (KAPA Biosystems, MA, USA) were used for library preparation. Samples were indexed using NEBNext Multiplex Oligos for Illumina (New England Biolabs, MA, USA). Qubit (Thermo Fisher, MA, USA) and TapeStation (Agilent, CA, USA) were then used to quantify and check the quality of the amplified DNA. The libraries were then pooled at similar molar concentrations and target enrichment (TE) was performed using NimbleGen SeqCap EZ system RNA probes (Roche, CH) as previously described [10]. Samples were then sequenced on the Illumina MiSeq platform (Illumina, CA, USA) using a v3 MiSeq Reagent Kit (Illumina, Illumina Centre, Cambridge, UK).

2.4. Bioinformatic Analyses

NGS data were analysed using an in-house Unix-based analysis and genotyping pipeline. In brief, raw sequence reads in the form of fastq files were cleaned and quality

checked and genotyped using a KMER based approach. A BLAST guided programme, Tanoti, was then used to map the sequences to the relevant reference sequences in order to generate consensus genomes. Whole genomes were accepted, where 90% of the genome was covered, and then aligned using MAFFT (multiple alignment program for amino acid or nucleotide sequences) Version 7.313 [11]. Finally, a maximum likelihood phylogenetic tree was constructed using RAxML [12]. The tree was constructed using a general time reversible nucleotide substitution model with 1000 bootstraps. Genetic distance between the sequences was calculated using MEGA X [13], and using a bootstrap method for variance estimation. HCV GLUE software (http://hcv.glue.cvr.ac.uk, accessed 13 June 2021) was used to evaluate and analyse resistance-associated mutations present in the sequenced samples [14]. Aligned, consensus amino acid sequences were inspected within NS3 and NS5A genes to identify mutations unique to HCV from the individuals who failed treatment. ClusterPicker [15] was used to define closely related sequence clusters that were less than 10% different to each other across the whole genome. Whole genomes generated as part of this study have been submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/, accessed on 18 July 2022). Appendix B lists the assigned accession numbers.

2.5. Sub-Genomic Replicon (SGR) Constructs

A sub-genomic replicon (pJFH-1) encoding a Gaussia luciferase gene [16] was modified to encode the NS5A amino acid substitutions identified as unique to the virus infecting individuals who failed treatment. The NS5A constructs containing polymorphisms that have previously been associated with resistance to pibrentasvir were also synthesised and used as controls. These polymorphisms included F28F+L31I and P29S+K30G.The background plasmid sequence, spanning non-structural proteins NS3 to NS5B of HCV, was that of JFH-1, which is a subtype 2a HCV sequence that was isolated from a Japanese individual with fulminant hepatitis [17]. This strain of HCV is known to replicate well in cell culture and is sensitive to DAAs. The constructs were manufactured by Invitrogen (ThermoFisher Scientific, UK) using a site-directed mutagenesis system. A pJFH-1/GND replicon, containing a self-inactivating mutation in the NS5B gene, was used as the negative control.

2.6. RNA Transcription and Electroporation

Replicon constructs were linearised with Xba1 (New England Biolabs, MA, USA) and then purified (Monarch DNA gel extraction kit, New England Biolabs, MA, USA). Purified DNA was then used as a template for RNA transcription (T7 RiboMAX Express Large Scale RNA Production System, Promega, WI, USA) followed by RNA purification (RNeasy Mini Kit, Qiagen, Hilden, Germany). RNA was electroporated (270V, 950 capacitance, ∞ resistance) in 4mm cuvettes (Molecular BioProducts, ThermoFisher Scientific, UK) at 2×10^5 cells per reaction. Electroporated cells were immediately chilled on ice to rest for a few minutes before being resuspended in 5mls of 10% foetal bovine serum DMEM (Gibco, ThermoFisher Scientific, UK). Cells were seeded into 96-well plates and incubated at 37 °C for 4 h.

2.7. Replication Capacity of SGR Constructs

To ensure the SGR constructs were efficiently replicating we collected supernatant at 4, 24, 48 and 72 h for luminescence reading. We then compared the replication efficiency to that of wildtype (JFH-1) by calculating replication capacity. This was calculated using the luminescence readings in the following formula: (Mutant 72 h/Mutant 4 h)/(JFH-1 72 h/JFH-1 4 h).

2.8. Sub-Genomic Replication Inhibition Assay

The 96-well plates were seeded with electroporated cells as mentioned above and then treated with Pibrentasvir (Cayman Chemical, MI, USA, CAS No. 1353900-92-1) in serial dilutions. Cells were incubated with drug for 72 h before culture medium was removed and luciferase assay performed (Pierce™ Gaussia Luciferase Flash Assay Kit,

ThermoFisher Scientific, UK). Relative light units (RLU) were calculated as the 72 h read divided by the 4 h read for each well. Maximum responses were set as the mean RLU of wells untreated with drug and the remaining RLU were normalised to this value and expressed as percentages. Drug response curves and IC50 values were calculated using non-linear regression (GraphPad Prism 9 software).

3. Results

3.1. Individual Characteristics

Between 2013 and 2019, 132 individuals infected with HCV genotype 2a were treated with G/P therapy. The median age was 48 years and 72% (95/132) were male. Outcome data were missing for nine individuals. Of those remaining, 91% (112/123) achieved SVR12. Table 1 summarises the demographic and clinical characteristics of this group. None of the individuals who failed treatment had liver cirrhosis. One individual developed HCC while being successfully treated with DAAs. None of the individuals who failed treatment had HCC prior to or post DAA therapy. Route of transmission was known for 76% (94/123), and of these, 70% (86/123) were people who inject drugs (PWID).

Table 1. Summary of demographic and clinical characteristics of HCV genotype 2 infected individuals treated with G/P between 2013 and 2019. * SVR = sustained virological response, ** IDU = injecting drug use. *** This includes other transmission risks including sexual transmission, tattoos and transmission through blood product administration. † Fisher's exact test used. †† Wilcoxon rank sum test used.

