



**University of
Nottingham**

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**Molecular Epidemiology and Antiviral
Resistance Characterization of Hepatitis C
Virus Circulating in Africa and Brazil.**

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**Thesis Submitted to the University of Nottingham for the
degree of Doctor of Philosophy in Virology**

February 2020

ABSTRACT

Hepatitis C virus (HCV) infection is a public health problem that is responsible for liver disease and hepatocellular carcinoma Worldwide. Remarkable achievements have been made in the study of molecular epidemiology of HCV in industrialized countries, and this information is proving invaluable in the design of appropriate HCV antiviral treatments. However, throughout sub-Saharan Africa, the understanding and knowledge of the prevalence and distribution of HCV genotypes and subtypes is woefully inadequate, and those studies that do exist have often utilised unreliable commercially available subtyping assays. In this study, we utilised serum samples drawn from 135 patients enrolled in the HCV Research UK cohort to delineate the molecular epidemiology of HCV in 32 countries situated across Africa. We used a Sanger population sequencing approach to define genetic diversity and resistance associated substitutions of HCV sequence. Subtyping was performed using the NS5B and NS5A phylogenetic analyses, which were then compared to results generated using a variety of on-line resistance-associated substitution (RAS) and subtyping tools as well as clinic data that had been generated using commercially available assays. The study revealed the presence of diverse subtypes within the study cohort, many of which harboured naturally occurring RAS. While the online tools performed reasonably well in terms of RAS and subtype prediction, phylogenetic analysis proved to be the most robust method. Critically, the commercial genotyping assays could not accurately subtype or genotype a large proportion of the HCV sequences. In order to develop a subtyping/RAS analysis procedure suitable for resource-poor settings, we also investigated the use of dried serum spot

(DSS) sampling for molecular epidemiological studies. Using serum samples collected in Brazil, we showed that this system was suitable for the collection, shipping, and processing of samples that were then suitable for downstream molecular epidemiological studies.

Together, this data highlights that more extensive molecular epidemiological studies of HCV are needed to enable informed decision-markings by the World Health Organisation and other relevant agencies and bodies in planning improved HCV genotyping assays /protocols for HCV genotyping and treatment regimens relevant to Africa and other low and middle-income countries (LMICs). Furthermore, DSS can serve as a valuable tool in the genetic and phenotypic characterization of HCV in an LMIC setting.

DECLARATION

I declare that this thesis is entirely my research work, except where otherwise stated and all the supporting literature and resources were duly referenced.

Dr Adeboyejo Kazeem Adeyinka

February 2020

DEDICATION

This work is dedicated in loving memory of my late parents Mr. T.A Adeboyejo and Mrs S.A Adeboyejo, for their love and support while alive.

ACKNOWLEDGMENTS

My sincere appreciation to Almighty God for the opportunity to start the Ph.D. and end with great joy and happiness.

My gratitude also goes to Professor Jonathan Ball, Dr. Alex Tarr, Patrick McClure, Dr. Barnabas King and Professor Will Irving for their immense contribution to the success of this research work.

I am also indebted to HCV Research UK for provision of the samples and other people like John McLauchlan, Paul Ellis, Sarah McDonald, and Jenni Benselin. I would also like to say special thanks to Yemisi Adedeji, Dr. Kehinde Sowunmi, Dr. Henry Ebili, Dr. Afrakoma Afriyie-asante, Dr Wakas, Dr. Patwast Jalal, Dr Mosaab Elsheikh, Mr Tunde Osunnuga, Mr Deji Lennox Ebimaro, Mr Owolabi Adesoye, Mrs Kemi Yusuph, Dr Apaa, Dr. Badejo, and Dr. Dennis.

I wish to express my appreciation to the entire virology group for the experience we shared as one big family.

I am grateful to the Nigerian Government and Olabisi Onabanjo University for offering me this scholarship through the Tertiary Education Trust Fund (TetFund).

I will forever be grateful to late Professor Afolabi Oluwadun of the Department of Microbiology and Parasitology for his immense support, advice, and encouragement when the chips were down.

Finally, I thank my sweetheart, Mrs. M.A Adeboyejo, for her unwavering support, motivation, love, and holding forth my home front during this program. Thanks to my children; Adedamola, Aderinsola Adetinuolawa,

Ademola for their understanding and perseverance, my uncle Dr. Adebajo and also my siblings Major Adegboyega Adeboyejo, Engineer Adetokunbo Adeboyejo, Simbo, Fadeke, Adedotun, Azeez, Adejoke and my entire family too numerous to mention.

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ABBREVIATIONS

°C- degrees Celsius

Aa-Amino acids

AASLD- American Association for the Study of Liver Disease

AHC- Acute hepatitis C

ALT- Alanine-aminotransferase

Anti-HCV- Anti-hepatitis C antibody

Anti-HBc- Anti-hepatitis B core antibody

Anti-HBs- Anti-hepatitis B surface antibody

APCs- Antigen presenting cells

ARFP- Alternate reading frame protein

ASGP-R- Asialoglycoprotein receptor

AST- Aspartate aminotransferase

ASV- Asunaprevir

BLAST- Basic alignment search tool

Bp- Base pair

bDNA- Branched DNA

CD36- Cluster of differentiation 36

CD81- Cluster of differentiation 81

cDNA- Complementary DNA

CHC- Chronic hepatitis C

CLDN1- Claudin-1

C-terminus- Carboxy terminus

CTL- Cytotoxic T cell

CD4+- CD4+cytotoxic T lymphocyte

CD8+- CD8+cytotoxic T lymphocyte

CSF- Cerebrospinal fluid

DAA- Direct-acting antiviral

DCV- Daclatasvir

DBS- Dried-blood spot
DSS- Dried-serum spot
DCs- Dendritic cells
DC-SIGN- Dendritic cell-specific intercellular adhesion-3-grabbing nonintegrin
DNA- Deoxyribonucleic acid
DEPC- Diethyl pyrocarbonate
E1- Envelope 1
E2- Envelope 2
EASL- European Association for the Study of the Liver
EC₅₀- B50% effective concentration
EDTA- Ethylenediaminetetraacetic acid
EHM- Extrahepatic manifestation
ELISA- Enzyme-linked immunosorbent assay
EMCV- Encephalomyocarditis virus
ER- Endoplasmic reticulum
FL- Full-length
GAG- Glycosaminoglycan
Gp- Glycoprotein
GT- Genotype
G/ml- Gram per millilitre
H77- Genotype 1 HCV strain
HBsAg- Hepatitis B surface antigen
HAV- Hepatitis A virus
HBV- Hepatitis B virus
HCC- Hepatocellular carcinoma
HCV- Hepatitis C virus
HCVR UK- Hepatitis C virus research United Kingdom
HCVcc- Cell-culture-derived hepatitis C virus
HCVpp- HCV pseudo-particle

Th1 cell- T helper 1 cell
Th2 cell- T helper 2 cells
HIV- Human immunodeficiency virus
Huh7- human hepatocyte-derived carcinoma cell line
HVR1- Hypervariable region 1
IDU- Injecting drug use
IFN- Interferon
IFN- α - Interferon alpha
IFN- γ - Interferon gamma
IFN- λ - Interferon lambda
IgVR- Intergenotypic variable region
IgM- Immunoglobulin M
IL4- Interleukin 4
IL5- Interleukin 5
IL8- Interleukin 8
IL10- Interleukin 10
IL28A- Interleukin 28A
IL28B- Interleukin 28B
IL29- Interleukin 29
IRES- Internal ribosome entry site
IgVR- Intergenotypic variable region
IVDUs- Intravenous drug users
JFH1- Japanese fulminant hepatitis clone 1
JAK/STAT- Janus kinase/signal transducer and activator of transcription
Kb- Kilobases
KDa- Kilo Dalton
LANL- Los Alamos databank
LDL- Low-density lipoprotein
LDLP-R- Low-density lipoprotein receptor

LDV- Ledipasvir

LEL- Large extracellular loop

LIMP2- Lysosomal integral membrane protein II

LMICs- Low and middle-income countries

L- Litre

LMs- Liver macrophages

MHC- Class I Major histocompatibility complex I

MHC- class II Major histocompatibility complex II

µl- Microlitre

ML- Millilitre

ML- Maximum likelihood

Mab- Monoclonal antibody

MEGA- Molecular evolutionary genetic tool

MSM- Men who have sex with men

NANBH- non-A non-B hepatitis

NAT- Nucleic acid test

NCBI- National centre for biotechnology information

NCR- Non-coding region

NGS- Next-generation sequencing

NJ- Neighbour-joining

NI- Nucleos (t)ide HCV NS5B polymerase inhibitors

NK cells- Natural killer cells

NKT cells- Natural killer T cells

NNI- Non-nucleos(t)ide HCV NS5B polymerase inhibitors

NR- Non-responder

NSP- Needle and syringe exchange program

NS- Non-structural

N-terminus- Amino terminus

Nt- Nucleotide

NTP- Nucleotide triphosphate

OCLN- Occludin

Omb- Ombitasvir

ORF- Open reading frame

P- Probability value

PTV- Paritaprevir

PBS- Phosphate buffer solution

PCR- Polymerase chain reaction

PBBML- Peripheral blood mononuclear leukocytes

PegINF- PEGylated interferon

PegIFN- α - PEGylated Interferon-alpha

PI HCV- NS3/4 protease inhibitor

PoC- Point-of-care

PWID- People who inject drugs

R- Responder

RASs- Resistance-associated substitutions

RdRp- RNA-dependent RNA polymerase

RBV- Ribavirin

RCT- Randomized controlled trial

RFLP- Restriction fragment length polymorphism

RIBA- Recombinant immunoblot assay

RPD3- Recombination detection program 3

RTV- ritonavir

RT-PCR- Reverse transcriptase-polymerase chain reaction

RNA- Ribonucleic acid

SSA- Sub Sahara Africa

SD- Standard deviation

SDS PAGE- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SGR- Sub genomic replicon

SOC- Standard of care

Sof- Sofosbuvir

SRB1- Scavenger receptor class B type 1

SsDNA- Single-stranded deoxyribonucleic acid

STI- Sexually transmitted infection

SVR- Sustained virologic response

TM- Transmembrane protein

UK- United Kingdom

UV- Ultraviolet

UTR- Untranslated region

US- United States

VEL- Velpatasvir

VIRP- Virus pathogen database analysis resource

VL- Viral load

VLDL- Very low-density lipoprotein

VOX- Voxiprevir

WHO- World Health Organization

WT- Wild-type

Nucleotide base	Standard Abbreviation
Adenosine	A
Cytosine	C
Guanosine	G
Thymidine	T
Uracil	U

Amino Acid	Standard Abbreviation
Alanine	A
Cysteine	C
Aspartic acid	D
Glutamic acid	E
Phenylalanine	F
Glycine	G
Histidine	H
Isoleucine	I
Lysine	K
Leucine	L
Methionine	M
Asparagine	N
Proline	P
Glutamine	Q
Arginine	R
Serine	S
Threonine	T
Valine	V
Tryptophan	W
Tyrosine	Y

1 CHAPTER ONE:

1.1 INTRODUCTION

Hepatitis C virus (HCV) is a hepatotropic pathogen of public health importance [1]. It was initially identified in cases of post-transfusion hepatitis in patients, who were negative for both hepatitis A and B virus, and was known as non-A non-B [2]. The genome of the non-A/non-B agent was then isolated and cloned from the serum of an experimentally infected chimpanzee in 1989 [3]. HCV infection contributes significantly to the cause of chronic hepatitis, liver cirrhosis, liver failure, and hepatocellular carcinoma [4]. Recent surveys have put the HCV burden globally at about 71 million, which translates to the global viraemic prevalence of about 1% [5]. While about 500,000 patients with chronic HCV infection die annually as a result of complications [6], about 2-3 million new cases are recorded every year [7]. In some developed countries such as in Western European countries, where sufficient data are available, about 5 million adults are living with chronic hepatitis [8]. Out of this, 38% have liver cirrhosis, and 44% develop primary hepatocellular carcinoma [9]. In the United States, about 3.2 million adults have chronic hepatitis, and the majority are baby boomers born between 1945 to 1965, who are 5 times more likely to be infected because doctors reused syringes [10]. It continues to be a significant cause of liver transplant in most advanced countries, especially the United States [11].

1.2 DIAGNOSIS OF HCV INFECTION

The initial diagnosis of HCV depends on the detection of anti-HCV antibodies and HCV antigen [12]. Other investigations include serum alanine aminotransferase level and liver biopsy [13]. The enzyme immunosorbent assays (ELISA) contains recombinant antigens and synthetic peptides that are formed from some portions of the core and non-structural HCV genome [14]. There have been a series of improvements over the years in increasing its sensitivity, specificity, and reduction in the window periods [15]. The increase in the number of antigens (core, NS3, NS4, NS5, E1 and E2) in the third generation ELISA has increased its broader spectrum in detecting anti-HCV antibodies, although the infectious window period is still a daunting issue [16]. Another limitation is its inability to detect anti-HCV antibodies (False-negative) in immunocompromised patients [17]. The fourth-generation assays are a further improvement to the third generation assays. Both antigen and antibody markers are combined in a single assay [18]. These assays are much more convenient and are thought to have reduced transfusion-transmission HCV in developed countries to as low as < 1 per million donations [19]. Nucleic acid test (NAT) assay is the gold standard in detecting the infection as early as the first week of exposure [20, 21]. The other supplemental test is recombinant immunosorbent assay (RIBA), which has high specificity and has the ability to identifying false-positives generated by other assays [22]. However, it is not commonly used in low resource countries due to costs [23]. Various NATs methods are now adopted for use in the molecular diagnosis (qualitative and quantitative) of HCV patients [24]. These methods employ PCR, branched DNA signal amplification, and transcription-mediated amplification [25, 26]. Numerous

commercial assays are now available to measure baseline HCV RNA and to monitor treatment response in HCV patients undergoing therapy [27]. The cost, sensitivity, specificity, and limit detection all play an essential role in the choice of these assays [28, 29].

1.3 NATURAL HISTORY AND CLINICAL MANIFESTATION OF HEPATITIS C VIRUS (HCV)

1.3.1 Acute infection

The development of multiplexed, flow-cytometric microsphere immunoassay, which measures the anti-HCV-IgG reactivity to the recombinant proteins (core, NS3, NS4, and NS5) is a step in distinguishing acute from chronic infection, although the study used HCV seroconversion panels instead of clinical samples [30, 31]. Another problematic aspect of HCV infection is the asymptomatic and non-constitutional nature of the disease, which, in some cases, also contributes to the difficulty in clinical diagnosis [32]. Conventionally, infection within the first six months of exposure is termed acute infection. During this period, patients may be asymptomatic, and the tendencies to progress to chronicity is high [33]. However, the patient can present with mild to moderate fever, fatigue, malaise, body pains, abdominal symptoms, and jaundice [34]. Anti-HCV test can be positive or negative depending on the phase of infection and whether the patient has seroconverted or not [35]. Most often, within 2 weeks of infection, HCV RNA may become detectable in the serum [36]. The hallmark of acute infection is an increase in serum alanine aminotransferase (ALT). ALT rises more than 10 folds above the normal limits, with or without a rise in total bilirubin levels [37]. Previous studies have shown that about 30% of patients with symptomatic infections

develop spontaneous viral clearance [38]. The mechanism involved is not well understood, but high-quality host immune response, as well as viral and epidemiological factors, are suggested to play critical roles in viral clearance [39].

1.3.2 Chronic infection

Chronic infections ensue when the patients fail to clear the virus after six months of exposure [40]. A significant number of studies have revealed that chronic infection occurs in approximately 80% of patients, while 20% may progress to liver cirrhosis within 20 years [41]. In this case, patients may present with weight loss, abdominal pain, ascites, muscle weakness, fatigue, pruritus, encephalopathy, and upper gastrointestinal bleeding [42]. Other complications of chronic HCV infection may include some extrahepatic manifestations such as membranoproliferative glomerulonephritis, cryoglobulinemia, and porphyria cutanea tarda [43]. Hepatocellular carcinoma is the ultimate sequelae of chronic hepatitis, and the risk is about 2-4% per year [44].

1.3.3 Co-infection

Hepatitis C co-infection is a common phenomenon in chronic HCV infections. It occurs when two or more heterologous HCV or distinct viruses coexist within an individual [45]. In most cases, the co-infecting viruses share the same route of transmission, namely parenteral, sexual, or vertical, although transmission efficiency may differ [46]. There is increasing evidence that multiple infections do occur as a result of simultaneous dual infection of two or more distinct HCV [47, 48]. Also, co-infections such as HCV/HIV and HCV/HBV are common occurrences [49, 50]. The prevalence and likelihood

of exposure vary from one region to the other. In developed countries, HCV/HIV co-infection is common among intravenous drug users (IVDUs) and men who have sex with men (MSM) [51]. In low and middle-income countries, blood transfusion and vertical routes play an essential role in its spread [52, 53]. The chronic HCV patients with HIV co-infection progress rapidly to liver cirrhosis and hepatocellular carcinoma [54]. In most developed countries, about thirty percent of HIV patients harbour HCV and this has significant implications for HCV treatment [52]. The prevalence of HCV/HBV co-infection is high among IVD, haemodialysis patients, and patients undergoing organ transplants [55, 56]. Patients with either heterologous HCV infections (Genotypes 1b+3a, 1b+4, 3a+4) or HIV co-infection are usually associated with faster disease progression and poor prognosis [57].

1.4 HEPATITIS C VIRUS STRUCTURE

HCV is an icosahedral enveloped virus with a diameter ranging from 55-65 nm [58]. The virus has two glycosylated proteins E1 and E2 embedded into the host cell-derived lipid bilayer membrane. Inside the lipid bilayer is the inner core protein that encapsulates a single-stranded positive RNA genome of about 9.6 kb in length (Figure 1). The core protein and the RNA genome form the nucleocapsid [59]. In the infected host, virion varies in density (1.15-1.17 g/ml) depending on its association with some plasma molecules.

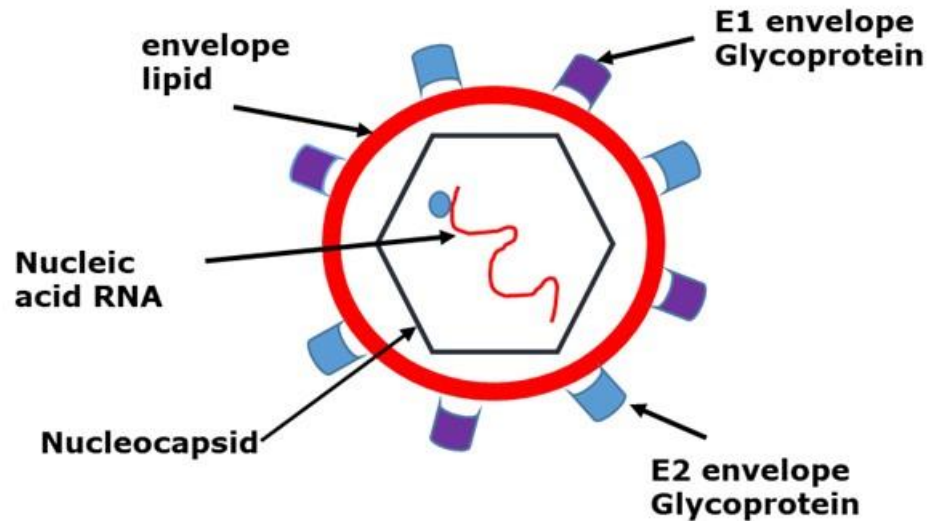


Figure 1: Structure of HCV virion

Schematic diagram of icosahedral symmetry of HCV virion consisting of lipid bilayer envelope studded with two envelope glycoproteins E1 and E2 heterodimer HCV RNA that encodes the viral proteins is encapsidated in the viral nucleocapsid.

It can bind to low-density lipoprotein or very-low-density lipoprotein to form infectious particles [60]. It has been found in association with immunoglobulin and can also exist as free particles [61]. Some HCV particles exist in human blood circulation as a naked form lacking envelop membrane. Their sizes vary significantly, ranging from 38-62nm in diameter with 1.27-1.34 g/ml buoyant density [62]. Their role in the pathogenesis of hepatitis C virus infection is still obscure.

1.5 ORGANIZATION OF THE GENOME AND PROTEIN FUNCTIONS

HCV genome is a single-stranded positive ribonucleic acid molecule, approximately 9500 nucleotides, and it resides in the capsid core proteins [63]. The genome has a single open reading frame (ORF) located between the 5' and 3' untranslated region(UTR) [64] (Figure 2). The 5' and 3' regions are highly conserved, and they possess about 341 and 230 nucleotides (nts), respectively [65]. The 5' UTR consists of four domains that form the major machinery in the interaction with the 40S ribosomal subunit. Domains II, III, IV, and the proximal end of the core constitute an internal ribosomal entry site (IRES), whilst domain III harbours a pseudoknot [65]. The function of IRES is an initiation of translation by binding to the 40S ribosomal subunit in the endoplasmic reticulum [66]. The ORF encodes for approximately 3000 amino acid (aa) residues, which are co- and post-translationally cleaved to produce 11 structural and non-structural proteins [67]. The 3' UTR is structurally delineated into three regions and is responsible for the termination of translation [68].

1.5.1 Structural proteins

The structural proteins include the core, frameshift proteins, p7, and E1E2 envelope glycoproteins.

The Frameshift protein, known as alternative reading frame protein (ARFP), was discovered in 1998 [69] and is generated from an ORF overlapping the core gene at nucleotide+1 [70]. Its role in the life cycle of HCV is not well understood, but studies have shown that antibodies specific to this protein can be detected in the sera of patients with chronic hepatitis and

hepatocellular carcinoma suggesting that this protein plays some roles in disease development[71]

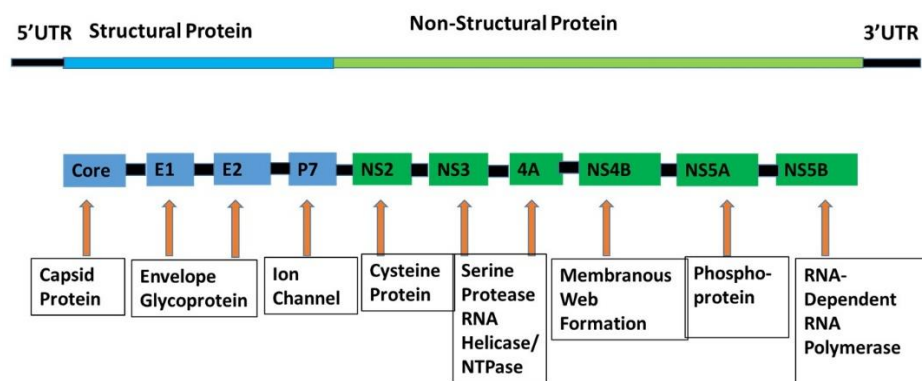


Figure 2: Genomic organization of HCV polyproteins

Schematic diagram of an open reading frame of HCV genome flanked proximal and distal by 5'UTR and 3'UTR, respectively. The positions of structural and non-structural proteins are illustrated. Adapted from Dubuisson *et al.*, 2007 [72].