	Treatment Outcome (N = 123)		p Value
	SVR * (N = 112)	**Failure (N = 11)**	
Gender			
Male	80 (71%)	11 (100%)	
Female	32 (29%)	0 (0%)	0.0648 †
Mean Age (standard deviation)	48.8 (±10)	47.5 (±10)	0.8731 ††
Mean HCV Viral Load (IU/mL)	5.88 (±0.9)	5.98 (±0.7)	0.8593 ††
HIV Status			
HIV negative	107 (96%)	11 (100%)	1 †
HIV positive	5 (4%)	0 (0%)	
Liver Status			
Non-Cirrhotic	96 (86%)	11 (100%)	0.356 †
Cirrhosis (>12KPa on transient elastography)	16 (14%)	0 (0%)	
Transmission			
IDU **	78 (69%)	8 (73%)	
Other ***	10 (9%)	0 (0%)	0.6724 †
Unknown	24 (22%)	3 (27%)	

3.2. Phylogenetic Analysis

A maximum likelihood tree was constructed with reference strains from different genotype 2 subtypes. Appendix A shows the full genotype 2 clade and reveals that the circulating HCV subtypes in the clinical cohort included both subtypes 2a and 2b. The subtype 2a cluster that includes individuals P19 and P20, who failed treatment, is shown in Figure 1. This cluster of sequences was distinct to all other sequences withing the subtype 2a clade, as analysed in ClusterPicker (https://hiv.bio.ed.ac.uk/software.html, accessed 19 May 2022). Individuals P6 and P10 were successfully treated with (G/P); P4, P7, P8 and P9 were not treated with (G/P). We noted that there was a sequence from an individual in Edinburgh from 2013 and two other sequences from UK individuals (sequence accession numbers KY320321 and KY620322), also from 2013, that formed part of this genotype 2a cluster, suggesting circulation of this subtype 2a clade since at least 2013 outside of the NHS GG&C area.

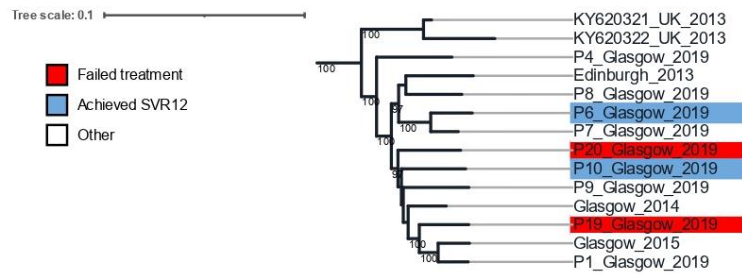

Figure 1. Maximum likelihood tree of whole nucleotide HCV genomes constructed with 1000 bootstrap replicates. This tree shows the Glasgow subtype 2a phylogenetic cluster that includes treatment failures P19 and P20, and P6 and P10 who were both successfully treated. Non-highlighted tree branches represent other sequences, from Scotland, in this 2a cluster, and two HCV sequences downloaded from GenBank [18], also from UK.

3.3. Resistance-Associated Substitutions

Resistance-associated substitutions (RASs) were analysed using HCV GLUE [14]. None of the known NS3 RASs (A156T/V, D168E/V) previously associated with glecaprevir resistance in HCV genotype 2a [19] were noted in the individuals who failed therapy. The known NS5A RAS L/M31M was identified in HCV sequences from P19 and P20 as well as the other sequences in the same subtype 2a cluster. The proportion of sequences with leucine and methionine at position 31 was 15% (22/147) and 85% (125/147), respectively, in all genotype 2a sequences from GenBank, curated by HCV GLUE. We compared available whole genome sequences from the individuals that failed (G/P) treatment (P19 and P20) to HCV sequences from individuals that were successfully treated (P6 and P10) and noted that NS5A polymorphisms R353K and P407L were present in P19 and P20, but not in P10. These were taken forward for in vitro resistance analysis. We next looked to see if the two NS5A polymorphisms were present in the other sequences within the subtype 2a cluster. Out of the remaining 10 sequences, 9 (90%) and 7 (70%) had the R353K and P407L polymorphisms, respectively. Finally, we checked to see how common these two NS5A polymorphisms were in all subtype 2a sequences by analysing an alignment of all subtype 2a NS5A sequences from HCV GLUE. The proportion of sequences that had the R353K and P407L polymorphisms were 11/257 (4.3%) and 138/257 (53.7%), respectively.

3.4. Replication Capacities of Sub-Genomic Replicon (SGR) Constructs

We tested the efficacy of pibrentasvir against SGRs harbouring the above-mentioned polymorphisms in NS5A alongside L31M polymorphism which was observed in P19 and P20 as well as P6 and P10. We first checked the replication capacities of the SGR constructs, the results of which are shown in Figure 2. The control SGR constructs (harbouring polymorphisms F28S+L31I and P29S+K30G) had greater replication capacities (median replication capacities of 1.3 and 1.2, respectively) than JFH-1 (wildtype). The same effect was seen with the SGR construct harbouring mutation P407L in NS5A (replication capacity of 1.4). The SGR construct with mutation R353K had a similar replication capacity to JFH-1.

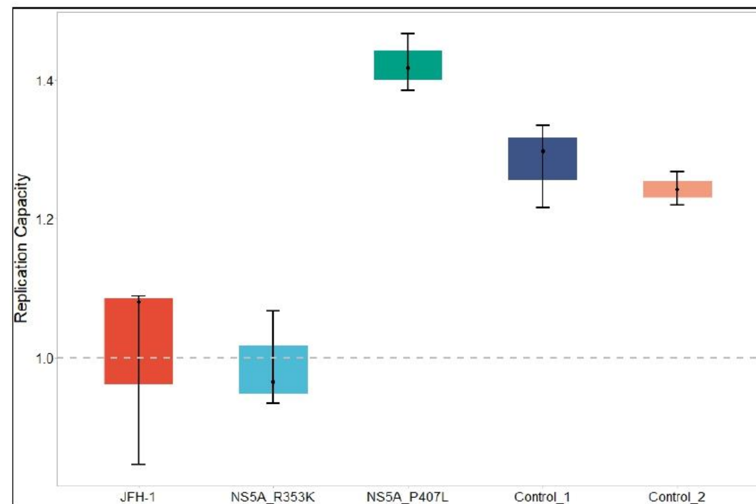

Figure 2. Replication capacities of JFH-1 (wildtype), SGR constructs harbouring polymorphisms unique to P19 and P20 (R353K and P407L), and controls. Control_1 harbours the polymorphisms F28S and L31I. Control_2 harbours the polymorphisms P29S and K30G.

3.5. In Vitro Efficacy of Pibrentasvir

The efficacy of pibrentasvir in replicons expressing R353K was tested with a serial dilution of pibrentasvir, in order to determine mean fold change in IC_{50}. Figure 3 shows the effect of polymorphisms R353K and P407L when compared to JFH-1. The IC_{50} value for R353K (IC50 = 2.42×10^{-3}, 95% CI = 9.54×10^{-4} to 6.546×10^{-3}, R^2 = 90.5%) was 2-fold higher than the IC_{50} value for JFH-1 (IC50 = 9.24×10^{-4}, 95% CI = 6.68×10^{-4} to 1.28×10^{-3}, R^2 = 99%). The IC_{50} value for P407L was comparable to that of JFH-1.

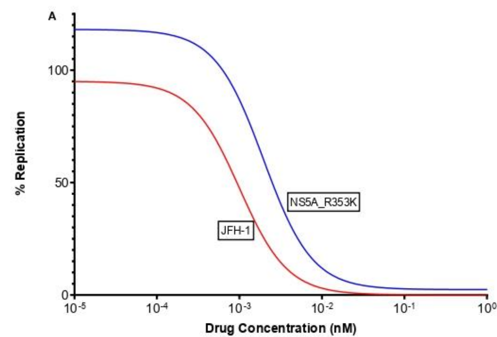

Figure 3. *Cont.*

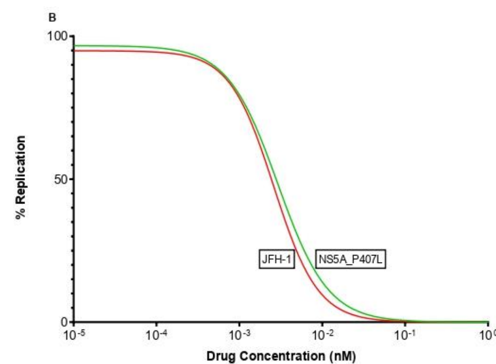

Figure 3. IC50 drug response curves shown for SGR constructs. The constructs harbouring NS5A_R353K is shown in panel (**A**) and NS5A_P407L in panel (**B**).

4. Discussion

In this study, we describe a cluster of HCV subtype 2a infection in 123 individuals who were treated with G/P and achieved a lower than optimal SVR rate of 91%. The EASL guidelines do not routinely recommend baseline HCV resistance testing except in cases of acquisition of infection in areas harbouring subtypes that have a low barrier to resistance [20]. This study aimed to identify mutations associated with lack of SVR12 following G/P therapy, which is considered a pan-genotypic regimen, after an increase in cases of relapse following this treatment in a single health board. Both drugs are also second generation in their respective drug classes and have a higher barrier to resistance than the first-generation drugs. Phylogenetic analysis of the cohort revealed a cluster with more than 90% similarity to each other across the whole genome, suggesting a localised transmission of subtype 2a infection in individuals in Glasgow and Edinburgh. This clade has likely been circulating in Scotland for at least 10 years.

The SVR rate of 91% in this group of individuals is slightly lower than reported in clinical trials of G/P combination treatment [21–23]. We identified a combination of two mutations within NS5A (R353K and P407L) in individuals that failed therapy; R353K in combination with L31M is associated with low–moderate resistance to G/P therapy while P407L is associated with an increase in replication capacity. The R353K polymorphism was present in 79% (11/14) of the sequences in this cluster, but only in 4% (11/257) of all published subtype 2a sequences that include the NS5A region. When R353K is combined with L31M it has a 2-fold higher IC_{50} value than wildtype when tested in an in vitro SGR replicon assay. The most common previously reported polymorphisms associated with resistance to DAAs in genotype 2 are at sites 24, 28–31, 92 and 93 [24–28]. These are all sites within domain 1 of NS5A, which is primarily involved in RNA replication. Crystal structures have been solved for NS5A domain 1 [29]. Site 353 is an unusual site to be associated with resistance as it occurs within the linker region between domains 2 and 3, closer to domain 3, which is involved in virion assembly [30,31] and shows in vitro RNA binding capacity [32]. It may have been undetected in more limited sequencing studies that did not employ whole genome sequencing methods.

While this study was not designed, nor powered, to look at clinical or demographic factors associated with treatment failure, we noted that males were over-represented in the treatment failure group. All 11 individuals that failed treatment were male and 71% (80/112) of individuals that achieved SVR were male (Fisher's exact test $p = 0.0648$).

In this study, we report a lower-than-expected SVR rate among a group of individuals with chronic genotype 2a HCV infection treated with G/P. A polymorphism at site 353 in NS5A was found to be more common in this strain than in other subtype 2a sequences and showed an increase in IC_{50} in an in vitro SGR replicon assay when challenged with

pibrentasvir. The combination of L31M and R353K was present in both cases of treatment failure. The P407L mutation also detected in this cluster is associated with an increase in replication fitness.

Author Contributions: R.S. wrote the manuscript, performed the in vitro assays and data analysis. E.C.T. and C.D. conceptualised the study and reviewed the data analysis and manuscript. S.T.B., E.S.P. and R.F. raised the suspicion of a challenging to treat 2a cluster and provided the clinical data. R.G., A.B.-S., S.J.S. and A.M. provided the clinical samples and associated data and also conducted HCV RT-qPCR. L.T., V.J.E.v.V., M.N.C.B. and A.F. carried out the library preparation and next generation sequencing. All authors have read and agreed to the published version of the manuscript.

Funding: R.S. and E.C.T. were funded by the MRC (MC UU1201412).

Institutional Review Board Statement: Ethical approval was granted by the National Research Ethics Service Committee, East Midlands (11/EM/0323) and from the West of Scotland Research Ethics Committee (12/WS/0002).

Informed Consent Statement: Patients were recruited by informed written consent (11/EM/0323, 12/WS/0002). Additional samples were obtained for sequencing without consent under NHS GG&C biorepository ethics approval using non-identifiable samples.

Data Availability Statement: Genomes have been uploaded to GenBank. Accession numbers are listed in Appendix B, Table A1. Aggregated clinical data is presented in the paper but individual data are not available due to ethical restrictions on the use of identifiable information.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Appendix A

Figure A1. Maximum likelihood tree of whole nucleotide HCV genomes constructed with 1000 bootstrap replicates, showing the whole HCV genotype 2 clade using sequences from GenBank and cases. Tip labels with a background colour distinguish between individuals from Glasgow and those outside of Glasgow. Non highlighted tip labels are reference sequences, downloaded from the Flaviviridae study group, International Committee on Taxonomy of Viruses (https://talk.ictvonline.org/ictv_wikis/flaviviridae/w/sg_flavi/54/hepaciviruses, accessed on 27 April 2022), which has a curated alignment of all reference HCV geno-subtype genomes, and full-length genotype 2 sequences downloaded from GenBank [18].

Appendix B

Table A1. Table of accession numbers assigned to whole HCV genome sequences submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/).

Sequence Identifier	Accession Number
P1	OP022902
P2	OP022903
P3	OP022904
P4	OP022905
P5	OP022906
P6	OP022907
P7	OP022908
P8	OP022909
P9	OP022910
P10	OP022911
P11	OP022912
P12	OP022913
P13	OP022914
P14	OP022915
P15	OP022916
P16	OP022917
P17	OP022918
P18	OP022919
P19	OP022920
P20	OP022921

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Articles

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.

Funding Medical Research Council, Wellcome, Global Challenges Research Fund, Academy of Medical Sciences, and PHARMBIOTRAC.

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Lancet Microbe 2024

Published Online https://doi.org/10.1016/S2666-5247(24)00041-7

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

Articles

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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

See Online for appendix 2

Study design and participants

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).