P7 is an integral membrane protein of two domains linked by a small cytoplasmic loop [73]. It is hydrophobic, 63 aa long, and is located between E2 and NS2 proteins [74]. The function of P7 is obscure, but it has been found to modulate membrane permeability in support of virus entry [75]. This activity has been confirmed in some studies in which

Amantadine, Rimantadine (multiple ion channel blockers) were used to hinder its activity [76].

The core protein is a component of the viral nucleocapsid, and it binds to the RNA genome. The host signal peptidase cleaves the C-terminal of polyprotein to generate an immature core [77]. Further cleavage of the C-terminal of the core by the host signal peptide peptidase results in the

formation of the mature core protein [78]. This mature core usually contains 191 aa with a molecular mass of 23 kDa (p23), although sizes ranging between 17-23 kDa have been detected. The 21 kDa form, however, seems to be predominant [79]. Existing knowledge has shown that the core protein has 3 distinct domains. The first domain is the N-terminal hydrophilic of about 120 aa. It has several positive charges, 3 nuclear localization signals, and it is involved in RNA binding and nuclear localization [80]. The second domain is the C-terminal hydrophobic domain of about 50 aa. It associates freely with the endoplasmic reticulum(ER), outer mitochondrial membrane, and lipid droplets. This domain is responsible for the proper folding of the core protein [81]. The third domain has about 20 aa and functions as a signal peptide for the downstream glycoprotein E1 [82]. Studies have shown that when the hepatitis C virus core protein is expressed in a cell culture model such as mammalian, bacterial, insect, or yeast cell, it can generate nucleocapsid-like particles [83]. Various roles have been assigned to HCV core protein; these include pro and antiapoptotic functions [84], and hepatocellular carcinoma in transgenic mice [85].

The envelope E1 and E2 glycoproteins are transmembrane proteins (TM) type 1, produced by the activity of the host signal peptidase on polyproteins [86]. E1 (193-383 aa) and E2 (384-746 aa) glycoproteins have C-terminal transmembrane domains, and N-terminal ectodomains that form non-covalent heterodimers [87]. During the synthesis of E1 and E2 glycoproteins, their ectodomains are translocated into the endoplasmic reticulum lumen while their transmembrane domains are attached to the endoplasmic reticulum membrane. It has a greater consequence on the form and function of the proteins. Recently, studies have shown that E1 and

E2 have large covalent complexes which are stabilized by several highly conserved cysteine residues that form 4 and 9 disulfide bonds, respectively [88]. Furthermore, the E1 and E2 glycoproteins are highly glycosylated and form about half of the mass of the whole protein [89].

Three hypervariable regions have been observed within the E2 envelope glycoprotein sequence of the ectodomain [90]. The first hypervariable region (HVR1; 384-411) is the immunodominant region, and it is responsible for eliciting type-specific neutralizing antibodies [91]. Studies have also suggested that the second hypervariable region (HVR2; 400-485), and the intergenotypic variable region (IgVR; 570-580) modulate antibody recognition and contribute to immune evasion [92]. However, recent studies have revealed that they are involved in the formation of E1E2 heterodimer, CD81 binding, and virus entry [91]. The TM domain of these glycoproteins consists of a short C-terminal hydrophobic anchor of <30 aa residues long [93]. This membrane consists of two- hydrophobic spans demarcated by a small segment containing two or one conserved positive charged residues [94]. Studies have shown that there could be a change in one membrane spreading topology in the TM domain if there is a re-orientation of the C-terminal of the domain after cleavage by signal peptidase [93]. Transmembrane proteins have been linked to various functions such as membrane anchoring, E1E2 heterodimerization, and endoplasmic reticulum retention signal [95].

1.5.2 Non-structural proteins

The non-structural proteins are NS2, NS3, NS4A, NS4B, NS5A, and NS5B

NS2 is a hydrophobic, nonglycosylated, transmembrane protein located between P7 and NS3 proteins [96]. It possesses two internal signal sequences, which can be found within 839-883 and 928-960. These signal sequences have been found to target this protein to the membrane of the endoplasmic reticulum [97]. Its function is not so clear in the life cycle of the virus because its deletion does not abolish HCV replication *in vitro* [98]. Nevertheless, studies have shown that it is involved in apoptosis inhibition and gene transcription modulation [99].

NS4A is believed to be a cofactor to NS3, and it forms a complex that anchor the protein to the endoplasmic reticulum where replication is thought to take place [100]. NS4B is an integral, hydrophobic protein (27 kDa) of about 261 aa residues [101], containing an N - terminal portion (1 aa-69 aa), a central portion having 4 predicted transmembrane passages (70 aa-190 aa) and a C-terminal portion (191 aa-261 aa) [102]. The protein is known to form oligomers. The oligomerization is a result of the association between the N-terminal and the C-terminal conserved elements [103]. Studies have shown that mutations in the oligomerization of NS4B usually hinders the membranous web formation [104]. The membranous web is known to be the site of viral replication where the protein modulates NS5B RNA dependent polymerase activity and host signal transduction translation [105]. NS4B is also highly antigenic, and it elicits both humoral and cell-mediated immune responses. However, it is a potential target for antiviral therapy [106].

NS5A is a monotropic phosphoprotein (447aa) that consists of an N-terminal amphipathic helix anchor and three domains, separated into two low complexity sequences [107]. The first domain (36-213 aa) and second domain (250-342 aa) have been directly linked to RNA replication whilst the third domain (356-447 aa) plays a major role in viral assembly [108], and core protein interaction [109]. Furthermore, it is also believed to modulate the interferon (IFN) response, acts as an antiviral target and this role has implications in the treatment of HCV [110].

NS5B encodes the RNA dependent RNA polymerase (RdRp) enzyme of about 68 kDa with almost 591 aa residues [111]. This enzyme is responsible for synthesising the positive-sense RNA via a negative sense intermediate [112]. Studies have shown that it has a crystal structure being described as “finger, palm and thumb.” It is formed by N-terminal 530 aa, which contains themes that are common among all RNA dependent RNA polymerases [113]. This enzyme binds to cyclophilin B, a cellular peptidyl-prolyl cis-trans isomerase, which regulates hepatitis C virus replication [114]. RdRp enzyme has been a major target for anti-HCV therapy because of its active role in viral replication [115].

1.6 HCV LIFE CYCLE

The development of cell culture-derived HCV (HCVcc), and HCV Pseudoparticles (HCVpp) has revealed the complex mechanism involved in the HCV life cycle [116, 117]. The process is multifactorial, involving virus attachment, entry, fusion, uncoating, RNA translation, Post-translation processes, Replication, assembly, and release [118]. Both the viral (glycoproteins) and host (receptors/coreceptors) factors play an essential

role in the successful generation of infectious HCV. Hepatitis C virus on entry into a susceptible host circulates and binds to receptors [119]. (Figure 3). Many receptors have been identified, characterized, and are thought to play various roles in virus attachment and entry as described in much detail below [120]. The virus binds to the host cell receptor through the immunodominant glycoprotein receptor E2 [121]. It was thought that heparin sulfate proteoglycans and low-density lipoproteins are the initial coreceptors that aid cellular entry. HCV then enters the cell by clathrin-mediated endocytosis and fuse to the endosome [122]. The fusion of the virus to the endosome is low pH-dependent, and this results in un-coating and release of the virus genome into the cytosol [123].

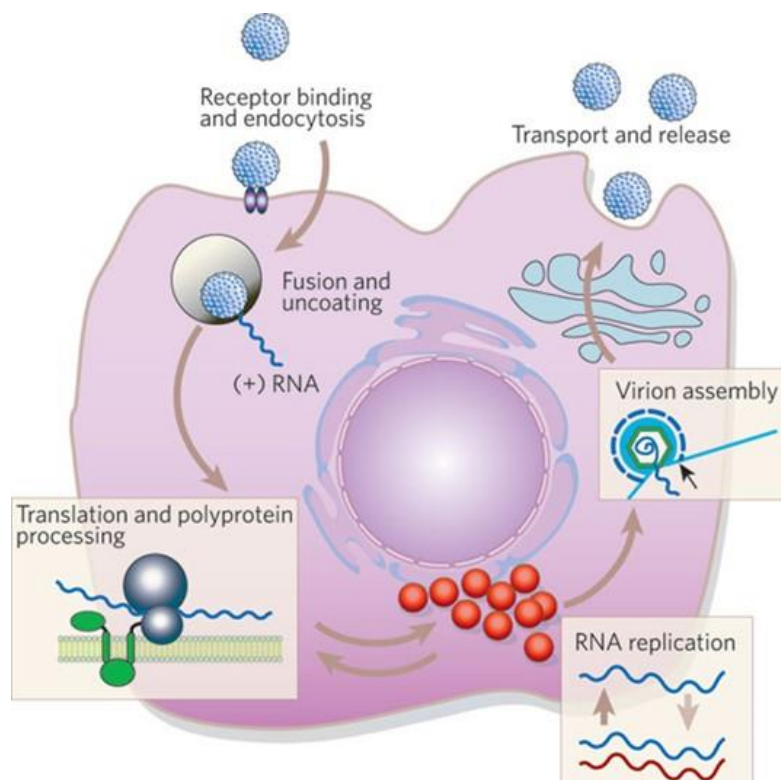


Figure 3: HCV life cycle. The first phase of the life cycle is attachment, then entry by endocytosis and uncoating. The HCV genome functions in three primary roles: translation, replication, and packaging into nascent virion[119].

The virus genome is a positive sense, and its translation into a polyprotein is mediated through interaction between the internal ribosomal entry site (IRES), eukaryotic initiation factors 2 and 3 and other cellular proteins in the endoplasmic reticulum [124]. The positive-sense RNA strand serves as a template for the production of the negative strand replicative intermediate, which is used to produce nascent positive-sense RNA. The replication of these various genome progenies takes place in a complex vesicle that forms a membranous web alongside with some non-structural viral proteins in the cytosol [125]. The fate of the positive sense RNA is of three folds; translation into a polyprotein, or serve as a template for further RNA replication, or assembly into new virion progenies [118]. Virus assembly occurs in the cytosol and budding of the virion is through the

endoplasmic reticulum membrane [126]. The maturation of the virion is achieved through trafficking along the endoplasmic reticulum and Golgi apparatus [127]. The infectious viruses are thereafter released through the host cell membrane [66]. The HCV life cycle has significantly increased current knowledge of how these various steps can be utilized for therapeutic options [128].

1.7 HCV RECEPTORS

Multiple cellular receptors are involved in attachment to the putative HCV E2 glycoprotein molecules before final entry into the host cell [129]. These receptors not only involve in binding; some take part in the internalization of HCV particles [130]. Early studies revealed that the entry of HCV into cells is complex, involving binding to distinct domains of multiple receptors [131]. These binding molecules include: CD81 [132], Scavenger receptor class B type1 (SRBI) [133], Claudin-1, Occludin [134, 135] Low density lipoprotein (LDLP) [136], Dendritic cell-specific intercellular adhesion-3-grabbing nonintegrin (DC-SIGN) [137], Glycosaminoglycans (GAGs), Asialoglycoprotein receptor (ASGP-R) [138, 139]. However, this interaction is not usually straightforward since some host resistance factors were found to interfere and block the interactions between HCV glycoproteins and some of the cellular receptors. A good example is EWI-2wint that inhibits signaling pathways required for HCV entry with CD81 [140].

1.7.1 CD81

CD81 is a cell surface protein with a molecular weight of about 26 kDa; it is a member of the superfamily tetraspanin with four hydrophobic transmembrane domains [141]. A series of investigations has led to the detection of structural and functional components of CD81 [132]. The prominent large extracellular loop (LEL) of its domain interact and bind directly with the E2 glycoprotein dimer [142]. Studies suggest CD81 is expressed in most cells except platelets and red blood cells. They mediate signal transduction, motility, adhesion, activation, and metastasis on the surface of most cells [143]. It is believed that CD81 participates both in the early phase of HCV entry mediated by low PH before endocytosis and late phase following internalization [144]. The development of human monoclonal antibodies raised against CD81 has increased the knowledge and better understanding of the mechanism involved in binding and conformational changes in E2 glycoproteins [145].

1.7.2 SRB1

Scavenger receptor class B type I (SRBI) was discovered as a high-density lipoprotein (HDL) receptor in 1993 [146]. It is a member of lysosomal integral membrane protein II (LIMPII) and CD36 group that plays a vital role in steroidogenesis [147]. Further investigations revealed that a hepatoma cell line deficient in CD81 receptors was able to bind recombinant E2 glycoprotein indicating that it may play an essential role in HCV cell entry since CD81 and SRB1 were believed as binding partners [133]. However, in CD81- expressing hepatocytes, SRBI antibodies have shown to effectively inhibit HCV pseudoparticle in infectivity assay, indicating that SRBI is required for HCV infection [116].

1.7.3 Claudin-1

Claudin-1 (CLDN1) is a member of a 24 tight junction molecule that is essential for HCV entry [148]. Its molecular weight varies between 20-27 kDa with complex secondary structures. It was the first HCV receptor discovered to have been expressed in non-hepatic 293T cells, in contrast to CD81 and SRB1 [149]. CLDN1 is a tetraspanin consisting of two extracellular loops and three intracellular domains with highly conserved motif W-GLWC-C that resides in the first extracellular loop [150]. Present evidence suggests that the proximal part (I32 and E48) of the first EL is responsible for the palmitoylation, which is directly involved in HCV entry [139]. Most antibodies that inhibit this target site seem to have prolonged the inhibition time, and this suggests the possibility of CLDN1 playing a significant role towards the end of the entry phase [151].

1.7.4 Occludin

Occludin (OCLN) is an integral membrane protein that forms part of the complex structural adhesion components known as tight junction molecules [152]. It is a co-receptor that is required for HCV entry. Though occludin was found not to play a significant role in the initiation of HCV attachment to target cells, its knockdown reduced HCV infection. Structurally, it preserves the integrity of cell polarity by demarcating basolateral from apical domains [153]. Functionally, it allows selective movement of some ions and solutes [154]. Occludin has been reported to play a vital role in intracellular interaction with E2 and also been found as a late entry co-receptor molecule [155].

1.7.5 Low-Density Lipoprotein Receptor (LDLP-R)

Low-Density Lipoprotein receptor transports cholesterol rich-Lipoprotein majorly through cells by clathrin-mediated endocytosis [156]. It is a type 1 transmembrane protein of about 839 amino acid residues. It is a complex structure consisting of 7 lipoprotein acceptor units-ectoderm, 3 epidermal growth factor domains, and a β -propellor domain [157]. Interaction of LDLP-R with LDLP was thought to be ionic- driven between charged residues of lipoprotein acceptor and apolipoproteins E and B [158]. The receptor-ligand complex permeates through the cells form a low PH endosome that adopts conformational changes [159]. This conformation enhances the release of bound lipoprotein and interacts with lysosomes [160]. Cholesterol esters are end products after undergoing lysis [161]. To further confirm the activity of the receptor, anti-LDLP-R with anti -lipoprotein B and E raised against these molecules were found to inhibit endocytosis [136].

1.8 MODEL SYSTEMS OF HCV REPLICATION

Understanding the HCV life cycles and viral-host interactions have been a significant problem faced by HCV researches in the past due to the lack of robust cell cultures for HCV propagation and limited animal model [162]. *In vitro* and *in vivo* model systems are required to study HCV pathogenesis, antiviral therapy, and vaccine development [163]. Lohmann *et al.*, in 1999, first reported a molecularly cloned genotype 1b HCV replicon system [112]. This was genetically modified sub-genomic HCV RNA, which replicates autonomously *in vitro*. It was achieved by replacing the structural genes protein-coding region with the neomycin-resistant gene in the upstream while the downstream was tagged with a second internal ribosome entry

site derived from the encephalomyocarditis virus (EMCV) that promote translation of the non-structural coding region [164]. This method has helped in the study of HCV RNA replication in the hepatoma cell lines [165]. However, the major setback of this replicon was the inability to replicate without adaptive mutations efficiently, and also viral entry and assembly cannot be studied. As a follow up to sub-genomic amplicon, in 2005, a full-length replicon derived from HCV genotype 2a Japanese fulminant hepatitis clone 1 (JFH1) was described [166]. This strain replicates more efficiently both in Huh 7 cells and non-hepatic cells, and more importantly, they did not require any adaptive mutations. However, the infectious efficiency was low, and it could not be propagated in naïve, Huh 7-cells. In the same year, Zhong *et al.*, reported JFH1 consensus clone that replicates at high titre in the range of 10^4 - 10^5 infectious unit /ml compared to the original JFH1 on Huh 7-derived cell lines [105].

In 2003, the HCVpp system was developed as a surrogate HCV infection system that provided valuable information on viral entry and neutralization [116]. HCVpp are produced from expression vectors encoding full-length E1E2 together with either a retroviral or lentiviral packaging genome carrying a reporter gene [167, 168]. These components are transfected into 293T cells resulting in the secretion of HCVpp containing a viral reporter gene, which allows a read-out of the level of infectious units per mL in the supernatant [169]. With the use of the HCVpp system, all the major six HCV genotypes have been investigated in terms of both viral and cellular entry factors [170]. More importantly, several neutralizing antibodies against HCV glycoproteins and cellular receptors have been studied in an attempt to provide HCV therapeutics, and for vaccine development [171,

172]. The secreted viral particles were capable of infecting naïve cells and chimpanzees [166]. This system not only led to the development of several chimeric HCVcc constructs representing diverse genotypes but also bring about *in vitro* assessment of antiviral therapy [173, 174].

In addition to the *in vitro* models described above, several researchers have developed a range of *in vivo* model systems. The use of chimpanzee as an animal model has been a massive task due to ethical constraints, limited availability, and high costs of these animals [175]. Consequently, a small animal model, a tree shrew (*Tupaia belangeri*), was discovered to have shown susceptibility to HCV infection displaying mild hepatitis and intermittent viremia [176]. Also, HCV RNA harvested from an inoculated animal can be effectively cultured in primary hepatocytes. A greater advance *in vivo* models is the genetically humanized mouse and human liver xenograft mouse models [177, 178]. These two methods have brought the HCV replication microenvironment closer to that in the human liver. It has revolutionized host-virus interaction, cellular immunity, preclinical analysis of antiviral drugs, and vaccine strategies [179].

1.9 IMMUNE RESPONSE TO HCV INFECTION

Infection, clearance, persistence, and progression of HCV into chronicity depend largely on the interaction between the host immune system and the virus [180]. This mechanism is rather complex in humans and poorly understood, but it involves a highly integrated innate and adaptive immune response [181].

1.9.1 Innate immune response

The innate immune response as the first line of defense mechanism plays a vital role in surveillance, recognition, and clearance of the viral infection [182]. It involves both cellular and humoral components which, though are less robust and non-specific compared to adaptive immune response, play a significant role in the outcome of HCV infection [183]. In humans, innate immune responses are mediated via cytokines and antigen presenting cells such as natural killer cells (NK), natural killer T cells (NKT), dendritic cells (DCs) and liver macrophages (LMs) [184]. Like other viral infections, initial HCV infection is characterized by rapid onset of high viral titres, which can be detectable within two weeks without any evidence of liver damage or antibody production [185]. At this stage, type 1 Interferon (INF) produced by HCV infected cells kick start the immune response. The type 1 interferons (INF; α/β) inhibit HCV replication via an intermediate, 2'-5' oligo adenylate synthetase [186]. Similarly, they activate NK cell, cytotoxic T cell (CTL) and also promote the expression of major histocompatibility complex (MHC) class II on the surface of antigen-presenting cells that eliminate infected cells by cytolysis [187]. Destruction of infected hepatocytes has also been found to stimulate NK and NKT cells to produce type 2 interferon (INF γ), which activates hepatic macrophages and local inflammation [188]. The hallmark of the innate immunity is to downregulate signaling pathway that promotes HCV replication and also initiates adaptive immune responses [189].

1.9.2 Adaptive immune response

The adaptive immune response consists of antibody-producing B cells and potent T cell responses (CD+4 T helper cells, CD+8 cytotoxic T lymphocyte) tactically responsible for the elimination of the remaining HCV if the fate of infection is viral clearance [190]. Seroconversion of immunocompetent patients with virus-specific antibodies can be detected in about 6-8 weeks following infection [191]. The role of antibody in HCV infection still needs further investigations. In acute infection, the antibodies produced by activated B-cells are both neutralizing and non-neutralizing, targeted at the hypervariable region (HVR1) of E2 glycoprotein [192, 193]. However, cross-neutralization has also been reported [194]. Available evidence has shown that there is a correlation between rapid induction of cross-reactive neutralizing antibodies with spontaneous clearance [195, 196]. About 20% of individuals able to clear HCV infection, both the neutralizing antibodies and potent T-cell responses are responsible [197]. To further show the significance of neutralizing antibody, Sagnelli *et al.*, reported increase replication of HCV in a patient receiving rituximab-based therapy as a result of B cell depletion [198]. Also, patients with primary hypogammaglobulinemia have been shown to exhibit a severe form of HCV infection [199].

The T cells of CD4+ and CD8+ detection during the acute phase of HCV infection have been found to have an excellent prognostic value [200]. The CD4+ activities differentiate into type 1 helper T cell (Th1) and type 2 helper T (Th2) cell. The Th1 cells produce interferon gamma, alpha, and interleukin 2 that activate cytotoxic T lymphocytes [181, 190]. The Th2 cells secrete

IL4, IL5, and IL10 that activate B-cells to produce neutralizing antibodies [201].

Interestingly, Th2 downregulates the activities of Th1, and it has been suggested that imbalances between these two pathways may likely contribute to the cause of chronic infection in HCV patients [202]. CD4+ T cells recognize HCV antigens presented by antigen presenting cells (APC) in association with MHC class II and also directly activate macrophages and CD8+ cells [203]; thereby acting as both effector and regulatory molecules. CD8+T cell, on the other hand, is MHC class 1 restricted; these cells directly lyse infected target cells and secrete IFN gamma and TNF alpha, which inhibits HCV replication without cell lysis [204].