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 **TrimGalore** see https://github.com/FelixKrueger/TrimGalore

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

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

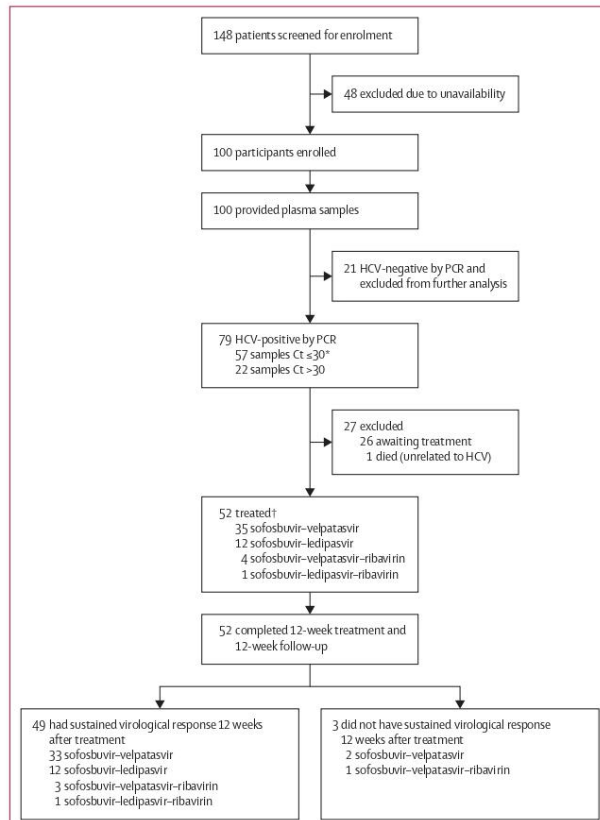


***Figure 1:* Trial profile**

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

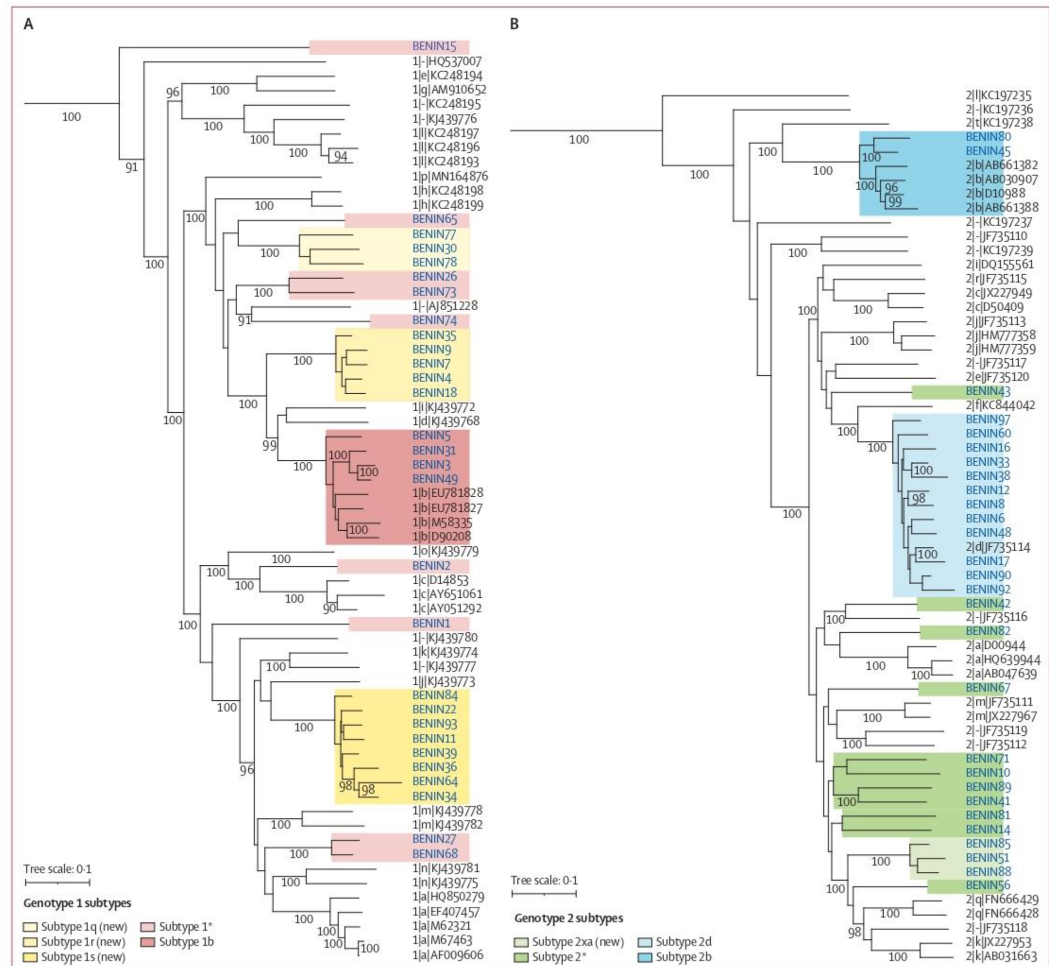
We aimed to recruit 100 participants to the study. We calculated that 36 participants in each genotype group (genotype 1 or genotype 2) would be required to give 80% power of detecting a 20% difference in SVR12 from a baseline of 95%, with a significance level (α) of 0·05. Patient data and SVR12 rates 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

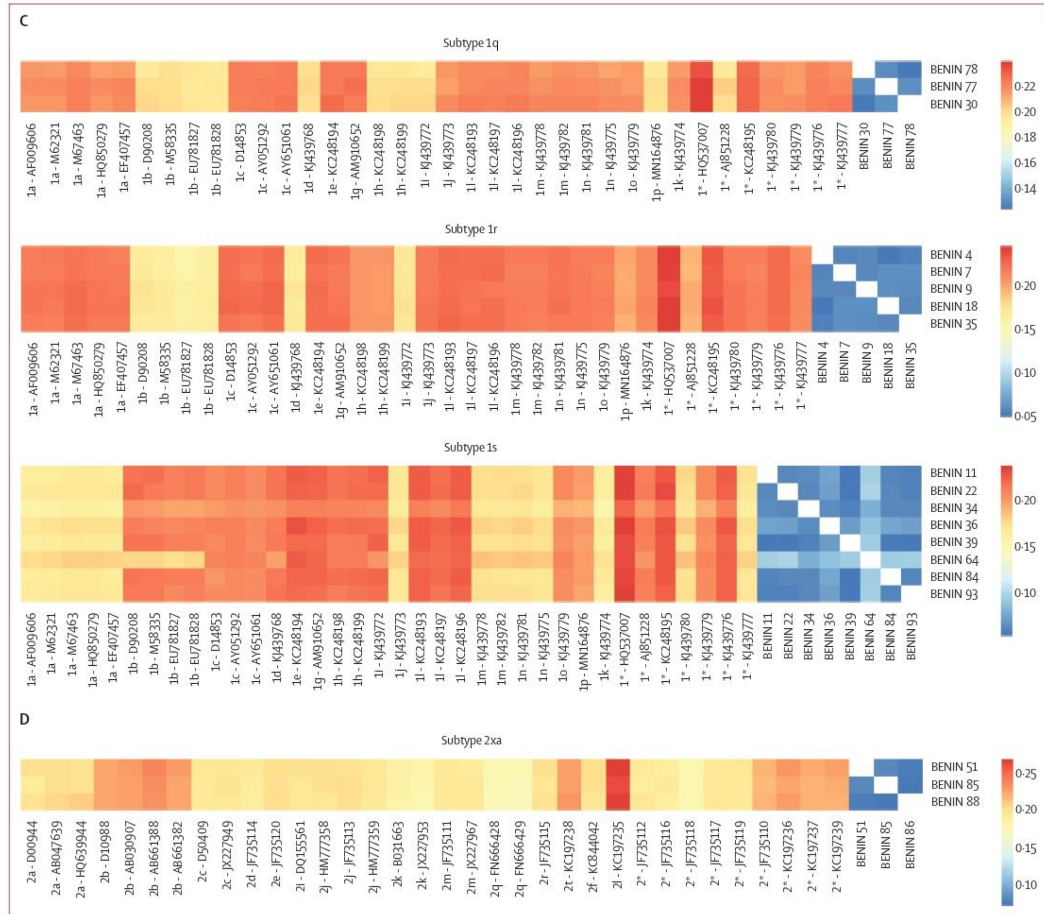


Figure 2: **Genomic diversity of HCV in Benin**

(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.

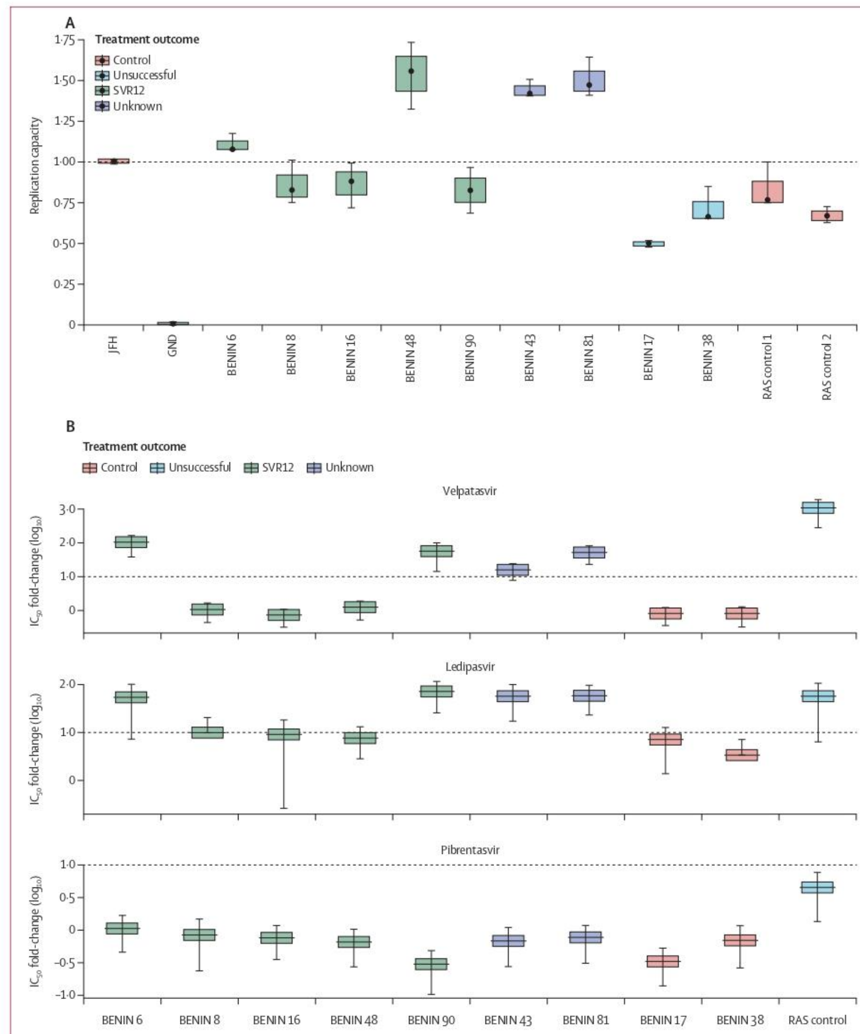


Figure 3: **In-vitro assays of replication and response to treatment**

(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.

Acknowledgments

This study was funded by the UK MRC (MC_UU_12014/1 and MC_UU_12018/12), Wellcome (102789/Z/13/A), Global Challenges Research Fund, Academy of Medical Sciences (GCRFNGR7\1149), and PHARMBIOTRAC. This award is jointly funded by the MRC and the UK Foreign, Commonwealth & Development Office (FCDO) under the MRC/FCDO Concordat agreement and is carried out in the frame of the Global Health EDCTP3 Joint Undertaking. Direct-acting antiviral agents were donated by Gilead Sciences. We thank Donald Smith of the ICTV Flavivirus working group for confirming novel HCV subtype assignments. During the preparation of this work the authors used ChatGPT4.0 in order to improve readability of the text. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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MAJOR ARTICLE

Aiming at the Global Elimination of Viral Hepatitis: Challenges Along the Care Continuum

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A recent international workshop, organized by the authors, analyzed the obstacles facing the ambitious goal of eliminating viral hepatitis globally. We identified several policy areas critical to reaching elimination targets. These include providing hepatitis B birth-dose vaccination to all infants within 24 hours of birth, preventing the transmission of blood-borne viruses through the expansion of national hemovigilance schemes, implementing the lessons learned from the HIV epidemic regarding safe medical practices to eliminate iatrogenic infection, adopting point-of-care testing to improve coverage of diagnosis, and providing free or affordable hepatitis C treatment to all. We introduce Egypt as a case study for rapid testing and treatment scale-up: this country offers valuable insights to policy makers internationally, not only regarding how hepatitis C interventions can be expeditiously scaled-up, but also as a guide for how to tackle the problems encountered with such ambitious testing and treatment programs.

Keywords. elimination; hepatitis care continuum; policy; viral hepatitis.

Viral hepatitis was responsible for 1.3 million deaths globally in 2015 and is now the seventh leading cause of mortality, rising from the 10th cause in 1990 [1, 2]. The full burden of disease encompasses not only mortality, but also reduced quality of life for patients (through cirrhosis and associated complications), financial costs of care for individuals and health care systems alike, and economic costs to society as a whole. Despite this burden, viral hepatitis has only in recent years received the attention it merits. Now, with a World Health Organization (WHO) elimination strategy published [1], the international community is at last focused on tackling the twin epidemics of hepatitis B virus (HBV) and hepatitis C virus (HCV).