1.9.3 Evasion of the immune response

Over the years, many studies have investigated the mechanisms and the factors that are responsible for the persistence of HCV infection in about 80% of patients that developed chronic hepatitis [205, 206]. The mechanisms to evade the immune systems affect the two arms of the immune system (both humoral and cell-mediated). The persistence of infection in chronic HCV patients have been attributed to a failure in three major components (neutralising antibodies, CD4+T, and CD8+ T cells) of the antiviral adaptive immune response [190]. T-cells mediate both protective and pathological roles in HCV infection by killing infected cells through IFN- γ and IL2 production [207]. Escape mutation is one of the widely reported mechanisms of HCV evasion of neutralizing antibodies [208]. HCV has a high replication rate of about 10^{10} - 10^{12} HCV virions/ day; however, with the lack of proofreading capacity of RNA dependent RNA

polymerase, populations of mutational variants generated are capable of escaping immune response [209].

Furthermore, sequence changes in the HVR1 of E2 glycoproteins of HCV have been reported to contribute to escape from neutralizing antibodies [210]. Similarly, Dowd *et al.*, showed that when individualized pseudoparticles expressing successive envelope sequences were used to measure neutralization by autologous sera, antibodies neutralizing earlier sequence variants were detected at earlier time points than antibodies neutralizing later variants; indicating clearance and evolution of viral variants in response to pressure from neutralizing antibodies [195]. Furthermore, a CD81 receptor binding site on E2 glycoprotein is a target for broadly neutralizing antibodies [211]. This region is highly glycosylated, and these specific glycans are known to shield the CD81 binding site, and mutation at this site inhibits neutralization [212].

HCV also persists in patients through modulation of the immune system either by downregulating or interfering with signaling pathways that inhibit HCV replication [213]. HCV core protein has been found to interfere with Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathways hence downregulating signaling cascade activity of the IFN system [214]. Also, NS5A downregulates the protein kinase R (PKR) expression promoting the secretion of IL8 that inhibits the activity of IFN [215, 216]. Furthermore, HCV core proteins (and NS4A/B) liberated in the course of infections bind with gC1qR on T cells, thus inhibiting its activation and secretion of IFN gamma [217]. In the same manner, the expression of MHC class I molecules on the infected cell surface requires trafficking via the endoplasmic reticulum-Golgi system; this is blocked by NS4A/B which

render cytotoxic T lymphocyte (CTL) inertia [218]. Hepatocytes, the liver cells are the primary replication sites of HCV; however, extrahepatic sites are now found to be other reservoir areas for persistence [219]. There is now evidence that HCV replicates in the Peripheral blood mononuclear leukocytes (PBML) [220], dendritic cells [221] and even in the cerebrospinal fluid (CSF) [222]. Recently, HCV was discovered to traffic from cell-cell via tight junction in order to evade neutralizing antibodies [223].

1.10 HCV GENOTYPES AND GENOTYPING

HCV exhibits considerable genetic diversity within its genome. This divergence was attributed to a lack of proofreading mechanism display by RNA dependent RNA polymerase [224], high number of virions replication per day, and random genetic drift [225]. Also, high genetic diversity in E2, especially HVR1 and HVR2, which are under constant immunological pressure by neutralizing antibodies [226]. The significant diversity in this genome has been employed in phylogenetic analyses to classify HCV into 8 genotypes and more than 66 subtypes [227]. Genotypes and subtypes differ by between 30-35% and ~15% at the nucleotide level, respectively [228]. Within an individual infected with HCV, genetically distinct but closely related strains that differ by about 5-9% in their nucleotide sequence exist, and these are often referred to as quasispecies [209]. Another mechanism known to generate genetic variation in HCV is recombination, which occurs as a result of different genome segments derived from parents of different genotypes, subtypes, and even intrastrain [229]. The discrepancy in the genome of these recombinants is determined by phylogenetic analysis of selected HCV segment (core and NS5B) which

can be validated via boot scan Simplot (RDP3) that defines a recombinant breakpoint [230]. Whilst several recombinants have been described (2k/1b, 2i/6p, 1a/1c/, 1b/1a); this is still considered to be a rare process [231].

Nucleotide sequence variability differs considerably across the genome; some segments are highly conserved (5' noncoding region, core), some are relatively conserved (E1 and NS5B) while some are highly variable (E2) [232]. Those regions that are highly and relatively conserved often used for genotyping [233, 234]. Therefore, a successful robust genotyping PCR based assay requires a variable region flanked by conserved regions when designing primers. Commercial genotyping assays utilize different strategies such as reverse hybridization, real-time PCR, DNA sequencing [235, 236]. These commercial assays differ in efficiency and accuracy in assigning genotypes and subtypes in clinical practice [237]. These assays target different HCV genome regions (Figure 4) [238, 239].

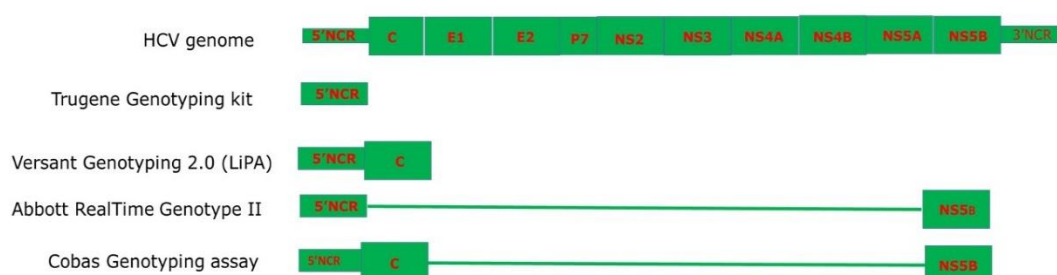


Figure 4: HCV genome organization and genome regions employed in the design of commercial genotyping assays.

Chueca *et al.*, evaluated the accuracy of three commercial assays: Trugene HCV genotyping kit-based on sequence analysis of 5'UTR (Siemens), VERSANT HCV Genotype 2.0 assay-based on 5'UTR and CORE (Siemens) and Real-Time HCV genotype II –based on 5'UTR and NS5B (Abbott) using NS5B sequencing as a reference. They observed varying discordance of 34% for Trugene and 15% for Versant HCV 2.0. The Abbott assay was able to subtype 1a and 1b accurately but could not subtype all other subtypes [240]. Also, Fernandez *et al.* compared the Cobas R HCV genotype assay, which targets three HCV genes (5'UTR, CORE, and NS5B) with NS5B sequencing as a reference [241]. This assay was able to achieve 98% concordance with the reference; however, it was unable to identify some subtypes 1a, 3a, and 4d present in clinical samples.

Furthermore, another study carried out by Chantratita *et al.*, described a commercial 6 HCV genotyping 9G test based on one test tube reaction where immobilized ssDNA oligonucleotide is allowed to hybridize with the complementary Cy5 labelled ssDNA in the PCR product. This test amplified and genotyped 5'UTR of 6 HCV genotypes (GTs 1a, 1b, 2, 3, 4, and 6). It was reported to be 100% in agreement with the genomic sequencing [242].

Genotype Real-time Light cyclers are a technique that uses conserved sequences from mostly 5'UTR, core, and NS5B fragments of the HCV genome, which were used to design primers and hydrolysis probes labelled with fluorophores [235]. The reaction can take place with a self-quenched probe in a single reaction coupled with melting curve analysis run in a real-time PCR machine that allows a two –melting temperature code to be generated where specific subtypes can be identified [243]. It is a quick

method with little contamination as all test takes place in a tube. However it can misclassifies genotype 6e as genotype 1[244].

Genotype LiPA involves the hybridization or reverse hybridization of PCR products with genotype-specific probes using different regions of HCV genome [245, 246].The biotinylated amplicon generated from RT-PCR amplification of the specific HCV region (5'UTR and core) were hybridized to immobilized oligonucleotide probes [247]. The probes bound to a nitrocellulose strip by a poly (T) tail are specific for the 5'UTR and core region of different HCV genotypes. Alkaline phosphate labelled conjugate (streptavidin) bound to the biotinylated hybrid. The chromogen (substrate) react with the streptavidin-alkaline phosphate complex forming a colour precipitate that appears as a visible banding pattern on the strip. In the case of genotype determination, the assay strips are aligned with the versant HCV genotype 2.0 assay reading card and this is compared with the line pattern on the versant HCV genotype 2.0 assay. Interpretation chart or bayer LiPA –scan HCV software v20 can be used to scan the assay strips on a flatbed scanner. Again this method also has a limitation in the identification of some HCV subtypes [248].

Other genotyping methods include serotyping [249] and restriction fragment length polymorphism (RFLP) [250], all of which have a varying degree of accuracy and tend to be less popular.

Serotyping is an indirect typing technique that relies on HCV infected patients to produce type-specific antibodies to multiple recombinant antigens or synthetic peptides derived from various parts of the HCV genome[251]. In most assays, the genotype-specific antibodies are

directed to the epitopes of the NS4 coding region of the genomes of genotypes 1 – 6 [252]. The advantage is that the score is easy to interpret using the criteria such as specific serotype, multiple serotypes, no type-specific antibodies or nonreactive [253]. The efficiency of the assay depends on the immunocompetent of the infected HCV patients, and the humoral immune responses of individuals vary considerably. There is a tendency to produce a false-negative result during the window period after infection [254].

Restriction fragment length polymorphism is based on non-selective amplification of HCV cDNA using suitable conserved primers [255]. The amplicon produced is subjected to restriction endonucleases which cleave the nucleotide sequences at specific sites that allow detection of the genotype/subtype through restriction patterns visualizable on agarose/polyacrylamide gel [256]. RFLP is easy to carry out, inexpensive. However, it is time-consuming, and also prone to misidentification of some novel HCV subtypes [257].

Genomic amplification, followed by sequence and phylogenetic trees, remains the gold standard for genotyping and subtyping [258]. Several studies have focussed on different regions (5'UTR, CORE, E1, NS3, NS5A, and NS5B) of the HCV genome to perform sequence and phylogenetic analyses in order to assign HCV genotypes/subtypes [259-262]. Most significantly, Hraber *et al.*, made a phylogenetic comparative analysis of these regions (polyprotein, 5'UTR, and NS5B) with whole genome (Figure

5) and discovered that the NS5B gene was able to accurately classify HCV to subtype level, in contrast to 5'UTR that is highly conserved and unable to resolve HCV at the subtype level [263]. Technical challenges encountered with the use of the sub-genomic NS5B phylogenetic analysis include good primer selection, presence of rare subtypes which confound analyses, intergenotypic recombination, and mixed infections [264]. The use of next-generation sequencing for genotyping and sequence analysis may provide one solution to these technical challenges [265, 266].

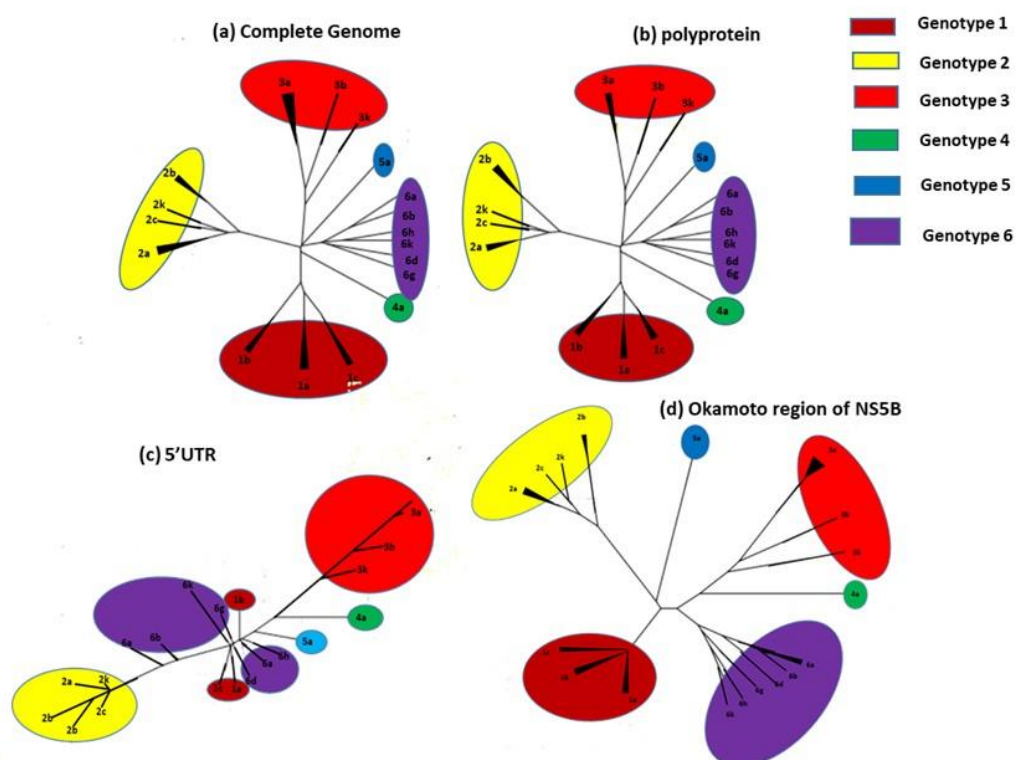


Figure 5: Unrooted neighbour-joining phylogenetic tree reconstructed from (a) complete HCV genome, (b) polyprotein, (c) 5'UTR, and (d) the Okamoto region of NS5B. The relative topology of each gene in the subtype assignment is more emphasize than the scale [263].

Whole gene sequencing (WGS) appears to define a comprehensive viral genotype/subtypes and other information on the three different potential DAA resistance at once [267]. It is capable of determining mixed infections, minor populations of RAS and can also be used efficiently to detect recombinant forms of HCV; although, recombinant forms are a rare occurrence [266]. WGS does not require labourious optimization of Sanger sequencing of each gene which often is time-consuming. However, WGS is still scarce; emerging and economic evidence is limited [268].

1.11 TREATMENT OF HCV INFECTION

Generally, the management of HCV is dictated by the host and viral factors [269]. Host factors include whether the patient is treatment naïve, treatment-experienced, and whether they display either compensated or, decompensated cirrhosis. Other patients' factors are obesity, insulin resistance, and race (IL28 Polymorphism). Viral factors include the viral load, subtypes/genotype, and pre-existing resistance-associated substitution (RAS) [270].

1.11.1 Previous HCV treatment

The first therapy instituted for the treatment of HCV infection was 48-week interferon monotherapy, which only achieved sustained virologic response (SVR) of 29% [271]. This therapy was genotype-dependent and was most effective in genotype 2 and 3 but poor for genotype 1. Following the poor performance of this monotherapy, ribavirin, a broad-spectrum antiviral guanosine analogue was introduced as a combination therapy that improved SVR to 40% [272]. In 2001, alpha-interferon was replaced with PEGylated interferon, which has polyethylene glycol polymer side chain that

not only improves its half-life but also increases the binding affinity to human type 1 interferon receptors [273]. The duration of this therapy is either 24 weeks or 48 weeks, depending on the genotype, but this still only resulted in 40-50% SVR [274]. Not only were these treatments relatively ineffective, but they were also associated with severe adverse reactions. PEGylated interferon causes bone marrow suppression (neutropenia and thrombocytopenia), flu-like symptoms (fever, headache, arthralgia, and myalgia), neuropsychiatric disorders (irritability, apathy), and autoimmune syndrome such as autoimmune thyroiditis [275, 276]. Ribavirin causes haemolytic anaemia, and all of these side effects are responsible for dose modification or poor drug compliance [277, 278]. The low SVR, coupled with the side effects of these drugs lead to the quest for alternative treatments.

1.11.2 Current treatment

An ideal anti-HCV drug regimen is expected to have high efficacy, is well tolerated, has short treatment duration, has oral dosing, be affordable, has limited drug-drug interaction and possesses a high barrier to resistance [279]. Unfortunately, it has been difficult for any drug to meet all these criteria. The primary goal of current direct antiviral agents (DAAs) combination therapy was the exclusion of the PEGylated interferon due to its toxicity [280]. DAAs have lesser side effects and toxicities compared to PEGylated interferon and ribavirin therapy. Some minor symptoms such as anaemia, headache, fatigue and gastrointestinal discomfort have been observed in few cases [281]. Furthermore, some level of nephrotoxicity [282] and hepatotoxicity [283] have been reported with combination therapy involving sofosbuvir in which renal function test determination is required

before the initiation of this therapy. In 2011, the first-generation direct-acting antivirals introduced were inhibitors of virally encoded NS3/4A protease (Boceprevir and Telaprevir) [128]. These DAAs, when combined with PEGylated interferon and ribavirin, improved the cure rate. The efficacy of this combination therapy was corroborated in two studies conducted on HCV genotype 1 patients that did not respond initially to PEGylated interferon and ribavirin. These patients were able to achieve 66% SVR when treated with boceprevir and 88% with telaprevir at 32 weeks and 48 weeks, respectively [284, 285]. Also, in 2013, less toxic second-generation DAAs consisting of RNA dependent RNA polymerase and NS3/4A inhibitors (Sofosbuvir and Simeprevir) were introduced and licensed. This drug combination improves the cure rate to about 90%, though it tends to be genotype selective, showing the greatest effectiveness for genotype 1 [286] and genotype 4 [287] infections. In 2014, another class of DAAs, ledipasvir, ombitasvir, daclatasvir (NS5A protease inhibitors), ritonavir (booster of protease inhibitor) and paritaprevir (NS3 Protease inhibitor) was introduced in combination with RdRp inhibitors (Sofosbuvir/Dasabuvir) in various formulations [288]. Most of the approved DAAs were based on the clinical trials of genotypes/subtypes prevalent in the western world, as reviewed in Bastos *et al.*, [289]. For instance, the use of sofosbuvir and velpatasvir in a fixed-dose for 12-week in patients with HCV genotype 1-6 without cirrhosis or with compensated cirrhosis was recommended based on phase 3 (ASTRA-1, ASTRA-2 and ASTRA-3) trials [290-292]. In the way, the efficacy of sofosbuvir, velpatasvir and voxilaprevir was demonstrated in the POLARIS-3 study [293]. Furthermore, the pan-genotypic glecaprevir and pibrentasvir formulation commonly used in western countries are based

on many clinical trials which include ENDURANCE and SURVEYOR trials [294, 295]. Sulkowski *et al.*, 2014 evaluated genotypes 1, 2, or 3 patients (n=211) with daclatasvir plus sofosbuvir, and a high SVR rate were observed [296]. These two classes of DAAs, when combined with or without ribavirin achieved more than a 96% cure rate [297]. Sofosbuvir and RBV combination therapy provided the first interferon-free regimen used effectively for genotype 2 and genotype 3 for 12-week and 24-week respectively [298].

Drug compliance can be problematic with extended dosing regimens. In order to shorten the duration of treatment from 12 weeks to about 4-8 weeks and at the same time providing pan-genotypic activity, single-pill regimens such as grazoprevir/MK8742, Sofosbuvir/G55816, and BM5791325/DAC/Asunaprevir were introduced in late 2015 /-early 2016 with nearly 100% cure rate [299]. A triple co-formulation of sofosbuvir, velpastavir-voxilaprevir, was found to achieve SVR rates >95% following 8 weeks of daily dose [300]. However, whilst the cure rate of these DAAs drugs has improved tremendously over the years, resistance to DAAs has been a significant challenge [301, 302]. Therefore, optimal treatment of HCV requires high efficacy of drug regimen, which helps patients to live a quality life, reduce morbidity, and prevent the spread of infection [303]. Therefore, national and international societies have recommended HCV treatment guidelines on existing evidence-based publications [304, 305]. The treatment recommendation of these societies is mostly the same and placed considerations for individual patients. The treatment modality include whether the patient is treatment naïve, treatment-experienced, presence of cirrhosis (decompensated or non-decompensated) or absent of

cirrhosis, degree of fibrosis, previous failed treatment, type of treatment failure, HCV/HIV co-infection, HCV/HBV co-infection, HCV/TB co-infection, chronic kidney disease, previous renal transplant, HCV genotypes/subtypes, presence of baseline mutation and others. To date, there are 8 different DAAs formulations approved for the treatment of HCV [306]. They are either pan-genotypic or genotype/subtype specific (Table 1). However, those that are even pan-genotypic, their efficacy varies by genotype, which in some cases require accurate genotype/subtype for proper treatment.

Table 1: Currently approved direct-acting antiviral regimen

Regimen	Genotype	Duration (wk)	Comments
Genotype-specific regimens			
Elbasvir/ grazoprevir (Zepatier)	1a, 1b, 4	12-16	Resistance testing recommended before use in genotype 1a and Ribavirin added if RAS to NS5A present and duration increased from 12 to 16 weeks
Ledipasvir/sofosbuvir (Harvoni)	1a, 1b, 4, 5, 6	8-24	
Paritaprevir/ritonavir/ombitasvir/ Dasabuvir (Holkira Pak)	1, 1b	8-24	Ribavirin must be added for genotype 1a
Paritaprevir/ritonavir/ombitasvir (Technivie)	4	12	Ribavirin must be added
Sofosbuvir +daclatasvir (Sovaldi +Daklinza)	1a, 1b, 3	12-24	
Pan-genotypic regimens			
Glecaprevir / pibrentasvir (Maviret)	1-6	8-16	
Sofosbuvir/ velpatasvir (Epclusa)	1-6	12	
Sofosbuvir/ velpatasvir /voxilaprevir (Vosevi)	1-6	12	Treatment depends on country guidelines

Brand names of direct-acting antiviral drugs are in parentheses.
Wk=week Adapted from Shah,2018 [306].

By convention, double combination with fixed-dose pan-genotypic regimens is preferable to minimize side effects and avoid drug-drug interactions where possible [307]. The specific choice of therapy regimen is largely complex, as indicated previously (base on genotype/subtypes,

complications of HCV infection, and special considerations such as comorbidities). Table 2 shows the current recommendation regimens for the treatment of HCV-monoinfected or HCV coinfecting patients with chronic hepatitis C without cirrhosis, including treatment-naïve patients who failed on a treatment based on PEGylated IFN α and ribavirin. This guideline is 2018 recommendations of the European Association for the study of liver for treatment of HCV infection in Europe. Guidelines differ from country to country based on each country's peculiarity and evidence base on *in vitro* and *in vivo* trials.

Table 2: Treatment recommendations for HCV-monoinfected or HCV coinfecting patients with chronic hepatitis C without cirrhosis, including treatment-naïve patients who failed on a treatment based on PEGylated IFN α and ribavirin (treatment-experienced, DAA-naïve patients).