At a recent conference (the first "Chronic Viral Hepatitis in Africa" conference, Egypt), clinicians and researchers from across the globe discussed the challenges of reaching WHO elimination targets. Considering the hepatitis care continuum (Figure 1) [3, 4], we identified several key areas in which progress must be made before elimination will be reached. We focused on those areas in which improvements are possible (using tools currently available) and on those aspects of treatment and care where the impact of changes in policy or strategy is potentially greatest. This viewpoint is a distillation of these discussions and is written with the aim of informing policy at this critical moment in the formation of national and international viral hepatitis programs.

Received 6 September 2017; editorial decision 2 November 2017; accepted 16 November 2017.

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DOI: 10.1093/ofid/ofx252

HBV BIRTH-DOSE VACCINATION: CONFRONTING LOST OPPORTUNITIES

Scaling-up of infant vaccination has already had demonstrable impacts on global HBV prevalence [5]. Infant vaccination alone, however, does not prevent mother-to-child transmission (PMTCT). As the risk of chronic hepatitis B (CHB) infection is as high as 90% if infected perinatally [6], effective PMTCT is crucial to reducing incidence.

A key component of a comprehensive PMTCT strategy is birth-dose vaccination. Modeling studies have suggested that an 80% global scale-up of birth-dose vaccination plus infant vaccination, compared with scaling-up of infant vaccination alone, could avert 18.7 million new chronic infections over the next 15 years, highlighting its importance as a PMTCT tool [7]. Monovalent HBV vaccine is inexpensive (US$0.20 per dose), and birth-dose vaccination is likely to be cost-effective [8].

Despite such evidence, global HBV birth-dose vaccine coverage remains low, at 39% [9]. Moreover, vaccines are often administered beyond 24 hours of birth [10], when they are less effective in

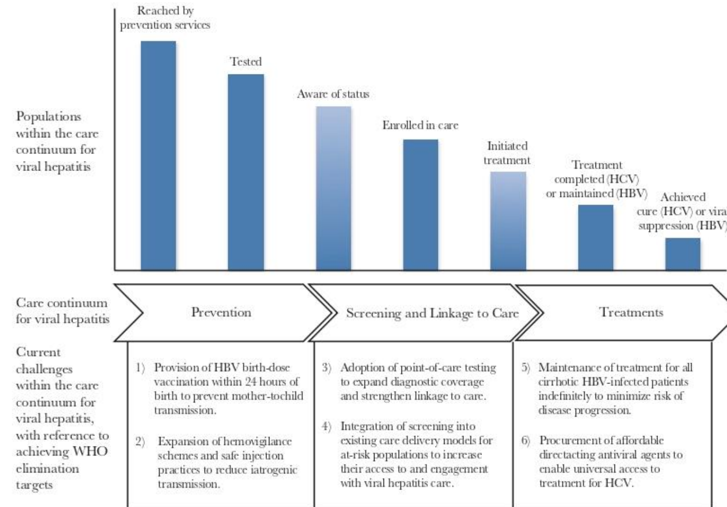

Figure 1. Overview of the WHO care continuum for viral hepatitis and the associated challenges encountered when aiming toward WHO elimination targets, adapted fromn Zhou et al. [3]. Populations within the care continuum for viral hepatitis are as defined within the Global Health Sector Strategy on Viral Hepatitis [4]. Abbreviations: HBV = hepatitis B virus; HCV = hepatitis C virus; WHO = World Health Organization.

PMTCT [11, 12]. There are several reasons for this low coverage: the monovalent vaccine is not funded by agencies like GAVI, as the cost of the vaccine falls below their funding threshold [13]; there are significant costs for vaccine delivery [14]; cultural factors may reduce access to health care by women in the postpartum period [15]; and hepatitis B interventions are rarely a public health priority [16], with only 9 countries in sub-Saharan Africa, for example, incorporating birth-dose vaccination into their national policies by 2015 [10, 17].

Innovative approaches exist to improve timely birth-dose vaccination administration, including increasing the number of health staff–attended births [18], ensuring coordination between immunization and maternal health services [19], expanding vaccine management systems [19], using prefilled injectable vaccines for home births [20], promoting awareness of the need for timely HBV vaccination [21], and simplifying the supply chain by, for example, using heat-sensitive labels to allow storage of vaccine outside the cold chain [22, 23].

A substantial scale-up in birth-dose vaccination coverage is pivotal to reaching WHO 2030 elimination targets [1]. This is long overdue, and acceleration of efforts requires a combination of political engagement by governments, financial commitment, and strategic planning to help countries reach these goals.

IATROGENIC TRANSMISSION: A COST-EFFECTIVE APPROACH TO CURBING TRANSMISSION

Blood and injection safety are fundamental to national viral hepatitis programs [1]. In Africa, for example, the risk of acquiring HCV from blood is 2.5 per 1000 units transfused, compared with 1 per 2–3 million units in high-income countries [24]. In addition, the reuse of injection equipment, inadequate sterilization procedures, lack of universal precautions, sharps injuries, and inadequate medical waste management systems all contribute to the burden of viral hepatitis. To reach WHO targets by 2030, all blood donations should be screened for HIV, HCV, HBV, and syphilis in a quality-assured manner, and 90% of injections should be administered using a safety-engineered device [25].

There are several options for reducing risk of transfusion-transmissible infections (TTIs). Centralized blood transfusion services targeting low-risk, regular, voluntary blood donors should be developed and integrated into health care systems [26]. Although more expensive than replacement donor systems [27], voluntary blood donors represent a safer, more sustainable approach [28] and should contribute at least 80% of all donations to transfusion services [29]. All donations should be screened for TTIs, with external quality assurance, using highly sensitive and specific assays [30, 31]. National hemovigilance systems supported by local transfusion committees enable ongoing surveillance for transfusion-related complications [32], but in low- and middle-income countries (LMICs), only 28% operate hemovigilance systems [33]. Blood safety programs can be cost-effective [34], but cost-effectiveness varies, and this should inform program design: adding antigen-antibody combination tests to reduce the serologically negative window period can be cost-effective [35], whereas TTI predonation

screening using rapid diagnostic tests (RDTs) is not considered to be cost-effective [36].

Improving injection safety is also key to reducing iatrogenic transmission: in 2010, approximately 1.7 million new cases of HBV and up to 315 000 new cases of HCV were attributable to unsafe injections [37]. Such infections can be avoided through the use of safety-engineered devices that protect health care workers from hazardous occupational exposures to bodily fluids [38], needle/sharps hygiene and safe disposal, and a ban on needle reuse [39]. Such measures must be delivered alongside education of health care workers in universal precautions and safe waste management systems. National policies for safe and appropriate use of injections are, furthermore, highly cost-effective [40]. Global bodies must take the lead in promoting and financing blood screening and injection safety initiatives to ensure that these cheap and effective interventions are implemented worldwide.