Patients	Treatment-naïve or experienced	Sofosbuvir/ledipasvir	Sofosbuvir/velpatasvir	Ombitasvir/paritaprevir/ritonavir and dasabuvir	Ombitasvir/paritaprevir/ritonavir	Greziprevir/elbasvir	Sofosbuvir and daclatasvir	Sofosbuvir and simeprevir
Genotype 1a	Treatment-naïve	8-12 wk, no ribavirin	12 wk, no ribavirin	12 wk with ribavirin	No	12 wk, no ribavirin if HCV RNA $\leq$ 800,000 (5.9 log) IU/ml or 16 wk with ribavirin if HCV RNA >800,000 (5.9 log) IU/ml ^b	12 wk, no ribavirin or 12 wk with ribavirin ^c or 24 wk, no ribavirin	No
	Treatment-experienced	12 wk with ribavirin ^a or 24 wk, no ribavirin						
Genotype 1b	Treatment-naïve	8-12 wk, no ribavirin	12 wk, no ribavirin	8-12 wk, no ribavirin or 12 wk, no ribavirin	No	12 wk, no ribavirin	12 wk, no ribavirin	No
	Treatment-experienced	12 wk, no ribavirin						
Genotype 2	Both	No	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
Genotype 3	Treatment-naïve	No	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
	Treatment-experienced		12 wk with ribavirin ^c or 24 wk, no ribavirin				12 wk with ribavirin ^c or 24 wk, no ribavirin	
Genotype 4	Treatment-naïve	12 wk, no ribavirin	12 wk, no ribavirin	No	12 wk with ribavirin	12 wk, no ribavirin	12 wk, no ribavirin	12 wk, no ribavirin
	Treatment-experienced	12 wk with ribavirin or 24 wk, no ribavirin				12 wk, no ribavirin if HCV RNA $\leq$ 800,000 (5.9 log) IU/ml or 16 wk with ribavirin if HCV RNA >800,000 (5.9 log) IU/ml	12 wk with ribavirin or 24 wk, no ribavirin	12 wk with ribavirin or 24 wk, no ribavirin
Genotype 5 or 6	Treatment-Naïve	12 wk, no ribavirin	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
	Treatment-experienced	12 wk with ribavirin or 24 wk, no ribavirin					12 wk with ribavirin or 24 wk, no ribavirin	

Note: ^aAdd ribavirin only in patients with RASs that confer high-level resistance to NS5A inhibitors at baseline if RAS testing available.

^bProlong to 16 weeks and add ribavirin only in patients with RASs that confer resistance to elbasvir at baseline if RAS testing available.

^cAdd ribavirin only in patients with NS5A RAS Y93H at baseline testing available[307].

1.11.3 Resistance Associated Substitution

By definition, RAS is any variation in the amino acid sequence compared to the virus consensus sequence, which is associated with resistance to any of the major three classes of DAAs currently in use [308]. Tables 3, 4, and 5 show common RASs in HCV NS3, NS5A, and NS5B respectively by genotype. There could be a change in an amino acid position associated with RAS, but in the absence of any known *in vitro* or clinical evidence to resistance, then it is designated or termed resistance-associated polymorphism [309]. RAS may be class-specific which, if present, will confer resistance to at least one of any DAAs in that class. They can also be drug-specific [310]. RAS may occur naturally before therapy (baseline RAS) [311], during treatment (Treatment-emergent or treatment-selected) [312], and even after treatment due to relapse or virologic breakthrough [313, 314]. The impact of RAS depends on the specific genotypes of infected patients and this can be evaluated by the *in vitro* fold change in the 50% effective concentration (EC_{50}) which may be from 2 - to >1000 - fold compared to wild type/reference replicon [315] (Table 6). Higher fold change is more likely to confer a clinical impact, thus the poor treatment outcome. Studies have shown that 15% cut off is able to detect the most clinically relevant baseline RAS [316, 317]. NS3 and NS5A RAS are most frequently encountered in patients resistant to DAA therapy containing

NS3/4A and NS5A inhibitors, respectively [318]. NS5A RAS are frequently selected in patients with failure of NS5A inhibitors containing regimens because they maintain a high replication competence (relative fitness) even in the absence of continuous drug pressure [319]. This reason allows them to remain the dominant viral quasispecies for prolonged periods. This is in contrast to NS3/NS5B RAS which is typically less fit and tends to disappear over several months, being overcome by more fit wild-type virus species [320]. Furthermore, NS5B has a high genetic barrier due to the highly conserved catalytic site region that nucleotides bind, rendering substitutions in this region rare and also make the virus replication-incompetent [321].

Table 3: List of reported NS3 class RASs known to reduce the susceptibility of different HCV subtypes to DAAs

Genotypes	NS3 class RASs, consensus amino acid position/substitution
GT1a	V36A/C/G/L/M, Q41R, F41L, T54A/S, V55A/I, Y56H, Q80H/K/L/R, S122GN/R, R155G/I/K/S/T/W, A156S/T/V, V158I D168A/C/E/G/H/K/N/T/V/Y, I/V170F/T/V
GT1b	V36A/C/G/L/M, Q41R, F43I, T54A/C/G/S, V55A, Y56H/L, Q80H/K/G/L/R, S122G/N/R/S, R155C/G/I/K/Q/M/S/T/W, A156G/F/S/T/V, V158I D168A/C/E/F/G/H/K/N/T/V/Y, I/V170A/L/T, M175L
GT2a	A156V, Y56H
GT3a	Q80K/R, R155K, R155K, A156V, D168E
GT4a	V36L, T54S, S122T, R155K, A156V, D168E, V170I
GT5a	V36L, Q80K, S122G, D168E
GT6a	V36L, Q80K, S122T, V170I

The letter preceding the number is the wild type amino acid residue, whereas those after number are the amino acid substitutions associated with the gene inhibitor resistance in question at that position.

For other subtypes not listed little or, no data are available

Table 4: List of reported NS5A class RASs known to reduce the susceptibility of different HCV subtypes to DAAs

Genotypes	NS5A class RASs ,consensus amino acid position/substitution
GT1a	K24G/N/R/T, K26E, M28A/G/I/T/M/V, Q30C/D/E/G/H/I/K/L/R/T/Y, L31I/F/M/V, P32L/S S38F, H58D/L/R, A92K/T, Y93C/F/H/L/N/R/S/T/W
GT1b	M28L/T/M, P29S, Q30G/H/P/R, L31F/I/M/V, P32L/S, P58D/S, E62D, A92K, Y93C/H/N/S
GT2a	K24A/G/N/R/S, M28A/C/F/G/L/S/T/V, Q30E/G/H/K/R/S//T, L31I/F/M/V, P32L, S38F, P58D/S, C92K/N/S/T, Y93C/F/H/N/S
GT2b	S24G/N/R, L28A/G/T, K30E/G/H/R/T, L31F/I/M, P32L, S38F, P58D, C92K/T, Y93C/F/H/N/S
GT3a	S24G/N/R, M28A/G/T, A30E/G/H/K/R/T, L31I/F/M/V, P32L, S38F, P58D/G, E92T, Y93C/F/H/N/S
GT4a	K24G/N/R, L28A/G/M/T/V, Q30E/G/H/K/R/S/T, M31F/V, P32L, S38F, P58D/L/S, A92K/T, Y93C/F/H/N/S
GT5a	Q24G/N/R, L28I/A/G/T, Q30E/G/H/L/K/R/T, L31F/I/M/V, P32L, S38F, P58D/S, A92K/T, Y93C/F/H/N/S
GT6a	Q24G/N, F28A/G/M/T/V, R30E/G/H/L/K/T, L31F/I/M/V, P32L/S, S38F, T58A/D/G/N/S, A92K/T, T93A/C/F/H/N/S

The letter preceding the number is the wild type amino acid residue, whereas those after number are the amino acid substitutions associated with the gene inhibitor resistance in question at that position.

For all other subtypes not listed, little or no data are available.

Table 5 List of reported NS5B class RASs known to reduce the susceptibility of different HCV subtypes to DAAs

Genotype	NS5B class RASs, consensus amino acid position/substitution
GT1a	L159F, S282T/R, L320F, L314H, C316H/N/Y, M414I/T/V, R422K, M423I/T/V, M426L, Y448C/H, Y448C, C451R, I482L, A486V, V494A/I, P495L, A553T, G554S, Y555H, S556G/N/R
GT1b	L159F, S282T, C316H/N/Y/M, S368T, N411S, M414I/T/V, R422K, M423I/T/V, C445F/Y, Y448C/H, C451S, I482L, A486V, V494A, P495L, V499A, A553V, G554S, S556G
GT2a	A15G, L159F, S282T
GT2b	L159F, S282T, V321I, V494S
GT3a	S282T, V321A/I, V494A
GT4a	S282T
GT5a	S282T
GT6a	S282T, V494A

The letter preceding the number is the wild type amino acid residue, whereas those after number are the amino acid substitutions associated with the gene inhibitor resistance in question at that position. For all other subtypes not listed, little or no data are available [322-324].

Table 6: Fold-change in EC₅₀ for known Resistance-Associated Substitutions for DAAs NS5A Inhibitors by Genotype

HCV Drug	Fold-Change								
	RASs in HCV Genotype 1a						RASs in HCV Genotype 1b		
	M28	Q30R	L31M	L31V	Y93H	Y93N	L31V	Y93H	Y93N
Daclatasvir	>100X	>1000X	>100X	>1000X	>1000X	>10,000X	<10X	>10X	>10X
Elbasvir	>10X	>100X	>10X	>100X	>1000X	>1000X	<10X	>100X	N/A
Ledipasvir	>10X	>100X	>100X	>100X	>1000X	>10,000X	>10X	>100X	N/A
Ombitasvir	>1000X	>100X	>3X	>100X	>10,000X	>10,000X	<10X	>10X	>10X
Pibrentasvir	>3X	>3X	>3X	>3X	<10X	<10X	>3X	>3X	>3X
Ruzasvir	<10X	<10X	<10X	<10X	<10X	<10X	<10X	<10X	<10X
Velpatasvir	<10X	>3X	>10X	>10X	>100X	>1000X	<10X	>3X	N/A

Abbreviation: EC₅₀, half-maximal effective concentration; HCV, hepatitis C virus, NA, data not available, RAS, resistance-associated substitution. RASs shaded in red are more likely to confer a clinical impact due to high fold-change. RAS shade in blue have an intermediate impact on efficacy while RAS shade in green are not likely to have a clinically significant impact [325].

The HCV NS3-Q80K variant is the most prevalent natural polymorphism to simeprevir [326]. The prevalence differs from one region to the other; it could be as high as 93.2% in China [327], 14% in the United States [328], and 19.8% in Europe [329]. Two or more simeprevir specific RAS (Q80K+K155K) do occur at baseline though it is of low prevalence [330]. When present, they further reduce SVR compared with a single mutant. The prevalence of NS5A RAS varies considerably among genotypes and also across most geographic regions [331]. The impact of NS5A RAS depends on specific drugs and RAS variants. For instance, a study that assessed *in vitro* impact of various NS5A RAS (28M/V, 30A/K/S/T/V, 31L/M, E92A and Y93H) on the activity of daclatasvir and ledipasvir on genotype 3 infected patients, showed that DCV is more potent (EC₅₀=0.52nM) than ledipasvir

($EC_{50}=141\text{nM}$) [332]. The implication of this is that the effectiveness of therapy depends on the combination of the other antiviral component(s) included in the regimen. Furthermore, double (L31M and/Y93H) [333] and triple (D168V+L31V+Y93H) [334] mutant combinations do occur in the real world. Although rare, they are strongly associated with treatment failure. S282T variant is the significant RAS associated with sofosbuvir resistance (nucleotide analogue, NS5B protease inhibitor) both at baseline and at virologic /treatment failure [335]. Interestingly, S282T is rare, even when detected, it could be overcome by extending treatment duration, and tend to disappear once the drug is discontinued [336, 337]. Treatment failure in patients that do not achieve SVR to prescribed DAAs is most often associated with RASs. Patients with baseline RASs, multiclass RASs, cirrhotic, decompensated patients are more likely to relapse or have viral breakthrough following treatment [338-340]. Consequently, it is essential to avoid suboptimal treatment by using highly potent DAAs with a high genetic barrier to resistance as the first line treatment [341]. Generally, it is advisable to do resistance testing for treatment failure patients before retreatment where the facilities are available [342]. However, the rule of thumb is to institute sofosbuvir, and any other drug from a class other than that was previously used, including ribavirin for 24 weeks [343]. For patients that fail NS5A inhibitors with multidrug-resistant variance, triple and quadruple DAAs regimen have been suggested [344]. However, it requires close monitoring since this is off label regimen with limited safety data to back it up.

1.11.4 Future treatment

The future treatment Of HCV infection is very promising due to the availability of all-oral DAAs that improve SVR up to >98%. It is also anchored on the present use of pan-genotypic single-pill regimens with a high genetic barrier [345]. Good examples of such formulations are maviret (glecaprevir and pibrentasvir and vosevi (sofosbuvir, velpatasvir and voxilaprevir) which are easier to adherence to [307]. The so call challenging to treat a group of patients such as cirrhotic patients, HCV co-infection, PWIDs, chronic renal disease patients have been brought under efficient regimens [346, 347] .To overcome the impact of resistance mutations, various attempts have been made to combine 3 or more DAAs with distinct mechanisms of action in order to improve the efficacy and shorten therapy duration in some groups of patients. Sulkowski *et al.*, in his "FOURward study" of 28 non-cirrhotic genotypes 1 patient with median baseline viral loads (9×10^6 IU/mL), used 4 different classes of DAAs with distinct mechanisms of action (daclatasvir, asunaprevir, beclabuvir, and sofosbuvir) for 4 weeks and 6 weeks respectively. The outcome was not encouraging due to a relapse in 77% of patients [348]. However, those with Non-SVR after 12 weeks of treatment were retreated based on the DAA resistance profile at failure, and all subsequently achieved SVR. Contrary to this, Lau *et al.*, reported good efficacy and safety of the triple regimen (sofosbuvir, daclatasvir, and simeprevir) for 12 genotype-1 patients for 3 weeks [349]. Although, he commented on the need for a larger sample size.

1.11.5 Prevention and control

Eradication of HCV seems possible and realizable if the level of prevention and control strategies are scaled up by different countries, and particularly

global intervention programme follow to a logical conclusion. HCV transmission differs from one geographic region to the other as illustrated above; in low and middle-income countries, blood and blood products screening, safe injection practices among health workers, proper sterilization of medical equipment, and increase health public awareness about the risk of infection from unsafe practices should be a priority. Intravenous drug use is the most frequently reported mode of HCV transmission in developed countries, and this is very common in rural and some communities such as prisons and detention centres. Efforts should be directed to needle exchange programs. More importantly, individuals with HCV infection should be counselled appropriately, efficiently treated, and monitored until SVR is achieved in order to break the infection transmission chain.

1.12 GLOBAL MOLECULAR EPIDEMIOLOGICAL PATTERN OF HCV

There is a wide variation in the temporal and spatial distribution of HCV globally [350]. The most of the data reported are based on seroprevalence on selected populations, such as blood donors [351], dialysis patients [352], intravenous drug users (IVDUs) [353], patients living with HIV [354], and prisoners [355]. The results from these reports are far less accurate compared with the whole community-based study. However, whole community-based studies seem not to be feasible because they require huge financial and material resources to embark upon [356]. In 2013, Hanafiah and colleagues classified the prevalence of HCV into high (>3.5%), moderate (1.5%-3.5%), low (<1.5%) [357]. Generally, the prevalence of HCV varies from continent to continent and even within

countries (Figure 6). The highest HCV prevalence rates are reported in Africa (2.9%) [358], and Asia (2.6%) [359]; lower prevalence rates are recorded in industrialized nations such as Western and northern Europe, North America and Australia [360-362]. For example, in Europe, it varies from Central, Western to Eastern Europe. It can be as low as 0.2% in Denmark [363] and, as high as 2.5% in Spain [364]. In the United States, the prevalence is moderate (1.7%) but can be up to 4.3% among intravenous drug users [365]. It was generally accepted that Egypt has a higher HCV prevalence of about 14.7% in the past [366]. However, a more recent epidemiological modelling studies carried out in 2013 revealed a more conservative estimate of 7.3% of the population with viremic state [367]. This reduction is attributed to the mortality of older age group who harboured the highest HCV prevalence. Other countries with high HCV prevalence are Pakistan and Taiwan with 4.7% and 4.4%, respectively [368].

HCV genotype distribution is of epidemiologic importance for global monitoring and surveillance [369]. Genotypes 1, 2 and 3 are found Worldwide and account for the most considerable number of infections acquired through blood transfusion [370].

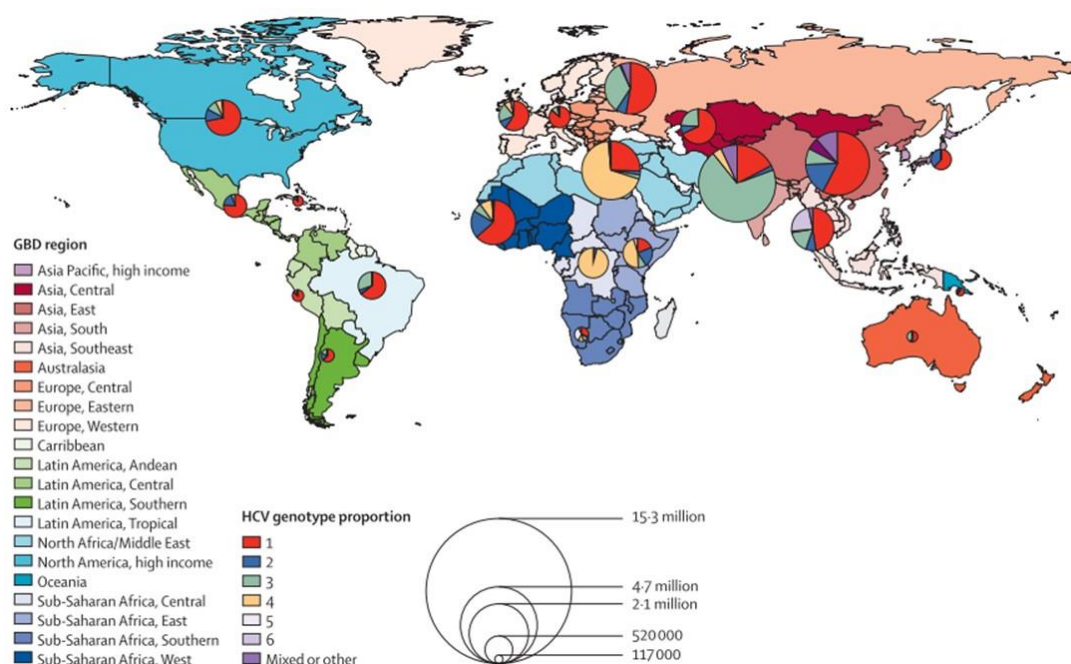


Figure 6: Global prevalence and genotype distribution of HCV infection in 2015. Adapted from Blach, 2017 [371].

Considering individual genotypes, genotype 1 is estimated to account for about 44% of all HCV cases; genotype 3, 25%; genotype 4, 15%, while other genotypes 2, 5 and 6 accounts for the remaining 16% [371]. In the UK genotypes 1 and 3 accounts for about 85% of all cases of HCV where genotypes 1, 2, 3 and 4 represent 45%, 10%, 40% and 5% respectively [372]. Genotypes 1 contributes 70% of all cases of HCV in the United States, while genotypes 2, 3, 4, 5 and 6 makes up 16%, 12%, 1%, <1%, and 1% respectively [373]. Genotype 4, 5, and 6 are not so common in the United States of America; some cases of these genotypes have been found in immigrants and intravenous drug users [374]. Genotype 4 is predominant in North Africa and the Middle East, while genotype 5 and 6

are prevalent in South Africa and Southeast Asia, respectively [375]. Phylodynamic and Phylogeographic analysis suggest the spread of subtypes 1a,1b,2a, and 3a during the 20th century [376]. In contrast, some subtypes are highly divergent (4l,4m,4n, and 4o), endemic, and somehow restricted to the geographic area of their origin [377]. However, globalization and the movement of people from one region to the other are now changing the epidemiologic pattern of HCV subtypes [374, 378].

In most of the industrialized countries like Europe, Canada, and Australia, and the United States, intravenous drug use still contributes the highest percentage of new HCV infections [372, 379]. In 1980 these countries adopted and implemented volunteer blood donor systems, and the discovery of a second-generation anti-HCV ELISA test for blood screening in 1992 helped significantly in the reduction of transfusion-transmitted HCV infection [370]. Despite the significant reduction in this mode of transmission, most of these countries still suffer from the previous transfusion-associated infections that occurred before the era of HCV detection [380]. Studies have shown that some genotypes and subtypes have a relationship with the mode of HCV transmission. For example, subtypes 1a, 3a, and 4 have been implicated in intravenous drug use related hepatitis, while genotypes 1b and 2 have been linked to blood transfusion and unsafe medical practices [254]. However, this relationship could be a founder effect rather than some subtypes being better adapted for a particular transmission route. In developing countries, blood transfusion, unsafe medical practices, and some cultural practices contribute significantly to the spread of HCV [381]. In some countries, both blood transfusion and IVD use are on par. For instance, a study conducted

in Anyang, China revealed that the risk factors such as IVDS, blood transfusion, and oesophageal ballooning were much higher in patients with HCV infection [382].

1.13 EPIDEMIOLOGY OF HCV IN AFRICA

Despite the high burden of this disease in Africa, there is a lack of data on the mortality and morbidity rate [383]. Most virus genotypes are found distributed heterogeneously across some of the major regions of the Africa continent [384, 385]. Interestingly, it has been suggested that most of the HCV genotypes originated from Africa [386]. Through the use of phylogenetic analysis, Candotti *et al.* were able to compare the HCV genotype 2 sequences from Ghana with the other strains from other regions of the world, and they found out that Ghanaian isolates were the most genetically diverse [387]. Based on these findings, they suggested Ghana as likely the origin of genotype 2. Similarly, genotypes 1, 4, and 5 have also been speculated to have originated from Africa through epidemiological and Bayesian phylogenetic studies of these strains [377, 388, 389].

The most studied country in North Africa is Egypt. Within this region, HCV prevalence also varies, and figures as low as 1.93% has been reported in Morocco [390]. The high prevalence of HCV in Egypt was attributed to the use of unsterilized or improperly sterilized syringes used during a massive campaign and treatment of schistosomiasis outbreak from 1960-1987 [391]. Genotype 4 accounts for more than 90% of cases of HCV infection in Egypt, and it has been the largest reservoir of this genotype in the world [392].

Southern Africa has one of the lowest HCV prevalence in the Africa subcontinent [393], where prevalence ranges from 1.1% in Zambia and South Africa to 1.6% in Zimbabwe and Namibia [394]. Figure 7 shows the seroprevalence of HCV in some Sub Saharan African countries. Genotype 5 is the predominant clade in Southern Africa, but recently, other genotypes and subtypes have been identified [395]. For example, a study carried out on the blood donors from different regions in South Africa showed that 40% had genotype 5, while genotypes 1, 2, 3, and 4 accounted for 33%, 13.8%, 7.7%, and 2.3% respectively [396].

Eastern Africa HCV prevalence is approximately 2.7% in Ethiopia, Tanzania, and Uganda and around 1.3% and 1.7% respectively, in Mozambique and Madagascar [397]. Unlike other Sub-African countries, the

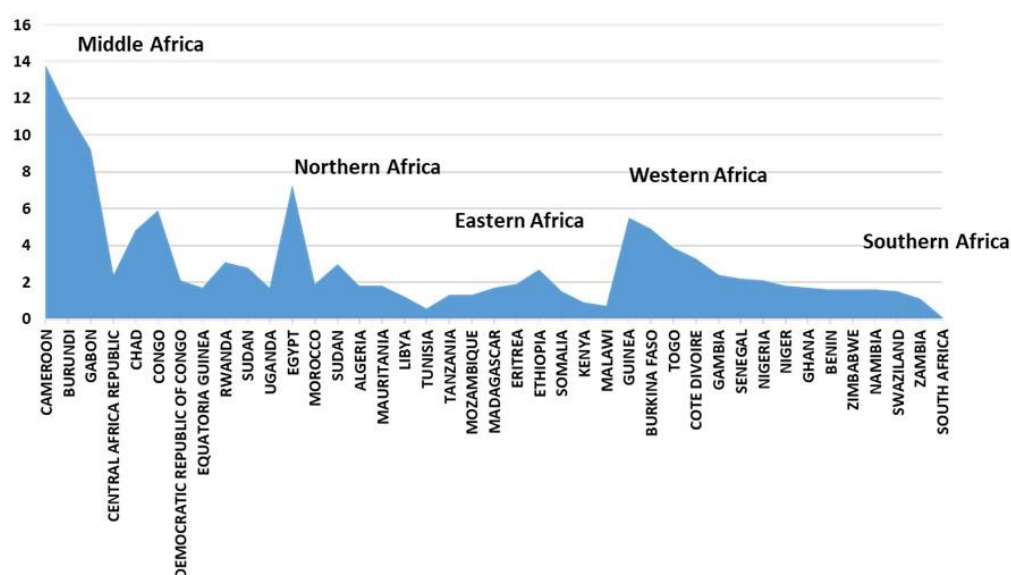


Figure 7: HCV seroprevalence in Africa. Adapted from PetruzzIELLO *et al.*, 2016, Elghrably *et al.*, 2016 [358, 391].

predominant genotype in Eastern Africa is GT4, but other genotypes (GTs 1, 2 and 5) are also reasonably well-represented [398].

Cameroon, Burundi, and Gabon have the highest HCV prevalence rates in the Middle African region of 13.8%, 11.3%, and 9.2%, respectively [389, 399]. Rwanda and the Democratic Republic of Congo have 3.1% and 2.1%, respectively [394]. In middle Africa, genotype 4 is said to be the predominant clade accounting for about 75% of infection [400]. In one cohort study carried out among the healthy Gabonese villagers, the most prominent risk factors discovered were the use of contaminated parenteral injections, past hospital admission, and age above 55 years [389].

The prevalence of HCV in West Africa also varies considerably. It is 4.4% in Burkina Faso with genotype 2 and 3, representing the highest prevalence of 56.3% and 15.6% respectively [351]. In another part of West Africa, the prevalence records were highly heterogeneous ranging from 3.2% (Ghana), 3.1% (Nigeria), 2.4% (Gambia), 1.9% (Mali) and 1% (Senegal) [401]. The highest HCV 2 genotypes are found in Ghana (85%) [402]. Within Nigeria, the prevalence differs from one zone to the other. In the North-central, a prevalence rate of 13.2% has been reported among people of a local community in Keffi [403], while 15% prevalence was reported among haemodialysis patients receiving care at a tertiary health facility in Maiduguri, North-Eastern Nigeria [404]. Furthermore, Forbi *et al.*, conducted a study in two remote communities in the North-central region of Nigeria and reported a 15% HCV prevalence rate, which is one of the highest so far recorded in Nigeria [405]. From his study, genotype 1 represented 85%, while genotype 2 only accounted for 15%. A similar study carried out among blood donors in the same north-central region of Nigeria

(Kaduna) revealed a 5.8% prevalence rate, and only genotype 1b was observed [406]. These prevalences differ significantly from the national prevalence number and this could have been due to population covered by the study and methods used.

1.14 AIMS AND OBJECTIVE.

Accurate HCV genotyping and subtyping are essential to determine clinical responses to anti-HCV therapy as treatment response, and more specifically, DAA-resistance is HCV genotype/subtype-specific. Furthermore, it is imperative to evaluate available commercial genotyping assays and online genotyping tools in order to devise diagnostic pathways that are appropriate to and achievable in low and middle-income country (LMIC) settings. Similarly, storage facilities of blood samples, transportation at the site of specimen collection, and the molecular study of HCV is a daunting task in LMICs. Hence we evaluated dried serum spot (DSS) as an alternative tool for blood sample collection for clinical diagnostic purposes and molecular epidemiological studies.

The following are the objectives of this research:

- (1) To investigate the distribution of HCV genotypes in individuals of African origin living in the United Kingdom.
- (2) To use Sanger sequencing with phylogenetic analyses approach to characterize SSA HCV strains.
- (3) To evaluate/verify the commercial genotyping assays and online genotyping tools compared to gold-standard phylogenetic analyses.

- (4) To determine the prevalence of resistance-associated substitutions in SSA HCV strains.
- (5) To determine the efficiency of DBS technology in the characterization of HCV strains.

2 CHAPTER TWO

2.1 MATERIALS AND METHODS

2.1.1 Study participants and Study site of HCVR UK Patients

Three hundred and thirty-nine HCV patients of African origin were identified and selected from over 11,800 chronic HCV patients in 2012-2014 enrolled in HCV Research UK. These patients included in our data were from 32 different countries attending 30 HCV treatment centres all over the UK. The clinical and demographic information were extracted from the data where possible, and this forms the baseline for our analysis. The available data showed that genotype determination was performed using three main commercial assays; Taqman Real-time (5'UTR), InnoLIPA (5'UTR+ CORE), and Abbott (5'UTR+NS5B). In few centres, where there were anomalies or failure to generate genotype, partial 5' NCR or NS5B Sanger sequencing was suggested as an alternative for genotype determination. Individual samples where we were unable to obtain HCV genotype (GT) data and other relevant clinical data were excluded from calculations in the final analysis. Informed consent was sought from each patient at the various recruitment centres, and studies were conducted in accordance with the Helsinki Declaration [407].

Out of 339 Pan- African birth HCV patients, we sought 135 of sub-Saharan African patients with RNA seropositive, and those who do not inject drugs (Figure 8). All the demographic and clinical details/information were extracted from the 339 baseline data. Serum samples for these patients were used for this study following clearance acceptance from the local ethics committee.

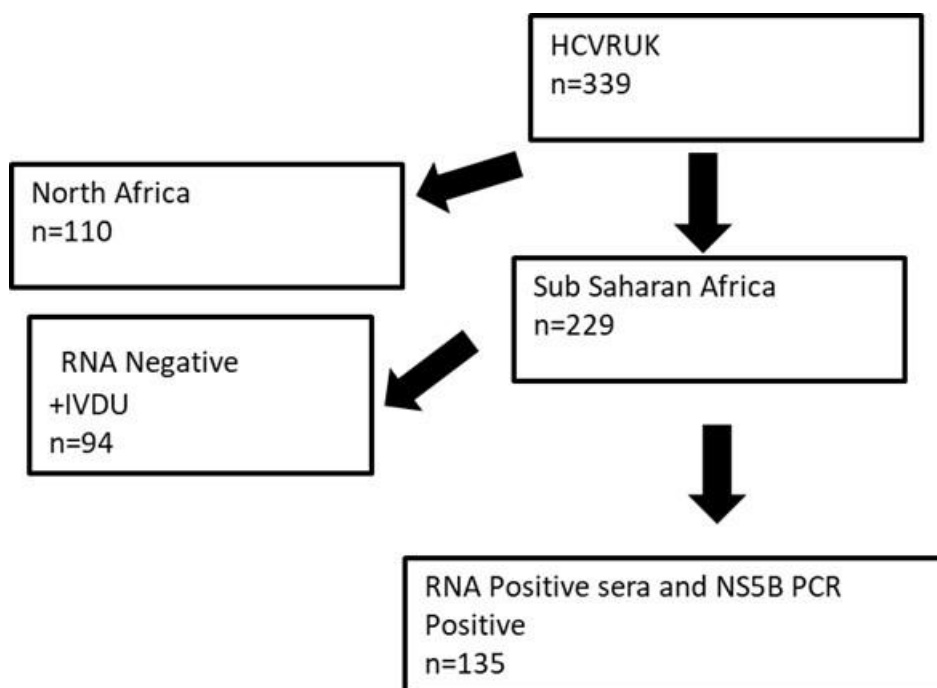


Figure 8: Illustration of the HCV UK database screening strategy for analysis.

2.1.2 Source of Sample from Sao Paolo, Brazil

Serum samples were obtained from seven specialized centres in Sao Paolo, Brazil. These centres receive blood for HCV Screening and treatment. The sera included in this study were collected between 2010-2017. Eighty HCV RNA positive serum samples with viral load ranging from 3.0×10^4 IU/mL to 5.3×10^7 IU/mL were collected, centrifuged, spotted into DSS, and transported to Nottingham, the United Kingdom for further analysis. The processing and analysis of samples were completed within five days of receipt of samples (Figure 9).

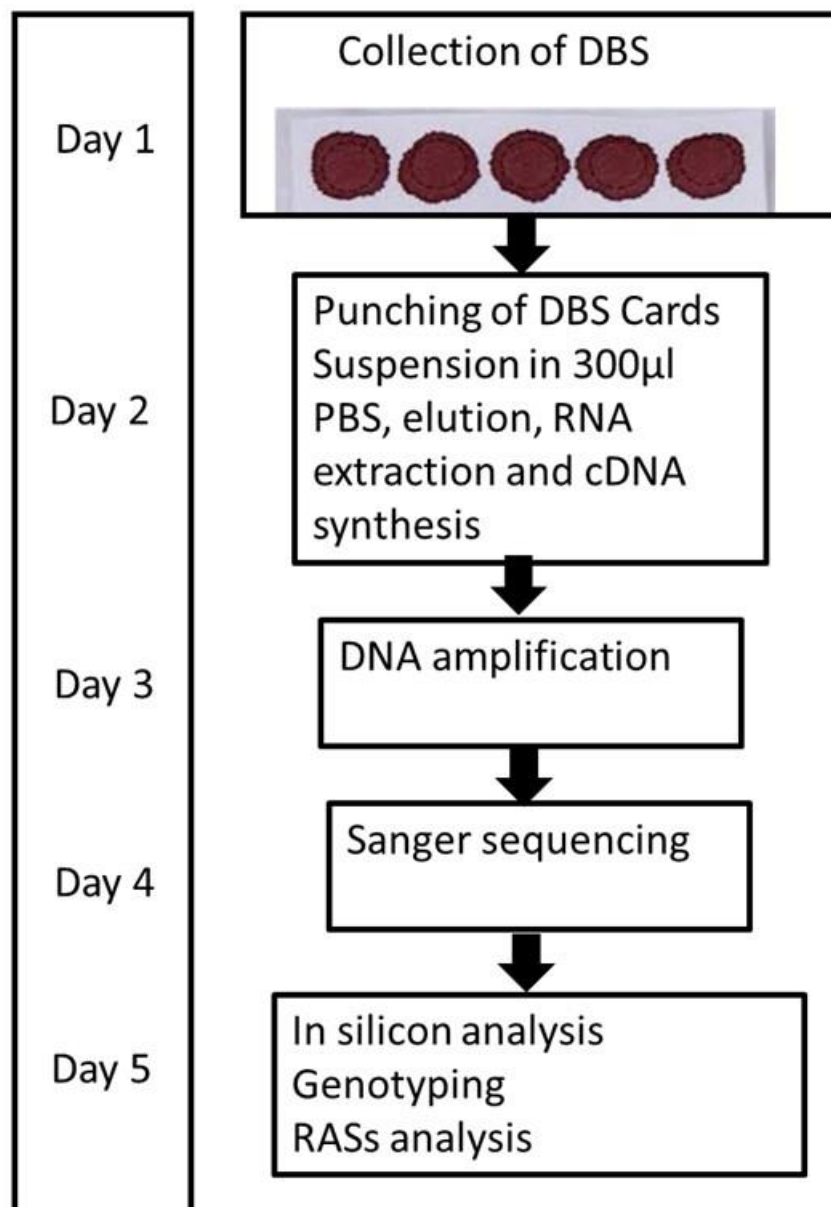


Figure 9: Flow diagram of the dried serum spot procedure for analysis.

2.1.3 Dried Serum Spot Preparation, and Elution

Thirty microliters of serum were spotted onto a Protein Saver™ 903 Card (Whatman Cardiff), then the cards were allowed to dry at room temperature for about 1 hour before use.

Each full DBS disk containing 30 µl of serum was cut using a 5 mm punch, and the resulting filter disk placed in a 2 ml tube containing 300 µl Phosphate Buffered Saline (PBS). The tubes were incubated with agitation at room temperature for 10 minutes, and then 140 µl of supernatant virus suspension was used for RNA extraction.

2.1.4 RNA Extraction

RNA was extracted from 140 µl of sera obtained from the patients or 140 µl each of the virus suspension from the DBS eluate. Extraction was carried out using QIAamp® MinElute® virus spin RNA extraction kit (Clontech) according to the manufacturer's instructions. Fifty µl HCV RNA eluted from each sample were used for cDNA synthesis.

2.1.5 cDNA synthesis

The eluted HCV RNA was reversed transcribed using the lyophilized EcoDry™ Premix Random Hexamer (Clontech® according to the manufacturer's instructions. Briefly, 20 µl of eluted RNA was pipetted into a lyophilized EcoDry™ reagent in 0.5 ml microtubes. This suspension from this tube was mixed thoroughly and transferred into a new 0.5 ml microtube. The 20 µl reactions were incubated at 42°C for 59 mins, followed by an inactivation step at 70°C for 10 mins, then stored at -80°C.

2.1.6 DNA Amplification

For reverse transcriptase-polymerase chain reaction (RT-PCR) of the NS5B HCV fragment, we used previously published highly conserved primers [398]. For the RT-PCR of NS5A, we designed two sets of in-house primers; one set relatively conserved pan-genotypic primers based on alignment of published full-length HCV sequence representing the six major HCV genotypes and the second sets were genotype-specific reverse primers (genotypes 1,2,3 and 4) all containing degeneracy based on alignment of multiple sequences of each genotype (Table 7). The PCR was performed using Hot Start Taq DNA polymerase (QIAGEN®) according to the manufacturer's instructions. For the NS5B coding region, the 338bp fragment was amplified. Briefly, the RT-PCR was performed by adding 8 µl cDNA template to a reaction mixture consisting of 5 µl x10 buffer, 2 µl 10mMol/1dNTP, 4 µl each of 10pmol of forwards and reverse primers respectively, 0.24 µl of Hot star Taq polymerase. The reaction was adjusted to 50 µl with sterile water. The PCR amplification was set at 95°C initial denaturation for 15 mins followed by 50 cycles of 95°C for 20 secs, 55°C in 20 secs, and 72°C for 30 secs and a final extension step at 72°C for 1 min. For NS5A amplification, 760 bp or 680 bp product size were generated depending on the NS5A primer set was used. We generated 132 sequences in all, the first set of the NS5A amplifications, using both the forward and reverse pan-genotypic primers, yielded 63 sequences. An additional 69 sequences were obtained using the pan-genotypic forward primer and genotype-specific reverse primers. The PCR reaction mix was the same as the NS5B with modifications to the PCR cycling conditions. Briefly, for the pan-genotypic primers and genotype-specific reverse primers, the initial

denaturation was at 95°C for 15 mins followed by the same 50 cycles of additional denaturation at 95°C for 20 secs, annealing this time was at 56°C for 20 secs, extension at 72°C for 1 min and final extension elongation at 72°C for 1min.

Table 7: RT-PCR Primers for NS5A and NS5B Amplification and Sequencing.

PRIMER NAME	DIRECTION	PRIMER SEQUENCE(5'-3')	POSITION
PanHCVNS5aF	SENSE	CAGTGGATGAACMGNCTNATHGC	6081-6103
PanNCVNS5aR	ANTISENSE	CCGGTTCNGGNTCRCANG	6823-6840
g1HCV_NS5aR	ANTISENSE	GGGTGAYGGGACYTGRCAYGG	6698-6678
g2HCV_NS5aR	ANTISENSE	TGGCAGGGGACATTTCCTATC	6527-6507
g3HCV_NS5aR	ANTISENSE	GCAGCYGGNACYTGRCANGG	6765-6747
g4HCV_NS5aR	ANTISENSE	CGAACCTCCACRTANTCCTC	6622-6603
NS5B Sn755	SENSE	TATGAYACCCGCTGYTTTGACTC	8256-8278
NS5B ASn 1121	ANTISENSE	GCNGARTAYCTVGTCATAGCCTC	8622-8644

Degenerate residues are indicated as M, N, H, Y, and V, and these represent the following nucleotides : (R=A+G, M=A+C, N=A+T+G+C, Y=C+T, H=A+T+C, V=G+A+C). The position numbering according to genotype 1a strain H77.

2.1.7 Analysis of PCR Products by gel electrophoresis

The amplified PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. The gel was prepared by boiling agarose (1.0%w/v) in 1x TAE (40 mM Tris, 0.11% acetic acid, 1 mM EDTA) (Sigma) with the addition of 5 µl/100 ml of ethidium bromide to the gel before casting. Gels were submerged in gel running tanks (Bio-Rad) containing TAE supplemented with 0.5 µg/ml ethidium bromide. A mix of 5 µl of PCR products and 2 µl of 6x loading buffer (50% glycerol in water, 0.002% bromophenol blue) alongside 100 bp Gene Ruler DNA ladder (MBI

Fermentas) were pipetted into agarose gel wells. The products were resolved at 90 volts for 36 mins. After that, the agarose gel was visualized using UV trans-illuminator (UVP) to confirm the presence and size of any PCR products.

2.1.8 DNA Sequencing

The PCR products successfully amplified were subjected to 1:10 dilution with water-RNAase-Free. The same set of the respective primers used for PCR amplification was used for sequencing. Sanger terminator chemistry sequencing was performed by Source Bioscience (Nottingham).

2.1.9 Sequence analysis and genotype determination

One hundred and thirty-five sequences (HCVR UK cohorts) and 66 sequences (Brazilian cohorts) were manually edited using MEGA 6 software [408] and subjected to basic local alignment search tool (BLAST) [409] to denote the sequence similarity to the previously reported genotypes/subtypes in the GenBank. The HCVR UK sequences were subjected to the Los Alamos database (LANL) [410], Oxford HCV Automated subtyping tool (version 2.0) [411], Virus Pathogen Database and Analysis Resource (VIRP) [412], Geno2pheno HCV Subtyping tool [413], NCBI genotyping tool [414], and HCV-glue tool [415]. Similarly, the Brazilian sequences were subjected to four of the above online tools (LANL, VIRP, Geno2pheno) and HCVComet [416] to predict genotypes/subtypes. The

assignment with the highest concordance among the genotyping tools was selected as predicted genotypes/subtypes.

2.1.10 Phylogenetic analysis

To further confirm and validate the predicted HCV genotype/subtypes, we performed phylogenetic analyses using reference sequences proposed by Smith *et al.*, 2014 [417]. The phylogenetic tree was constructed using MEGA 6 software by either using the Neighbour-joining or Maximum Likelihood method applying the Kimura two-parameter model for pairwise distance estimation or General Time reversible model, (GTR) respectively [408]. The reliability of the clustering of the clades was evaluated by bootstrap with 1000 replicates. Bootstrap $\geq 70\%$ was considered as a threshold for excellent confidence repeatability since this corresponds to a probability of $\geq 95\%$ that the corresponding clade is real [418]. Phylogenetic base genotyping predictions were observed, compared with the previous online genotyping tools and commercial genotyping assays. The reference strains used in this study were obtained from GenBank and LANL database. See table 8 for these references.