DIAGNOSTICS FOR HCV: TACKLING THE BOTTLENECK

The advent of highly efficacious direct-acting antiviral (DAA) treatment has revolutionized the therapeutic landscape for chronic HCV infection, but less attention has been paid to screening and diagnosis. Given the nature of the infection, asymptomatic HCV-infected individuals are unlikely to seek health care [41]. Consequently, WHO targets of 90% of active infections diagnosed by 2030 [1] are aspirational, outstripping the diagnosis coverage achieved even in those countries that have been most successful in identifying infected individuals, such as France, Australia, and Sweden [42]. Currently, a 2-step process for diagnosing active HCV infection is usually required: a serological test to screen for exposure, followed by an HCV RNA nucleic acid test (NAT) to confirm viremia [43]. This 2-step process inevitably leads to patient loss to follow-up (LTFU) [44–46]. Furthermore, in LMIC, NAT is economically challenging, and the specialized laboratory staff and equipment are often not available [47, 48].

Alternatively, serum HCV core antigen quantification (HCVcAg) can be used as a surrogate marker for HCV viremia. Testing HCVcAg is a relatively low cost (as low as US$10 per sample [49]), fully automated, and a commercially available assay that can be performed on the Abbott ARCHITECT platform, making it an attractive test for resource-limited settings [49, 50]. Employing HCVcAg testing while still dependent on a centralized testing facility can "uncouple" the sample collection from the testing site through the use of dried blood spot (DBS) samples [51, 52]: While whole blood and plasma samples require prompt transport to the laboratory or refrigeration, DBS samples can be stored at room temperature for several weeks [53]. The robustness of this sample storage technique makes it ideal for decentralizing testing, which is attractive for resource-limited settings [43]. Though testing DBS samples for HCVcAg has been shown to have reduced sensitivity compared with using serum samples [52, 54], the low cost and uncoupling of sample and testing site suggest a role for DBS testing in marginalized populations unlikely to present at centralized testing facilities [54].

The development and validation of RDTs has become a research priority. The WHO has prequalified 2 HCV RDTs: SDBioline (SDBioline, Gyeonggi-do, Republic of Korea) and Oraquick (OraSure Technologies Inc., Bethlehem, PA) [55, 56], an oral fluid-based point-of-care (POC) test with a comparable performance to third-generation enzyme immunoassays (EIAs) [57]. POC testing has been shown to improve HIV linkage to care in LMIC and to be cost-effective [58-60, S61]. Combination RDTs for HIV, HBV, and HCV have also been shown to increase uptake and receipt of results relative to laboratory testing [S62].

Finally, the adoption of pangenotypic DAA regimens may eliminate the requirement for genotype testing entirely [53]. All such developments can simplify and strengthen the diagnosis and treatment cascade, removing potential causes of LTFU, and will be crucial in reaching diagnosis and treatment targets. Active implementation of affordable POC testing at the primary care level will be essential to upscale identification and linkage to care of infected individuals.

PROVIDING CARE: ACCESSING HARD-TO-REACH HCV-INFECTED POPULATIONS

In most countries, anti-HCV prevalence is well below 10% in the general population [S63] but significantly greater in high-risk populations. The most studied population is PWID, in which anti-HCV prevalence can exceed 90% [S64], but men who have sex with men (MSM) and other recreational drug users are increasingly being recognized as significant at-risk populations [S65, S66]. Such populations are often difficult to reach for a variety of reasons, including stigma and the possibility of prosecution [S67, S68]. Programs specifically designed to address epidemics within these populations are critical to the success of disease burden reduction efforts.

Qualitative [S69, S70] and quantitative [S71–S73] research has shown that PWID are interested in engaging with health care services for HCV testing and treatment; there is clear evidence of successful treatment outcomes in this group [S74], yet treatment in PWID remains suboptimal [S75,S76]. Health care provider concerns can act as barriers to HCV treatment. Such concerns may include the presence of comorbidities, the belief that there will be adherence issues, and difficulties managing side effects [S77]. From a patient perspective, there are several factors that may reduce the likelihood of accessing HCV treatment, including negative experiences within health care systems [S78, S79], low literacy rates [S80, S81], inadequate communication with health care providers regarding the nature of the disease and treatment options [S79], and social factors such as discrimination, the threat of stigmatization, and criminalization [S81].

Reviews of treatment models in high-income countries suggest that effective approaches to improving rates of HCV diagnosis and treatment integrate HCV services into addiction care units [S82]. Focusing on treatment of addiction [S83] and encouraging

positive feedback between health care staff and PWID [S84] improve outcomes. Such multidisciplinary approaches reduce noncompliance even among homeless or active illicit drug users [S85]. Similar approaches can be utilized in general practitioner services; a study in the United Kingdom demonstrated how specialist nurses seconded to primary care facilities can offer screening to at-risk individuals, considerably improving HCV detection [S86]. An alternative approach may be treatment as prevention: Modeling suggests that it may be effective at curbing transmission in both PWID [S87] and MSM [S88].

In LMIC, many patients, in addition to those discussed above, are hard to reach through lack of health care services. In such scenarios, HCV screening could be integrated into existing care delivery models for HIV and tuberculosis (TB) [S89, S90]. Success of HIV treatment roll-out offers valuable lessons [43]: Community-based testing improves uptake among high–CD4 count individuals compared with facility-based approaches [S91], and we propose that the impressive improvements in HIV case finding could be replicated for HCV using similar methods.

TREATING HBV INFECTION: SETTING GLOBAL STANDARDS TO SIMPLIFY CARE

Achieving ambitious WHO testing and treatment targets for HBV requires careful consideration of the challenges of scale-up; discussion should focus on simplified models of care and methods of wide-scale testing and treatment, particularly in LMICs.

Treatment for CHB targets individuals with, or at risk for, advanced liver disease, and aims to suppress virus replication, halt disease progression, prevent complications, and avert HBV-related deaths [S92–S94]. Several antiviral agents approved for the treatment of CHB are available in developed countries, with regional guidelines outlining a continuum of care and recommendations on who to treat, how to treat, and when to stop treatment [S95–S96]. These documents, while based on evidence of drug efficacy and benefits of treatment, are not adapted for use in countries where CHB is endemic and HBV-related mortality is highest [2]. WHO guidelines for the prevention and treatment of CHB attempt to address this unmet need [S94]. Tenofovir is the recommended antiviral in resource-limited settings, with entecavir recommended in children aged 2–11 years. The guidelines encourage the use of clinical parameters (clinical diagnosis of cirrhosis) and/or noninvasive tests (APRI score > 2) to assess severity of liver disease.

WHO guidelines suggest that treatment should be targeted at those with the highest risk of disease progression, based on the detection of persistently raised alanine aminotransferase (ALT), and HBV DNA levels greater than 20 000 IU/mL in those older than age 30 years [S94]. All cirrhotics should be treated regardless of ALT levels, HBeAg status, or HBV DNA levels. The simplicity of administration of these antivirals, their tolerability and safety profiles, and their high barrier to resistance make them ideal for long-term use in regions where close monitoring and management of adverse effects may not be feasible.

While there is consensus that cirrhotics require lifelong treatment, the safety of stopping therapy in noncirrhotic patients remains less clear. Most international guidelines recommend discontinuation of therapy for noncirrhotic HBeAg-positive individuals who show evidence of HBeAg loss and seroconversion to antibody to HBeAg (anti-HBe), undetectable HBV DNA, and who complete at least 12 months of consolidation therapy [S94]. Discontinuation is only recommended when ALT and HBV DNA levels can be monitored, as a high proportion relapse after stopping treatment [S94, S97], placing an additional financial strain on health care systems. Antiviral therapy can be stopped in noncirrhotic HBeAg-negative individuals at least 12 months following loss of HBsAg and achieving anti-HBs status [S98]. This needs to be accompanied by sustained virological suppression and normalization of ALT, with off-treatment monitoring for relapse. With only a small proportion of patients achieving HBsAg loss, and with the need for regular monitoring for reactivation and flares after cessation of antiviral therapy in both HBeAg-positive and -negative individuals, we believe it is advisable at present to treat all patients indefinitely [S98]. The additional financial burden that such lifelong treatment imposes, however, as well as challenges to maintaining people in the cascade of care, must be acknowledged when adopting this approach.

An additional complication is access to treatment in LMICs: despite generic tenofovir costing less than US$50 per annum and being accessible to HIV-HBV co-infected individuals as part of antiretroviral therapy, many HBV-monoinfected individuals cannot access antiviral therapy or have to pay out of pocket. National health care systems need to ensure funded, sustainable access to antiviral therapy for HBV-mono-infected individuals, implemented in concert with diagnosis scale-up strategies.

Treatment programs need to be accompanied by wide-scale HBV testing, particularly in LMICs, where screening rates are low. Community-based testing and treatment for chronic HBV infection has been shown to be feasible: In The Gambia, the Prevention of Liver Fibrosis and Cancer in Africa (PROLIFICA) study showed that POC HBV tests perform well in African community settings [S99], and subsequently demonstrated that community-based screening could achieve high coverage and good linkage to care [S100] while remaining cost-effective [S101].

THE COST OF DIRECT-ACTING ANTIVIRALS FOR HCV: HOW PROGRESS CAN BE MADE

A 12-week DAA course costs in excess of US$70 000 in the United States today [S102]. With an estimated 71 million active HCV infections globally [1], such prices render any program aimed at global HCV elimination unrealistic. In 2016, the WHO reported a range of approaches adopted by several countries to demonstrate how barriers to treatment can be overcome [S103].

In LMICs where patents have not been filed or remain under examination, a market for low-cost, generic versions of patented

drugs can be created. Sofosbuvir is not patented in Egypt, and a 28-day supply currently costs under US$30 [S104]. India has used this approach to facilitate production of generics, but a recent decision to grant a patent to Gilead for Sofosbuvir may damage India's role as an HCV generics producer [S105]. Pharmaceutical companies have, in places, awarded voluntary license agreements to permit local companies to produce generics. As of August 2015, Gilead had 11 such agreements with Indian companies [S106]. Additionally, the originator company of Daclatasvir has signed an agreement with the Medicines Patent Pool to enable sublicensing to multiple generic manufacturers in 112 LMICs [S103]. Many middle-income countries are viewed as having market potential and are excluded from these agreements, including China, Brazil, and Thailand [S106]. More generally, complexities of voluntary licensing fragment the market, ensuring that pricing power remains in the hands of pharmaceutical companies [S107]. This problem can be circumvented by development of new therapies: The Drugs for Neglected Diseases Initiative (DNDi) has obtained the license for a new HCV DAA, ravidasvir. This has allowed it to begin production of the drug through an Egyptian firm, Pharco Pharmaceuticals, without the need to maximize profit [S108].

High-income countries can bulk purchase to reduce costs; no country has attempted this more ambitiously than Australia. The government agreed to a AU$1 billion (US$0.73 billion) program to treat 62 000 individuals, corresponding to per-treatment costs of around US$12 000 [S109]. The true novelty of the Australian approach is that if expenditure exceeds up-front cost, the price of drugs decreases, potentially to 0 [S109]. Australia has, in effect, created a subscription system, paying a fixed amount and treating as many Australians as it can.

What ultimately unites these approaches is their customized nature; some countries have had success in reducing prices, but others risk being left behind. A unified approach must be taken to ensure that high-quality, low-cost drugs are available regardless of location. Pooled procurement is one method for achieving this, an approach pioneered by the Global Fund to tackle the HIV, malaria, and TB epidemics [S110], and attempts are underway to apply this approach to viral hepatitis [S111]. Such schemes could be transformative; however, they will rely on capital and political will to achieve the scale required for the program to be a success.

Egypt as a Case Study

Egypt serves as a model for HCV diagnosis and treatment scale-up [S104, S112]. This country, with the world's highest HCV prevalence, has increased HCV treatment numbers to the hundreds of thousands [S103] and intends to treat 5 million patients by 2030 [S104]. It has achieved this by slashing HCV treatment costs and empowering dozens of diagnostic facilities. Furthermore, by simplifying the diagnostic process through implementing the first universal test and treat strategy, it has reduced the time from initial diagnosis to treatment to as little as one week in some centers [104]. Egypt offers an invaluable example to policy makers, not only as an exemplar of how viral hepatitis interventions can be scaled-up quickly and intensively, but also as a guide for how to tackle the problems that may be faced with such ambitious interventions.

Recommendations

Egypt demonstrates what can be accomplished with regards to HCV diagnosis and treatment, given sufficient political will, state coordination, and investment. Across the world, efforts to address viral hepatitis, both HBV and HCV, should focus on providing universal HBV birth-dose vaccination within 24 hours of birth, establishing hemovigilance schemes to prevent transmission of blood-borne viruses, providing nonreusable syringes and educating health staff in injection safety, adopting new diagnostic technologies to expand access to screening, specializing intervention efforts for vulnerable high-risk groups to reduce transmission where it is highest, treating all HBV-infected patients indefinitely to minimize potential harm caused by discontinuation, and collaborating across the continent to drive down HCV drug costs to ensure that, once treatment has been accessed, it is affordable.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

The authors are grateful to the Newton Fund, the British Council, the Mosharafa Foundation, the Science and Technology Development Fund, and the South African National Research Foundation for facilitating the workshop and for funding the conference at which this work was started.

Author contributions. I.W., A.I.G., C.W.S., M.W.S., Y.X.H., R.J.W., N.C., and S.T.R. organized the conference and conceived the article, along with its principal themes. A.H., A.G.L., C.R.J., E.B., G.N., I.W., R.S., S.N., and Z.M. wrote the initial draft. All authors critically reviewed the manuscript. A.H., S.T.R., C.W.S., Y.X.H., R.J.W., and M.W.S. edited and formatted the final submission.

Potential conflicts of interest. All authors: no reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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