Table 8: Reference strains used in the study obtained from GenBank and Los Alamos Database

Number	Accession Number	Number	Accession Number
1	HQ229470_gt1aTH	57	AY265433.gt2CM
2	JX463611_gt1aUS	58	AY632160_gt2CM
3	HQ850279.gt1aUS	59	EU710392_gt3aGB
4	EU255934_gt1aUS	60	JQ717256.gt3aIN
5	EU781827.gt1bUS	61	JF735121_gt3hCA
6	AF207758_gt1bJP	62	JF735126.gt3hSO
7	HM106869_gt1bIE	63	NC_009825.gt4aEG
8	AY051292.gt1cIN	64	FJ462435.gt4bCA
9	AB714160_gt1cID	65	KY608652_gt4cBE
10	KJ439768.gt1dMA	66	FJ462436_gt4cCA
11	AM910652.gt1gES	67	KY608652_gt4cBE
12	KY348757.gt1GQ	68	FJ462436_gt4cCA
13	AY632087.gt1eCM	69	DQ418786_gt4dUS
14	AY632086_gt1e_CM	70	KM587625_gt4dUS
15	KY608615_gt1gUS	71	AY743076_gt4dFR
16	AY632134.gt1hCM	72	AY743063_gt4fGA
17	AY632143.g1hCM	73	EF589160.gt4fFR
18	KY627918.gt1iDZ	74	FJ462432.gt4gCA
19	KY627918.gt1cET	75	GU054461.4hFR
20	KC248196.gt1lCM	76	FJ462438_gt4kCA
21	AY632114_gt1lCM	77	EU392173.gt4kCM
22	JQ679051_gt1NG	78	KY083754.gt4rFR
23	AY685035_gt1CM	79	JX227962_gt4rGB
24	AY834967_gt2aCN	80	KU883428_gt4rSA
25	DQ345614.gt2bMG	81	JX227972.gt4mGB
26	D50409.gt2cIT	82	JX227961gt4mGB
27	HM777358.gt2jVN	83	JX227960_gt4vGB
28	DQ345622.gt2kMG	84	DQ333684.gt5aZA
29	JF735115.gt2rCA	85	DQ333656_gt5a_ZA
30	EU246930.gt6aVN	86	EF108306.gt7aCA
31	KC248197 gt1lCM	87	M62321.gt1aUS
32	AY632116 gt1lCM	88	EU710204gt1aGB
33	KY6008615 gt1gUS	89	EF116020.gt1kCA
34	KY608616gt1gAU	90	EF116013.gt1kCA
35	KC248194 gt1eCM	91	EF116021.gt1jCA

36	KY844047.gt1cCN	92	AY754624.gt1iCA
37	AY051292.gt1cIN	93	KJ439772.gt1iDZ
38	D14853.gt1c1N	94	AY506585.gt1bRU
39	AJ851228.gt1GQ	95	EU155304.gt1bUS
40	MH666011.gt1dTN	96	D00944.gt2aID
41	AF037235.gt1mGQ	97	AF037236.gt1mGQ
42	KC844042.gt2fCN	98	JX227953.gt2kUK
43	KC844050.gt2fCN	99	KC197234.gt2kFR
44	JF735120.gt2eCA	100	AB047639.gt2aJP
45	JF735114.gt2dCA	101	JF735111.gt2mCA
46	HM777358.gt2j	102	JX227967.gt2mCA
47	JF735113.gt2jCA	103	DQ155561.gt2iVN
48	KC197230.gt2iFR	104	JX227949.gt2cUK
49	FN666428.gt2qES	105	KC197240.gt2iFR
50	FN666429.gt2qES	106	KC19735.gt2iFR
51	KC844048.gt2bcn	107	HQ850279.gt1aUS
52	AB559564.gt2bjp	108	AB69159gt3aJP
53	JF735122gt3kCA	109	AB691596gt3aJP
54	JF35124gt3CA	110	D49374GT3bJP
55	JF73125gt3iCA	111	D63821gt3kID
56	JX227954gt3gGB	112	FJ407092gt3iIN

The letters following the subtypes are countries of origin of the reference strains. The abbreviation letters indicate these countries : TH , Thailand ;US ,United State ; JP, Japan ; IE ,Ireland ; IN ,India ; ID ,Indonesia ; MA ,Morocco ; ES ,Spain ; GQ, Equatorial Guinea ;CM ,Cameroon ;NG ,Nigeria ; CN ,China ; MG ,Madagascar ;IT ,Italy ; VN ,Viet Nam ; CA ,Canada ; GB , Great Britain ;SO ,Somalia ; EG ,Egypt ; FR ,France ; BE ,Belgium ; GA ,Gabon ;SA ,Saudi Arabia ; ZA ,South Africa ;DZ, Algeria ; Mauritania, MR.

2.1.11 Analysis of resistance-associated substitution

Both NS5A and NS5B sequenced fragments of the study samples were evaluated for the presence of RASs by subjecting the sequences to the Geno2pheno software tool [413]. Upon submission of the refined open reading frame sequences, the tool aligned the input sequence data, identified the genomic regions, predicted the HCV genotype/subtypes, and

generated for each DAAs a drug resistance prediction reports which are easily interpretable. The amino acid sequence in NS5B corresponds to amino acid positions -228 to -339 (Though ,this does not cover all known NS5B RAS positions ; thus, NS5B resistance analysis is not complete and may be an underestimate) while that of NS5A encompasses complete residues -1 to -118.

2.1.12 Data Analysis

All data were correctly coded in the Excel and entered into SPSS version 24 for analysis. The different variables were treated appropriately, analyzed into mean, standard deviation, and proportions. The distribution was compared using the chi-square test, Fisher exact test, and the Mann Whitney U test. Descriptive statistics were used to generate inferences using a p-value less than 0.05 as significant.

3 CHAPTER THREE: MOLECULAR EPIDEMIOLOGY OF HCV IN AFRICA

3.1 Introduction

Significant progress has been made and still ongoing in the research on genetic diversity, transmission, therapy, and other aspects concerning the molecular epidemiology of HCV worldwide [419-421]. However, in developing countries most especially within the African continent where the prevalence has been documented to be high, there is a dearth of information in most countries [383]. In a few studies reported so far, the prevalence rates differ remarkably between different countries and regions. For instance, Sub Saharan Africa (SSA) has a prevalence rate of 3.8%, Central Africa (6.0%), West Africa (2.4%), South and East Africa (1.6%), and North Africa (0.6%-8.4%) [381, 422, 423]. Proper understanding and comparison of epidemiological data in these countries are not straight forward; disparities in diagnostic protocols, unclear definition of infections and the use of different methods are some of the challenges [424, 425]. The existing knowledge about the epidemiology of HCV in Africa is obtained from few communities or population-based studies on risk groups such as blood donors, sickle cell patients, pregnant women, health care workers, haemodialysis, patients, etc. which seem not sufficient enough to capture the true picture of the HCV burden [426-428]. Moreover, the molecular epidemiology of HCV keeps changing due to mass immigration and travels that have made the world a global village [429]. Therefore, continuous surveillance and monitoring are paramount. Due to the dearth of information about the molecular epidemiology of HCV in Africa most especially in Sub-Saharan Africa, we set to determine the distribution, risk

factors and the genetic diversity of HCV infection in patients of African origin living in the United Kingdom (UK). Data of these patients was requested for systematic investigation and analyses from Hepatitis C Virus Research United Kingdom (HCVR UK). HCV Research UK is a consortium that provides the infrastructure that links major UK liver centres responsible for managing HCV-infected patients across the UK.

3.2 RESULTS

3.2.1 Demographic features of patients and treatment outcome

Three hundred and thirty-nine chronic HCV patients of African origin were identified from the HCVR UK database. Of 339 patients from the cohort, males predominate with 66.4 % (225/339) while 33% (112/339) were female. (Table 9). The ages ranged from 23-87 years, with mean age, of 57.3 ± 13.3 years. The mean age for a male is 57.45 ± 13 , while that of the female is 56.9 ± 12.3 . There is no statistically significant difference between the mean ages of both sexes. The age group 50-59 has the highest proportion of infection of about 27.1% of the entire sample population. One hundred and twenty-seven (37.4%) of the patients had a risk factor of being born abroad, and 16.8% had infection through blood and blood products.

Table 9: Demographic characteristics and risk factors in 339 study patients.

Variable	Categories	No (%) ; N=339
Sex	Male	225(66.4%)
	Female	112(33%)
	Unknown	2(0.6%)
Age, years	20-29	8(2.4%)
	30-39	35(10.3%)
	40-49	60(17.7%)
	50-59	92(27.1%)
	60-69	87(25.7%)
	70-79	50(14.7%)
	80-89	7(2.1%)
Mean $\pm$ SD		57.3 $\pm$ 13.3
Gender/Mean age p-value		0.146
Source of infection	Blood/Blood products	57(16.8%)
	Born abroad	127(37.4%)
	Sexual	10(2.9%)
	Injecting drug use	35(10.3%)
	Perinatal exposure	1(0.3%)
	Dental	7(2.1%)
	Tattoo	2(0.6%)
	Surgery	5(1.5%)
	Needle stick	9(2.7%)
	Unsterilized needle	1(0.3%)
	Vaccination	3(0.9%)
	No known risk factor	33(9.7%)
	Data missing	49(14.5%)
HCV Viral Load level IU/mL	<6X10 ⁵	64(18.9%)
	6X10 ⁵ -8X10 ⁵	14(4.1%)
	>8X10 ⁵	91(26.8%)
	Data missing	170(50.2%)

Data frequencies are designated as “n” while percentages are in brackets — SD=Standard deviation.

Other known route of infections is intravenous drug use (35; 10.3%), sexual contact (10; 2.9%), dental care (7; 2.1%), needle stick (9; 2.7%) surgery (5; 1.5%) and vaccination (3; 0.9%). No known risk factor was established in 9.7% of the patients. Ninety-one (26.8%) of the subjects had a high viral load greater than 8x10⁵IU/mL, while 64 (18.9%) had less than

6X10⁵IU/mL and the remaining 14 (4.1%) had a viral load range between 6x10⁵-8x10⁵IU/mL.

3.2.2 Hepatitis C virus genotype distribution

HCV genotypes 1 to 5 and one mixed infections were found among the 297/339 (88%) subjects whose genotypes determined by commercial assays were available for analyses. The United Nations geoscheme for Africa was used in this study to subdivide the African continent into four macro-regions: North, East, West, Middle, and South [430]. The overall frequency distribution of the HCV genotypes is as follows: 40.1% (119) for GT1, 4.0% (12) for GT2, 10.1% (30) for GT3, 44.1% (131) for GT4, 1.4% (4) for GT 5 and 0.3% (1) for mixed infection. (Table 10).

Table 10: Regional distribution of HCV genotypes in the study.

Region Of birth	Hepatitis C Genotype							
	n(297)	GT1	GT2	GT3	GT4	GT5	MIXED	NOT KNOWN
Northern Africa	91	21(23.1%)	2(2.2%)	6(6.6%)	62(68%)	0(0%)	0(0%)	19
Southern Africa	47	24(51.1%)	2(4.3%)	15(31.9%)	4(8.5%)	2(4.3%)	0(0%)	2
Western Africa	47	34(72.3%)	5(10.6%)	0(0%)	8(17.0%)	0(0%)	0(0%)	6
Eastern Africa	72	28(38.9%)	0(0%)	9(12.5%)	32(44.4%)	2(2.8%)	1(1.4%)	12
Middle Africa	40	12(30.0%)	3(7.5%)	0(0%)	25(62.5%)	0(0%)	0(0%)	3
	297	119(40.1%)	12(4.0%)	30(10.1%)	131(44.1%)	4(1.4%)	1(0.3%)	42

The HCV genotype distribution was statistically significantly different among the regions ($p < 0.0001$). Genotypes 1 and 4 were found in all four regions. In the western region, GT1 was found in 72.3% of patients, followed by 51.1% in the south, 38.9% in the east, 30% in central Africa and 23.1% in the northern part. GT 4 was more prevalent in the north (68%), gradually decreasing in the central (62.5%), eastern (44.4%) and western Africa (17%). GT 3 was found in 31.9% of patients from the south, 12.5% from

the east and 6.6% from the north. GT2 was present in 10.6% of cases in the west, 7.5% in the central, 4.3% in the south and 2.2% in the north. GT 5 was rare in the study with a frequency of 4.3%, 2.8% in southern and eastern Africa, respectively. The only mixed genotype (1+4b) represents 0.3% of the entire sample. Figure 10 demonstrates the relationship between participants' HCV genotype and the suspected route of infection. Route of infection data was available for 272/339 (80.2%) of the participants. Genotype 1 contributed 9.9% to infection through blood/blood products, 7.7% of intravenous drug use, whereas genotype 4 accounted for 7.4% of blood product and 2.6% of intravenous blood use.

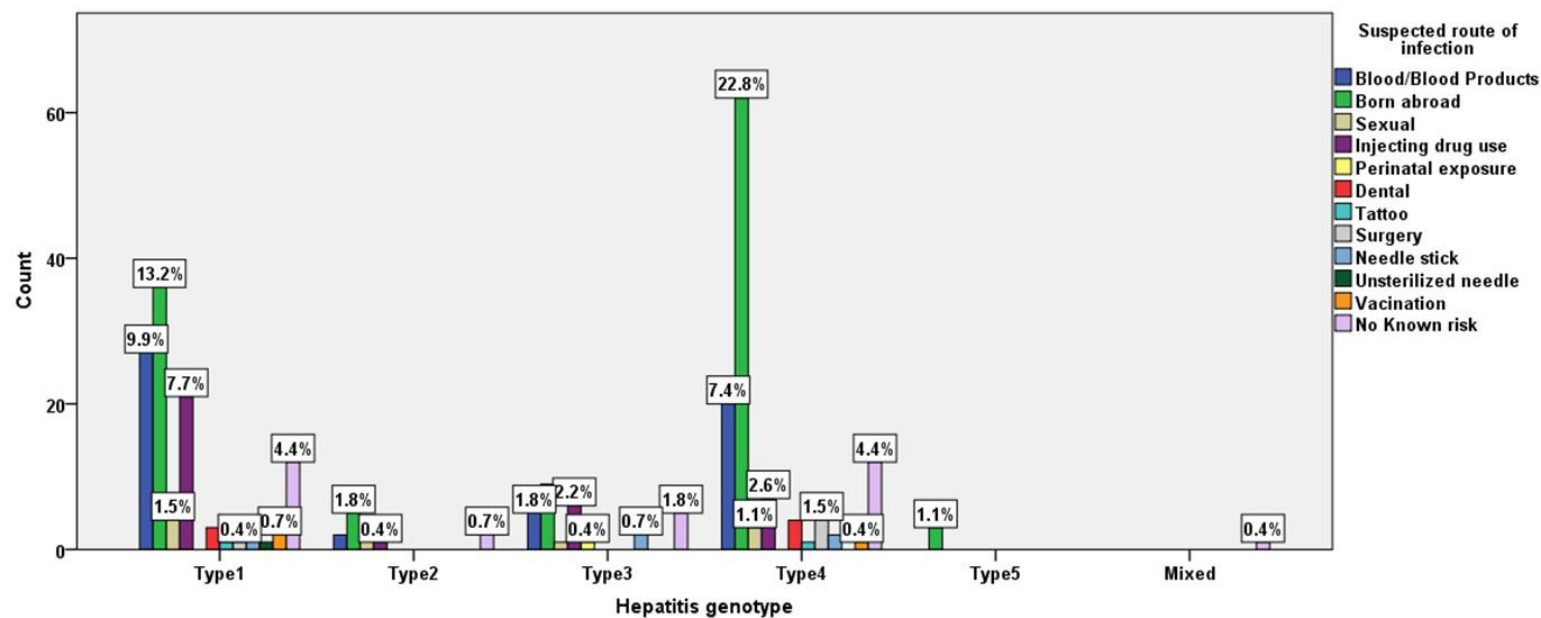


Figure 10: Distribution of HCV genotypes by suspected routes of infection.

Among the 32 countries (5 each in North and West, 13 in the East, 6 in the middle and 3 in the South), represented in the study, the prevalence and genotype distribution varies considerably (figure 11). The data are skewed, revealing that six countries have a substantial number of patients > 10 ; hence we use this as the cut off for genotype frequency distribution. No statistical significance detected when genotypes distribution was evaluated according to sex ($p=0.835$), age group ($p=0.738$), viral load ($p=0.332$), treatment outcome ($p=0.607$) and risk factors ($p=0.722$)

Table 11 shows the distribution of genotypes among the countries whose genotype is available for analysis. In the Northern region, Egypt has a substantial number of samples with 82%, representing GT4, followed by GT1 (17%) and the GT3 (1%). Other countries in this region are Algeria (GT1 and GT3), Libya (GT3, GT4), Morocco (GT1, GT2, and GT3), and Tunisia (GT1, GT3, and GT4).

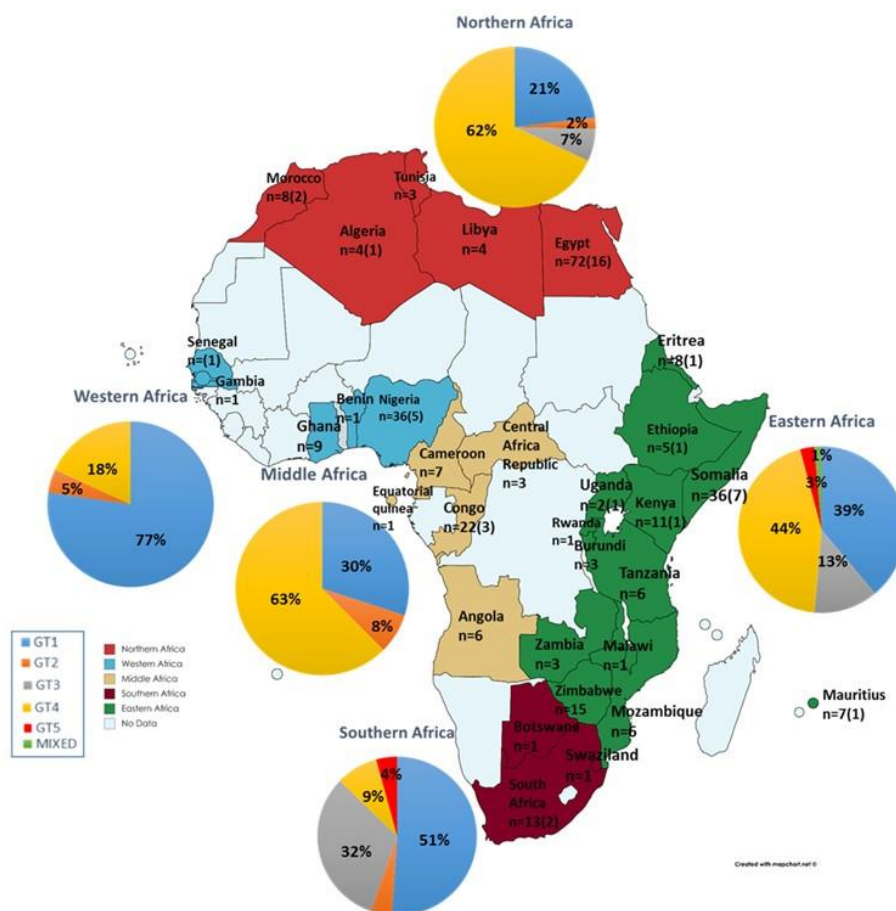


Figure 11: Map highlighting 5 regional divisions of Africa and 32 countries in the study according to the United Nations geoscheme for Africa [430]. The genotype frequency distribution in each region is represented in the pie chart. The "n" designates the frequency of the genotype data in each country while the figure in brackets represents the number of missing genotype data. The colour scheme for genotype frequency and the region is located at the side of the map.

Table 11: HCV genotype prevalence among the 32 study countries.

Region of birth	Country of birth	GT1	GT2	GT3	GT4	GT5	MIXED	TOTAL
		n(%)	n(%)	n(%)	n(%)	n(%)	n(%)	
Northern Africa	Algeria	3	-	1	-	-	-	4
	Egypt	12(17)	-	1	59(82)	-	-	72
	Libya	-	-	2	2	-	-	4
	Morocco	5	2	1	-	-	-	8
	Tunisia	1	-	1	1	-	-	3
Western Africa	Benin	1	-	-	-	-	-	1
	Gambia	1	-	-	-	-	-	1
	Ghana	4	5	-	-	-	-	9
	Nigeria	28(78)	-	-	8(22)	-	-	36
	Senegal	-	-	-	-	-	-	1
Eastern Africa	Burundi	-	-	-	3	-	-	3
	Eritrea	-	-	-	7	1	-	8
	Ethiopia	3	-	-	2	-	-	5
	Kenya	7	-	3	1	-	-	11
	Malawi	-	-	1	-	-	-	1
	Mauritius	5	-	1	1	-	-	7
	Mozambique	2	-	3	1	-	-	6
	Rwanda	-	-	-	1	-	-	1
	Somalia	15(42)	-	5(14)	14(39)	1(3)	1(3)	36
	Tanzania	3	-	1	2	-	-	6
	Uganda	-	-	-	2	-	-	2
	Zambia	1	1	-	1	-	-	3
	Zimbabwe	10(67)	-	3(20)	1(7)	1(7)	-	15
Middle Africa	Angola	5	-	-	1	-	-	6
	Cameroon	4	3	-	-	-	-	7
	Central Africa Republic	1	-	-	2	-	-	3
	Congo	1(5)	-	-	21(95)	-	-	22
	Equatorial guinea	1	-	-	-	-	-	1
	Sao Tome Principe	-	-	-	1	-	-	1
Southern Africa	Botswana	-	-	1	-	-	-	1
	South Africa	6(46)	1(8)	5(38)	-	1(8)	-	13
	Swaziland	-	-	1	-	-	-	1
	Total	119	12	30	131	4	1	297

The number of participants was skewed in relation to countries of origin. The genotype frequency >10 were selected for percentage genotype distribution.

In the western Africa data, the genotype distribution in this area includes Benin (GT1), Gambia (GT1), Ghana (GT1, GT2). Senegal has no genotype distribution data while Nigeria has 78% and 22% for GT1 and GT4, respectively.

The eastern region has the highest number of representative countries in this study. The following are their genotype distribution: Burundi (GT4), Eritrea (GT4, GT5), Ethiopia (GT1, GT4), Kenya (GT1, GT3, and GT4), Malawi (GT3), Mauritius (GT1, GT3, and GT4), Mozambique (GT1, GT3, and GT4), Rwanda (GT4), Tanzania (GT1, GT3, and GT4), Uganda (GT4), and Zambia (GT1, GT2, and GT4). In Somali data, GT1 represents 42%, GT3 (14%), GT4 (39%), GT5 and Mixed genotype 3% each. Furthermore, Zimbabwe data reveal GT1 (67%), GT3 (20%), and 7% each for GTs 4 and 5.

Middle Africa countries comprise Angola (GT1, GT4), Cameroon (GT1, GT2), Central Africa Republic (GT1, GT4), Equatorial Guinea (GT1) and Sao Tome Principe (GT4). Congo has 95% of GT4 and 5% for GT1.

The southern Africa region in this cohort was represented by three countries. Both Botswana and Swaziland have GT3, each. South Africa has GT1 (46%), GT3 (38%), and 8% each for GTs 2 and 5

3.3 DISCUSSION

For the first time, we report the prevalence and genetic diversity of HCV in patients of African origin residing in the UK. This plays a significant role in providing baseline data and the state of epidemiology for HCV patients of African origin in the UK .The sex ratio of almost 2:1 (male: female) in this

study was similar to most HCV epidemiological studies, both in Africa [431] and UK [432]. There have been conflicting reports on the reason for a high male to female ratio in most of the epidemiological studies of HCV. Some authors attributed it to increase risk activities in males, whereas many reported a high increase rate of viral clearance in females [433-435]. The highest prevalence of HCV infection was found among the age group 50-69 which was also in tandem with the study conducted by Niu *et al.*, 2016 on HCV infection in China [436]. This finding supports the effect of an increase in exposure with age.

The dominant source of infection aside from the risk of being born abroad was blood transfusion (16.8%). The transmission of HCV over twenty years was known to be through blood and blood products [437, 438]. Since then, proper screening of blood and blood products was given adequate attention in most developed countries which have yielded a good result. For instance, this has reduced transfusion-transmitted risk up to 1 in 7.7 Million in France, 1 in 2.2 Million in the USA and 1 in 4.6 Million in Canada (Hema Quebec) [439]. Surprisingly, in African countries, blood/blood product transmission continues to contribute to high HCV prevalence. Agyeman *et al.*, 2016 conducted a systematic review of the epidemiology of HCV in Ghana and reported 2.6% (95 CI=2.1% to 3.1%; I²=98.33%, $P < 0.001$) prevalence rates of infections among blood donors with a higher rate estimated for replacement blood donors than the voluntary blood donors [440]. Furthermore, the IVDUs accounted for 10.3%, which was high compared to what was known about this mode of transmission in Africa. A study in Uganda among HCV patients showed that IVDUs were rare [441]. However,

a study conducted in Morocco revealed a 60% high seroprevalence of anti-HCV antibodies among drug users and of the middle age [442]. Similarly, a systemic review of global epidemiology of people who inject drugs showed a high seroprevalence (63%) in Egypt, which is almost approaching the industrialized countries; Russia (96%), UK (54%), Canada and US (77% each) [443]. These findings reflect the emergence of intravenous injection as a contributing factor to a source of HCV transmission in Africa.

Ten of 339 (2.9%) obtained infection through sex, although studies have shown that sexual transmission of HCV is not effective [444] except in a co-infection state such as infections causing genital ulcerations [445] or sexual activities like anal sex, [446, 447]. A study in West Africa has implicated sexual transmission as effective, which was significantly higher (10%) among those that engage in sexual services [448]. Similarly, the prevalence of nosocomial infections in this study was 1.5% (surgery) and 2.1% (Dental). Transmission of HCV infection through nosocomial means is common in hospital environments where blood and blood products are not subjected to normal standard procedures during patient treatment [449]. In a study carried out by Enomoto *et al.*, 2001 in a surgical ward and dental hospital, the prevalence was 6% for surgical patients, and dental patients ranged from 8.7% to 16.7% [450]. There is also documented evidence that health workers harbour HCV infection, which can be a source of infections of the patients if universal precautions are not taken [451, 452]. The prevalence of unsterilized needle and vaccination was low; 0.3% and 0.9% respectively. This may be as a result of safer needle injection campaign since the reported cases of widespread HCV epidemic as a result of a

contaminated needle and syringes in Egypt and Pakistan [453]. However, exposure to contaminated injection from traditional practitioners is still a problem in Africa as reported in the patient cohort in Rwanda [454]. About 9.7% of patients in this cohort could not be attributed to a specific risk factor, which is also a common occurrence in HCV prevalence studies [455]. The viral load of the participants varies considerably; 26.8 % of the patients had HCV viral load greater than 8×10^5 IU/mL, which indicates a poor response to therapy. Studies have shown that the HCV RNA level is a predictor of end-stage liver disease death among patients with chronic HCV infection [456]. Higher viral load $> 6 \log$ IU/mL increases the risk of developing end-stage liver disease and hepatocellular carcinoma [457-459].

Northern Africa is represented by five countries in our study (Morocco, Algeria, Tunisia, Libya, and Egypt). Daw *et al.*, 2016, classified this region into Maghreb (Algeria, Libya, Morocco, Tunisia, and Mauritania) and Nile (Egypt and Sudan) where GT1, GT2, GT3 and GT4 were reported [422]. This is in support of our data, although the prevalence of these genotypes varies substantially; GT 4 was the most dominant in Egypt while GT1 was most prevalent in Algeria, Morocco, and Tunisia [460]. The sub-Saharan Africa data also showed variation in the prevalence of all the genotypes. The Endemicity of GT4 still exist in this cohort and predominate in central Africa, as previously reported [389]. Congo has GT4 (95%) predominantly and GT1 (5%). No previous data available for HCV genotype distribution in Congo but studies have shown that neighbouring country, the DRC has GT4 predominantly [377]. However, GT1 is more prevalent in Cameroon than

GT4 and GT2, which is a close neighbour to Congo [461]. In West Africa, Nigeria had a substantial number of data from the cohort (n=36) where GT1 and GT4 represent 78%, 22% respectively. This is in concordance with Oni *et al.*, 1996, that reported the prevalence of GT1 and GT4 [462]. This is in contrast to Forbi *et al.*, 2012 that reported GT1 and GT2 [405] and Purdy, GT2 only [463]. Patients of Somalian and Zimbabwean origin represent a significant number of a cohort from the Eastern region of Africa. Genotypes 1, 3, 4, and 5 were prevalent in the two countries except for only one mixed infection (GTs 1+4b) found in a patient from Somalia. Mixed infection is not only peculiar to Somalia; a recent study in Rwanda revealed the occurrence of mixed infection (GT1 and 4), which was reported with treatment failure [464]. Fewer previous studies in Zimbabwe only showed seroprevalence of HCV, no genotype distribution data [383, 465]. Similarly, few reported cases of genotype distribution were documented in Somalia predominantly GT3 [466]. South Africa met our 10 cuff-off (n=13); in the southern African region in which genotypes 1, 3, 4, and 5 were found among the patients. Genotype 5 is not a predominant genotype in our study, as reported in previous studies [467].

3.4 CONCLUSION

This study reaffirms the predominant of the hepatitis C virus genotype 1 and 4 in Africa. Surprisingly, Western Africa, which was known to be predominated with genotype 2, has a 9% prevalence in our data. Similarly, in southern Africa, genotype 5 has a low prevalence of 4% compared to genotypes 1 and 3 with 49%, 31% respectively. Furthermore, this data also shows an increase in the transmission of HCV infection through intravenous

drug use compared to previous studies. All these observations were inconsistent with the previous studies of HCV of Africa origin. This variation can be attributed to the difference in population study where this study is limited to HCV patients of African origin living in the UK. More importantly, genotype 1 and 4, which was known to be harder to treat, are much more prevalent in cohorts with poor response to PEGylated interferon and ribavirin, which is still in use in most parts of Africa [383]. This study will have a more significant impact on the strategic planning of HCV treatment of migrant communities in the UK and other Western countries.

4 CHAPTER FOUR: EVALUATION OF COMMERCIAL GENOTYPING ASSAYS IN SSA HCV INFECTED INDIVIDUALS WITH NS5B SEQUENCING AND PHYLOGENETIC ANALYSIS.

4.1 INTRODUCTION

In chapter three, I highlighted the genetic diversity of HCV across the African continent. These data were based on commercial genotyping assays. However, there is increasing evidence that the performance of these assays is poor, particularly at the subtype level [468, 469]. The NS5B genome sequence is highly divergent and therefore, sufficient enough for HCV genotyping and subtype determination [470]. Hence sequencing of this region followed by phylogenetic analysis is the gold standard and has been adopted for the HCV genotype and subtype assignment [471]. The main objective of this chapter was to evaluate the performance of the commercial genotyping kit used at the patients' treatment centres and compare them with the performances of six online tools and NS5B sequence phylogenetic analyses. We assigned genotype/subtype to the selected 135 SSA samples by partial NS5B sequence phylogenetic analyses. Therefore, the fragment of the NS5B gene (338 bp) representing positions 8283-8620 with reference to H77 (NC_004102) was amplified using previously published primers. The same primers used for PCR amplification were utilized to perform Sanger sequencing by Nottingham Source Bioscience. All the sequences were aligned using MEGA 6 software and subjected to six online tools and phylogenetic analysis.

4.2 RESULTS

4.2.1 Demographic Characteristics of patients

Of the 339 participants of African origin, 135 subjects who were from SSA were selected. The male gender made up 57.0% of the sample subset, while the female was 42.3%. (Table12). For the 339 and 135 participants, the mean age and standard deviation were 57.3 ± 13.3 and 58.5 ± 12.4 , respectively. Fifty-seven (42.2%) of patients had a risk factor of being born abroad, and 17% had infection through blood and blood products. Other known route of infections is sexual contact (5; 3.8%), dental (3; 2.2%), surgery (2; 1.5%), and needle stick (1; 0.7%). No known risk factor was established in 23.7% of the patients. Fifty-four (40%) of the patients had a high viral load greater than 8×10^5 IU/mL while 24 (17.8%) had less than 6×10^5 IU/mL and the remaining 5 (3.7%) had a viral load range between 6×10^5 - 8×10^5 IU/mL.

Table 12: Demographic characteristic and risk factors of 135 samples selected from 339 participants.

Variable	Categories	No (%);N=339	No(%);N=135
Sex	Male	225(66.4%)	77(57.0%)
	Female	112(33%)	57(42.3%)
	Unknown	2(0.6%)	1(0.7%)
Age, years	20-29	8(2.4%)	1(0.7%)
	30-39	35(10.3%)	9(6.7%)
	40-49	60(17.7%)	21(15.6%)
	50-59	92(27.1%)	40(29.6%)
	60-69	87(25.7%)	35(25.9%)
	70-79	50(14.7%)	26(19.3%)
	80-89	7(2.1%)	3(2.2%)
Mean $\pm$ SD		57.3 $\pm$ 13.3	58.5 $\pm$ 12.4
Gender/Mean age p-value		0.146	0.774
Source of infection	Blood/Blood products	57(16.8%)	23(17.0%)
	Born abroad	127(37.4%)	57(42.2%)
	Sexual	10(2.9%)	5(3.8%)
	Injecting drug use	35(10.3%)	
	Perinatal exposure	1(0.3%)	
	Dental	7(2.1%)	3(2.2%)
	Tattoo	2(0.6%)	
	Surgery	5(1.5%)	2(1.5%)
	Needle stick	9(2.7%)	1(0.7%)
	Unsterilized needle	1(0.3%)	
	Vaccination	3(0.9%)	
	No known risk factor	33(9.7%)	32(23.7%)
	Data missing	49(14.5%)	12(8.9%)
HCV Viral Load level IU/mL	<6X10 ⁵	64(18.9%)	24(17.8%)
	6X10 ⁵ -8X10 ⁵	14(4.1%)	5(3.7%)
	>8X10 ⁵	91(26.8%)	54(40.0%)
	Data missing	170(50.2%)	52(38.5%)

Data frequencies are designated as “n” while percentages are in parenthesis — SD: Standard deviation.

4.2.2 HCV genotype assignment by NS5B Sequence Analysis

The characterization of HCV sequences into genotypes and subtypes in this cohort was based on the results of the match between the partial NS5B sequence phylogenetic analyses and the six online tools.

A total of 135 partial NS5B sequences were derived from the SSA cohort, and these were subjected to phylogenetic analysis. To assist the visualisation of the trees, the dataset was analysed according to the United Nations African region (Figure 12-15). There were only seven sequences derived from individuals previously living in the Southern Africa region, of which 4 (57%) were GT1a, and the remainder were either GT1b, GT3a or GT5a (Figure 12).

Genotype 4r was the predominant subtype present in the 27 Middle African sequences, representing 26% of the sample set (Figure 13). This was followed by GT4k (19%), GT4c (11%), GT4h (7%), GT4f (7%), whilst GT4b, GT1a, GT1b, GT1l, GT1e, represented 4% respectively. Also, there were 7% (2/7) of unassigned genotypes located on the branches with low bootstrap support or that clustered with other unassigned subtypes.

Western Africa had predominantly genotype 1; 39% of these genotypes were not assigned to a specific subtype. Also, 8% of GT2 was not assigned to subtype. Other sequences in this region were characterised to subtype level. These included subtypes 1a (6%), 1b (8%), 1e (3%), 1l (17%), 2c (3%), 4k (3%), 4r (3%), 4t (6%), and 4v (8%) (Figure 14).

The Eastern region had the highest cohort representation, with 65 sequences (Figure 15). Subtype 4r (29%) predominated followed by subtypes 1b (17%), 3h (12%), 1a (11%), 4d (6%), 3a (5%) and 5a (3%). Other subtypes were 1g, 4b, 4c, and 4m with 2%, respectively. All of the sequences obtained from individuals from the Eastern regions could be assigned to a specific subtype.

Those that could not be classified to specific subtypes (genotypes 1? and 2?) were further evaluated using available full-length HCV subtypes in the GenBank. Genotypes 1? were aligned with 12 confirmed different subtypes and 3 unassigned genotypes (Subtypes 1a, 1b, 1c, 1d, 1e, 1g, 1h, 1i, 1j, 1k, 1l and 1m) whereas GT 2? were aligned with 13 confirmed subtypes (2a, 2b, 2c, 2d, 2e, 2f, 2i, 2j, 2k, 2l, 2m, 2q, 2r) including 3 unassigned genotype 2. Two reference sequences were selected for each subtype where possible. The reference sequences were trimmed to the same region as that analysed for the SSA cohort sequences. The Neighbour-joining tree (NJ) and Maximum Likelihood (ML) phylogenetic trees were reconstructed using MEGA 6 software and applying the Kimura two-parameter model and General Time reversible (GTR) model, respectively. None of the GT1? and GT2? Clustered with the known previously confirmed subtype sequences. Both NJ and ML phylogenetic trees had identical topologies, and the sample sequences were distributed throughout the tree, indicating diverse variability among the sample sequences as shown in Figures 16 and 17, respectively. The unassigned GT1 sequences clustered with unassigned sequences or sequences with low bootstrap support <70% from Nigeria, Cameroon, Ethiopia, and Spain. In the same way, unassigned GT2 clustered with sequences from China, Canada, and Cameroon.

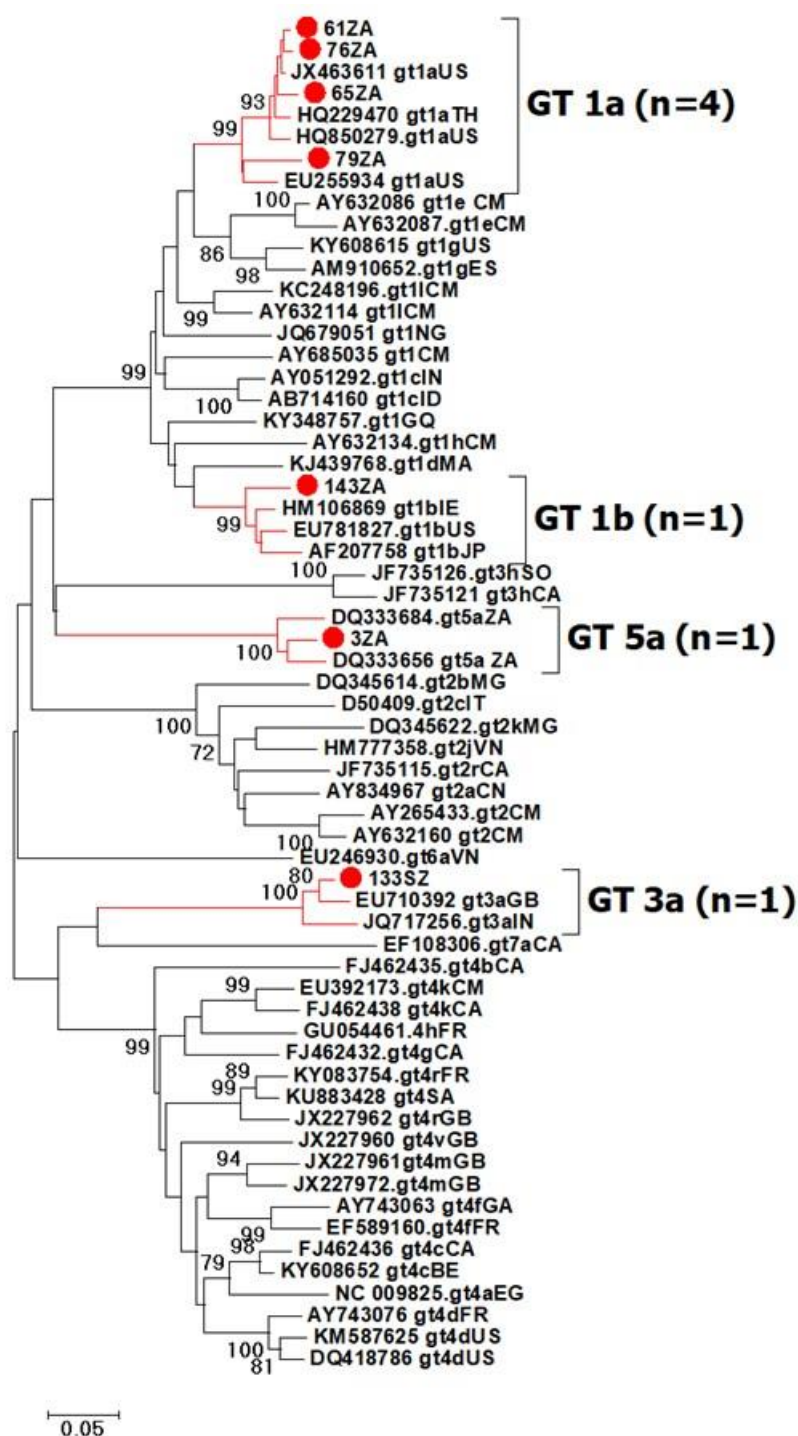


Figure 12: Phylogenetic analysis of 7 NS5B sequences from HCV patients of Southern African origin. The phylogenetic tree was constructed by the Neighbour-joining method. Reference sequences contained representatives of confirmed HCV subtypes obtained from the Los Alamos database indicated by accession number, genotype, and country of origin. Predicted subtypes are shown in red dots and branches. The number in brackets showed the frequency of subtypes. Bootstrap support >70% are specified in the inner node.

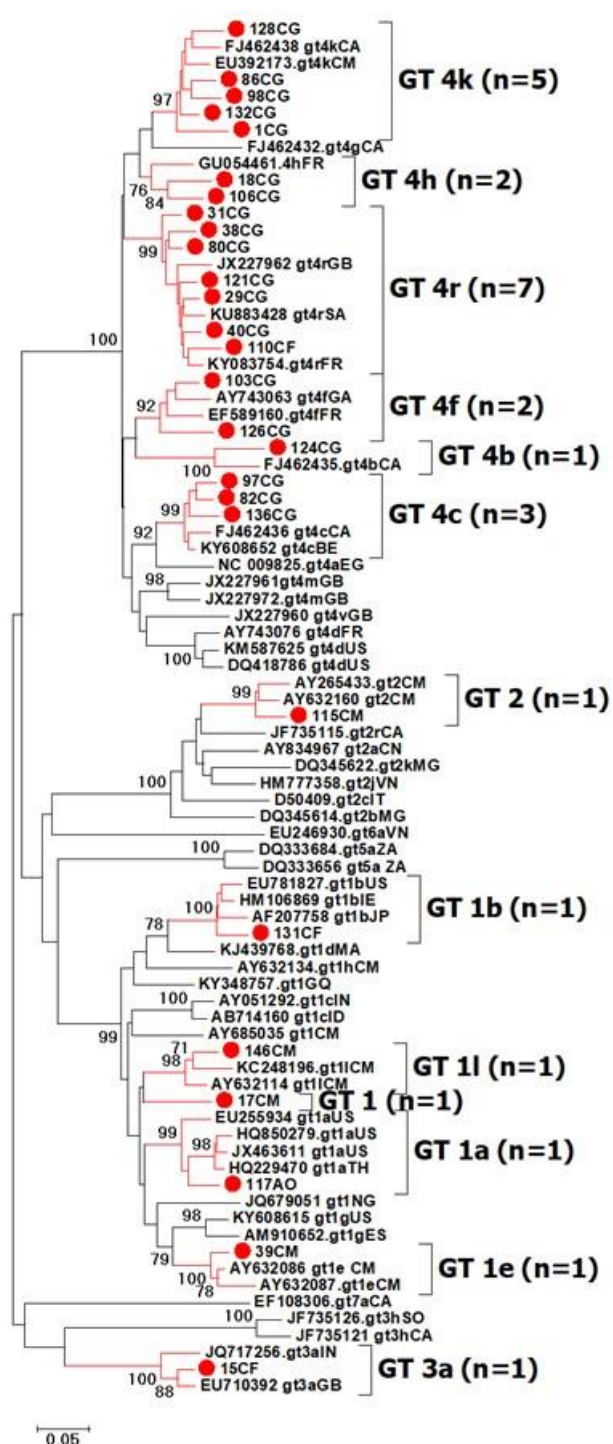


Figure 13: Phylogenetic analysis of 27 NS5B sequences from HCV patients of Middle African origin. The phylogenetic tree was constructed by the Neighbour-joining method. Reference sequences contained representatives of confirmed HCV subtypes obtained from the Los Alamos database indicated by accession number, genotype, and country of origin. Predicted subtypes are shown in red dots and branches. The number in brackets showed the frequency of subtypes. Bootstrap support >70% are specified in the inner node.

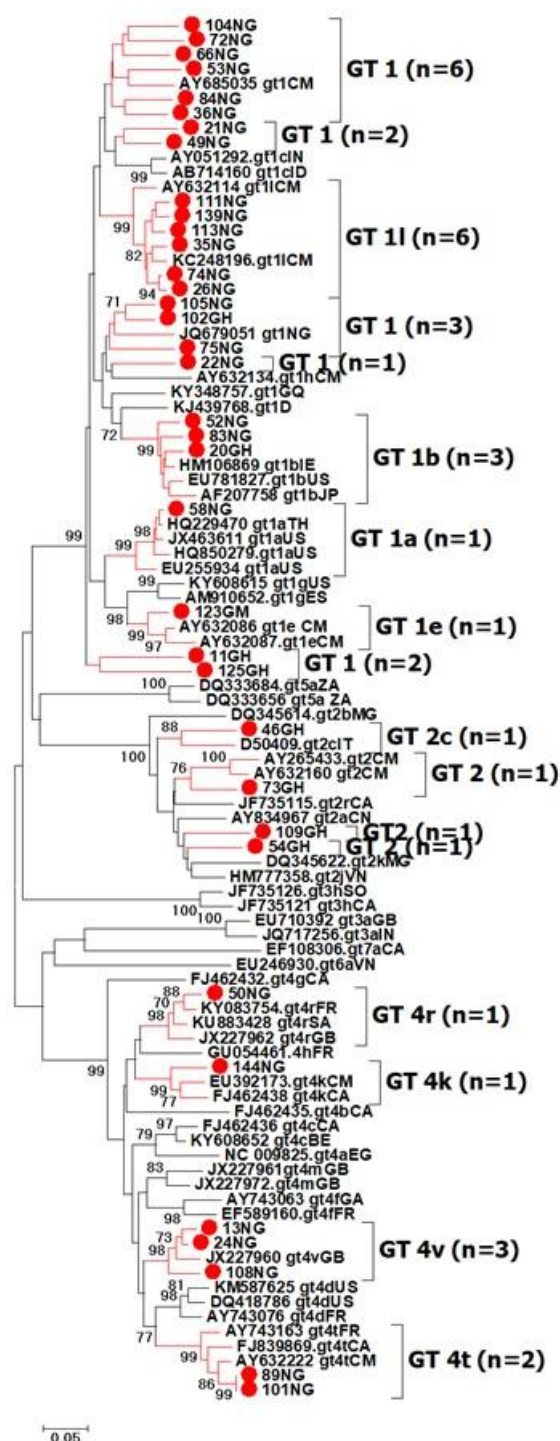


Figure 14: Phylogenetic analysis of 36 NS5B sequences from HCV patients of Western African origin. The phylogenetic tree was constructed by the Neighbour-joining method. Reference sequences contained representatives of confirmed HCV subtypes obtained from the Los Alamos database indicated by accession number, genotype, and country of origin. Predicted subtypes are shown in red dots and branches. The number in brackets showed the frequency of subtypes. Bootstrap support >70% are specified in the inner node.

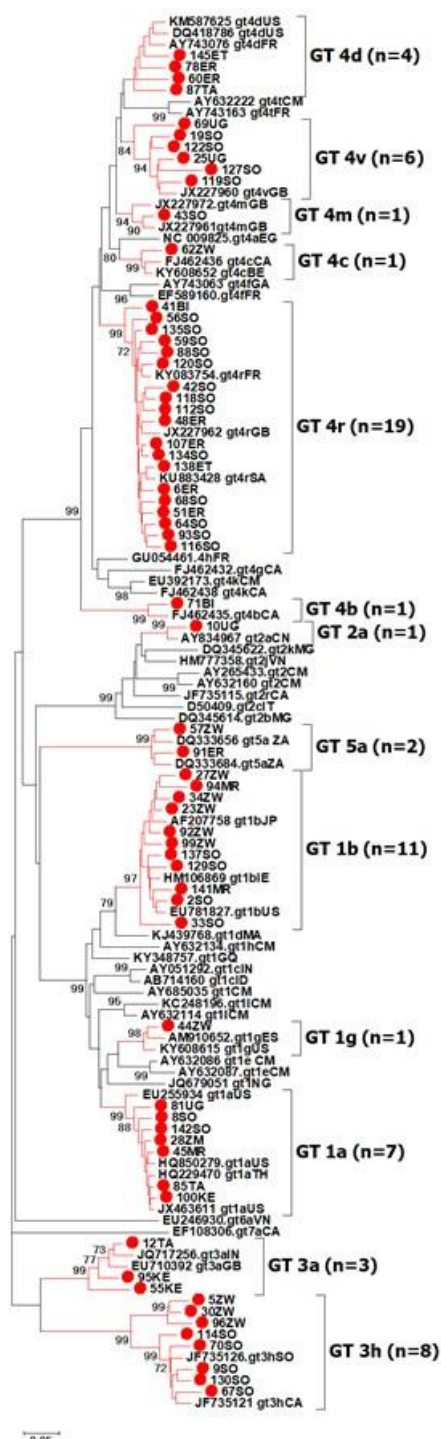


Figure 15: Phylogenetic analysis of 65 NS5B sequences from HCV patients of Eastern African origin. The phylogenetic tree was constructed by the Neighbour-joining method. Reference sequences contained representatives of confirmed HCV subtypes from the Los Alamos database indicated by accession number, genotype, and country of origin. Predicted subtypes are shown in dots and branches. The number in brackets showed the frequency of subtypes. Bootstrap support >70% are specified in the inner node.

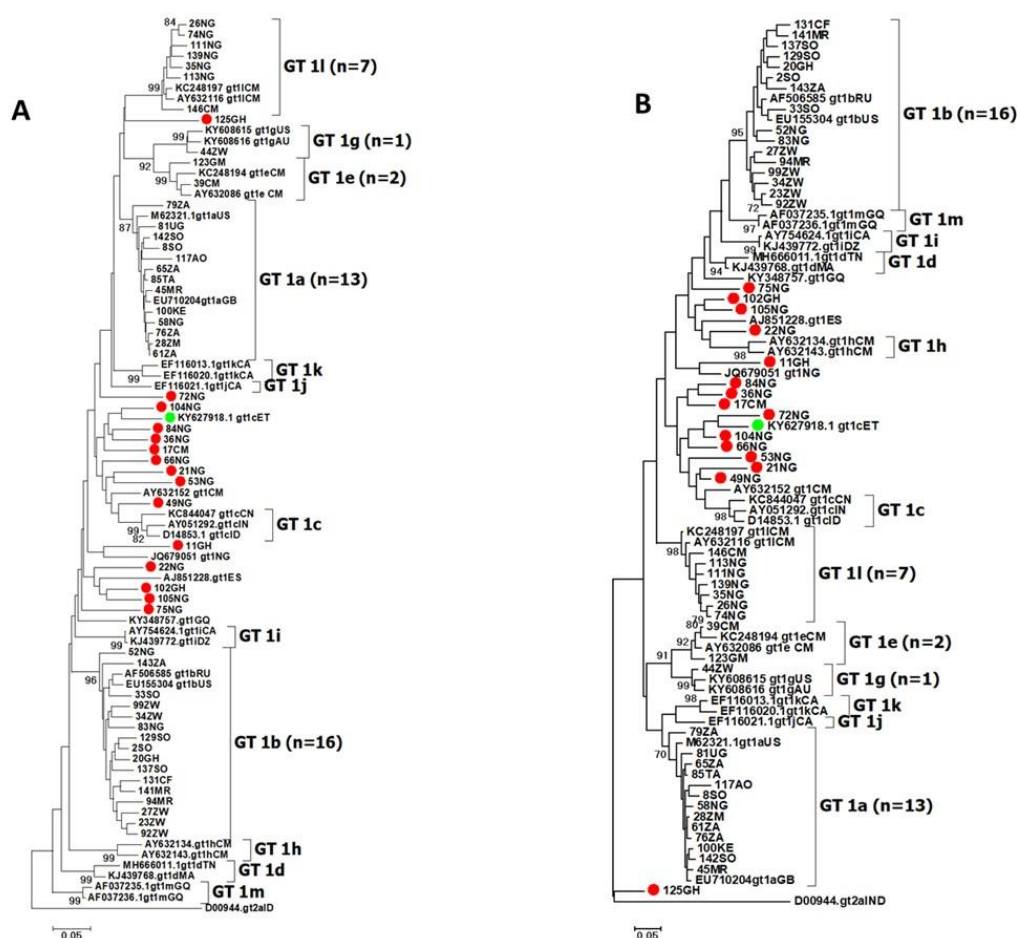


Figure 16: Phylogenetic Neighbour-joining (A) and Maximum Likelihood (B) trees reconstructed from 15 unassigned genotype 1 sequences. The study sequences are indicated in red dots while confirmed genotype 1 subtype reference sequence shown in green dot was assigned as subtype 1c in the previous publication but did not cluster with other subtype 1c reference sequences.

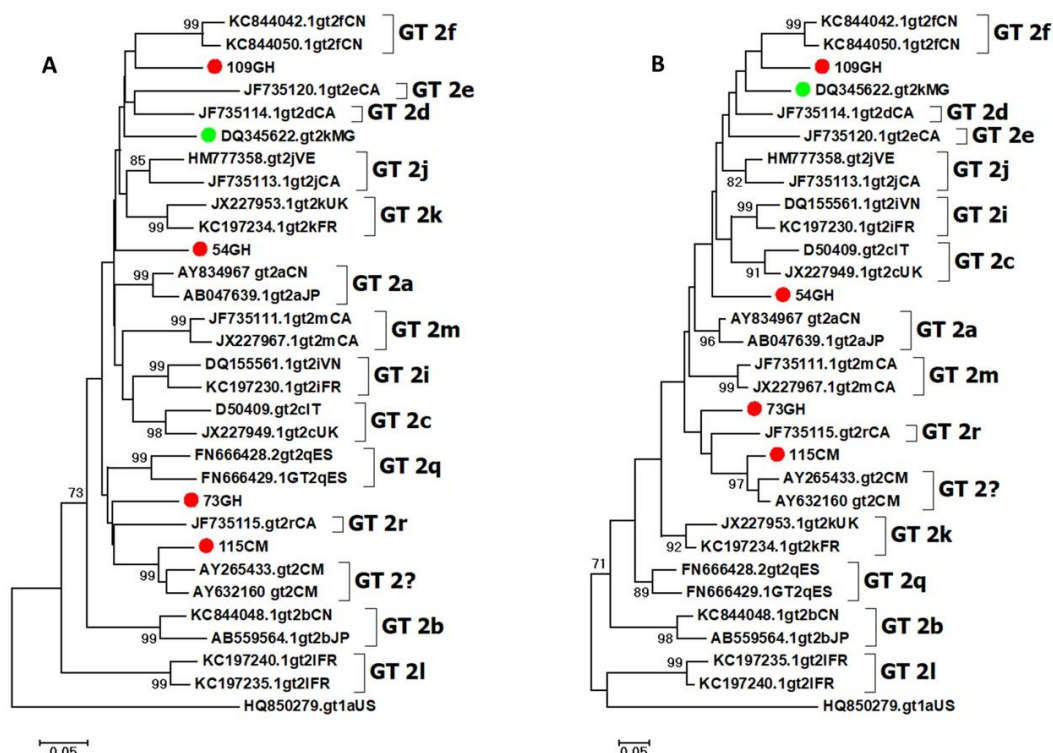


Figure 17: Phylogenetic Neighbour-joining (A) and Maximum Likelihood (B) trees reconstructed from 4 unassigned genotype sequences. The study sequences are indicated in red dots while confirmed subtype reference sequences pointed in green dot was assigned as subtype 2k in previous publication but did not cluster with other subtype 2k reference sequences.

To determine the concordance of these genotyping methods, we used the 121 samples that have data for all the genotyping approaches. Consensus genotype assignments varied considerably among the six genotyping tools, commercial assays, and phylogenetic analyses. Six online genotyping tools analyses and the commercial assay yielded concordance rates of 31% to 98%; HCVGLUE had the highest concordance rate of 98% while the commercial assays (Versant HCV genotype 2.9 assay, Taqman PCR Roche, and Abbott Real-time PCR using M2000 platform) had the lowest (31%). (Figure 18).

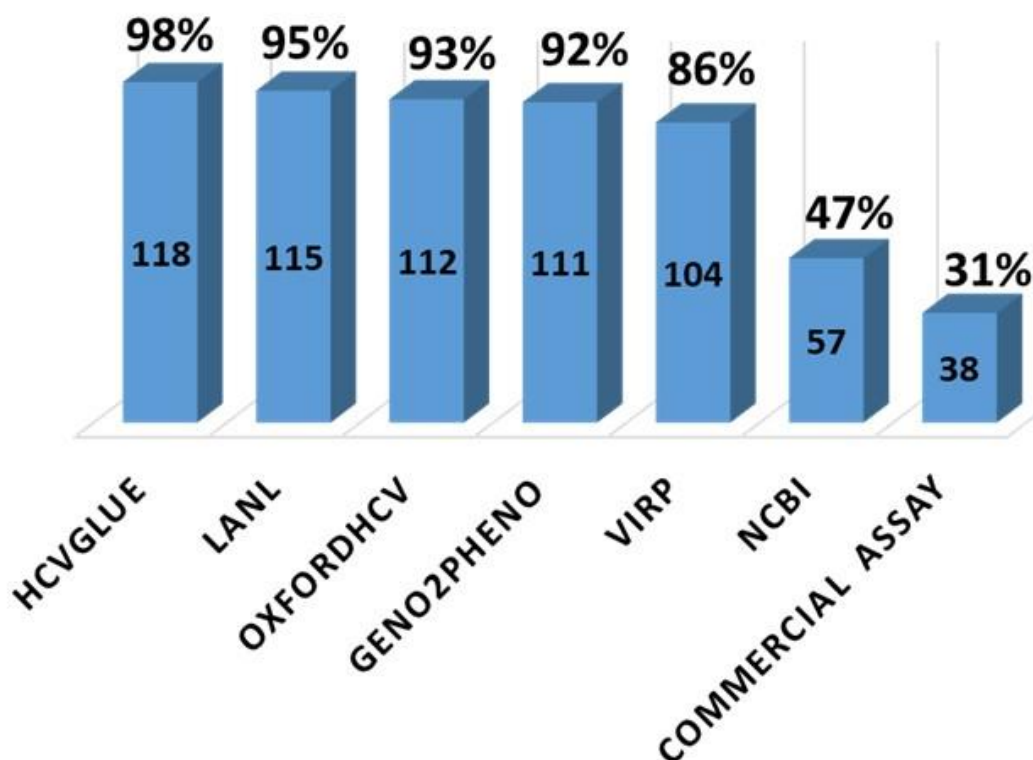


Figure 18: Percentage concordance of the six online genotyping tools and commercial assay on 121 sequences compared to Phylogenetic analysis

Abbreviations: LANL, Los Alamos Database; OxfordHCV, HCV Phylogenetic typing tool; VIRP, Virus Pathogen Database, and Analysis Resources; Geno2pheno HCV Genotyping Tool; NCBI, National Centre for Biotechnology Information Genotyping Tool; HCV-Glue Automated Genotyping Tool.

Overall, the 135 sequences were assigned to twenty-two different genotypes and subtypes. Nineteen (14.1%) of the sequences with HCV genotypes 1(GT1?) and 2 (GT2?) were not assigned to specific subtypes while 116 (85.9%) were identified to subtype level. Figure 19 demonstrates the distribution of HCV sequences into genotype/subtypes and they were distributed as follows: GT1? (Number of isolates n=15; 11.1%), GT2 (n=4; 3.0%), GT1a (n=13; 9.6%), GT1b (n=16; 11.9%), GT1e (n=2; 1.5%),

GT1g (n=1; 0.7%), GT1l (n=7; 5.2%), GT2? (n=4;3.0%) ,GT2a (n=1;0.7%),GT2c (n=1;0.7%), GT3a (n=5;3.7%), GT3h (n=8;5.9%), GT4b (n=2;1.5%), GT4c (n=4;3.0%), GT4d (n=4;3.0%), GT4f (n=2;1.5%), GT4h (n=2;1.5%), GT4k (n=6;4.4%), GT4m (n=1;0.7%), GT4r(n=27;20.0%), GT4t(n=2;1.5%), GT4v (n=9;6.7%) and GT5a (n=3;2.2%). Table 13 shows the distribution of genotypes/subtypes across the major 4 regions of SSA, where the samples were selected.

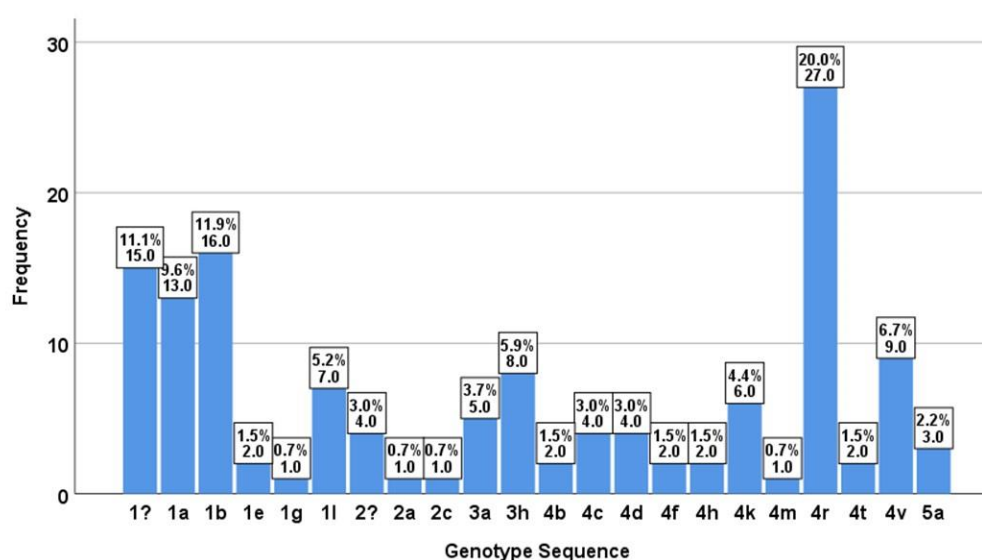


Figure 19: Genotype/subtype distribution derived from 135 sub-Saharan Africa sequences.

Table 13: Regional distribution of genotypes/subtypes derived from 135 study samples

Genotype	Southern Africa n (%)	Middle Africa n (%)	Western Africa n (%)	Eastern Africa n (%)	Total
1?	-	1(4)	14(39)	-	15
1a	4(57)	1(4)	1(3)	7(11)	13
1b	1(14)	1(4)	3(8)	11(17)	16
1e	-	1(4)	1(3)	-	2
1g	-	-	-	1(2)	1
1l		1(4)	6(17)	-	7
2?	-	1(4)	3(8)	-	4
2a				1(2)	1
2c	-	-	1(3)	-	1
3a	1(14)	1(4)	-	3(5)	5
3h	-	-	-	8(12)	8
4b	-	1(4)	-	1(2)	2
4c	-	3(11)	-	1(2)	4
4d	-	-	-	4(6)	4
4f	-	2(7)	-	-	2
4h	-	2(7)	-	-	2
4k	-	5(19)	1(3)	-	6
4m	-	-	-	1(2)	1
4r	-	7(26)	1(3)	19(29)	27
4t	-	-	-	2(6)	2
4v	-	-	3(8)	6(9)	9
5a	1(14)	-	-	2(3)	3
Total	7	27	34	67	135

N represents the number of sequences, while % in brackets indicate the percentage of sequences.

4.2.3 Comparison of HCV genotyping by NS5B sequencing and phylogenetic analysis with the genotyping result of commercial assays.

As previously mentioned above, the initial genotype assignment was based on commercial assays from patients' different treatment centres.

Out of 135 samples assigned to genotype/subtype by NS5B sequence phylogenetic analyses, 121 samples had commercial assay genotype data for analyses. The genotype data of the 121 samples are as follows: GT1? (n=14), GT1a (n=11), GT1b (n=15), GT1e (n=2), GT1g (n=1), GT1l (n=6), GT2? (n=4), GT2a (n=1), GT2c (n=1), GT3a (n=5), GT3h (n=7), GT4b (n=2), GT4c (n=4), GT4d (n=3), GT4f (n=2), GT4h (n=1), GT4k (n=6), GT4m (n=1), GT4r (n=23), GT4t (n=2), GT4v (n=8), and GT5a (n=2). The parameters for analysis were based on major and minor discrepancies in genotype assignment using NS5B sequence phylogenetic analysis as reference. Minor discrepancies were considered when a commercial assay failed to assign a specific subtype to an isolate (failed to subtype) or assigned a different subtype (mis-subtyped) — for example, subtype 1a assigned as 1b. A major discrepancy was designated as mis-genotyped if there was a mismatch across genotype. For example, when subtype 3a was identified as 4r. However, If the commercial assay correctly identified isolates genotype/subtype, they were labelled as correctly genotyped and subtyped, respectively [241, 472].

The comparison between commercial and phylogenetic-based subtyping for the 121 SSA sequences is presented in figure 20. In this cohort, 14 (12%) of the sequences were assigned as GT1? by NS5B sequence phylogenetic analysis. Five (36%) of these sequences were correctly assigned by

commercial assay, while the other 64% (9) were mis-subtyped either as subtype 1a, 1b, or 1c. For other genotypes 1 sequences that were identified at the subtype level, the ability of the commercial assays to assign them accurately varied considerably. For subtype 1a, 64% (7) were assigned to the right subtype (rightly subtyped), 18% (2) were mis-subtyped, and 18% (2) were not assigned subtype (failed to subtype). Eighty percent (12) of subtype 1b samples were assigned to the right subtype, 7% (1) were mis-subtyped, and 13% (2) were not identified to subtype level. One subtype 1e sequence was correctly identified to the subtype level whilst the only other GT1e sequence was mis-typed. None of the GT1g or GT1l sequences were correctly identified to subtype level (either they were mis-subtyped, or they could not be identified to subtype level). By contrast, all the sequences (100%) with GT 2? and subtype 2c were correctly identified. Furthermore, 12 sequences with genotype 3 were identified; 5 sequences had subtype 3a while 7 had subtype 3h. Out of 5 sequences with subtype 3a, 2 (40%) were rightly subtyped, another (2) 40%, could not be identified to subtype level, and 1 (20%) was mis-genotyped. In the case of subtype 3h, 2 (28%) were rightly identified to subtype level, another 2 (28%) could not be assigned to a specific subtype, and 3 (44%) were mis-genotyped.

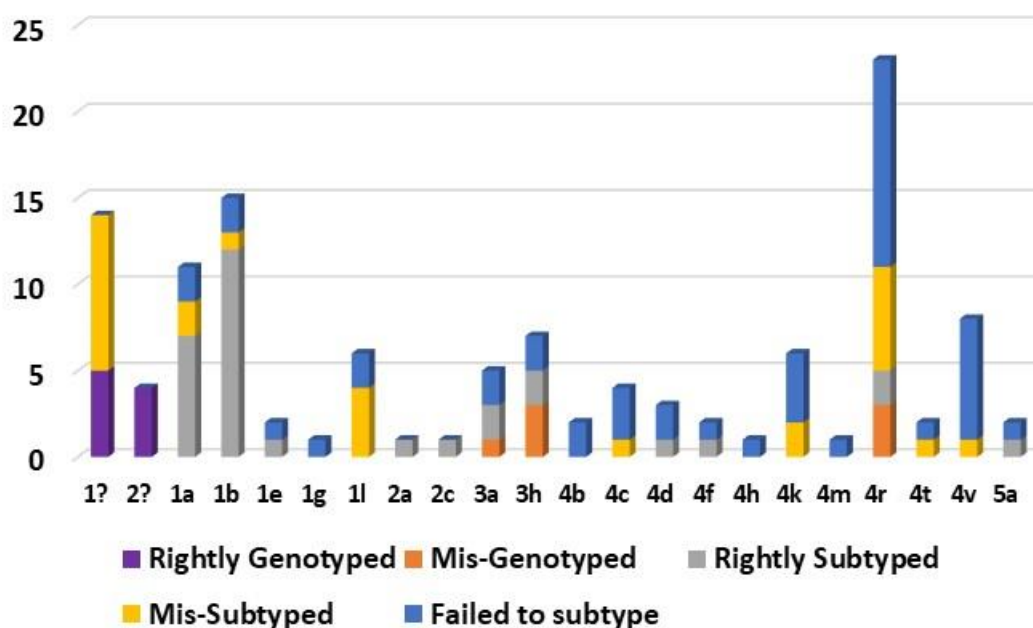


Figure 20: Overall discrepancies in the assignment of genotype/subtypes in 121 study samples by commercial assay. Only 2 of 23 subtype 4r are correctly assigned to subtype. None of the subtypes 1l, 4h, 4m, and 4v was rightly assigned to subtype. Mis-genotype: Wrong classification across genotype e.g., GT1a assigned as GT4r; Mis-subtyped: Wrong classification within subtypes, e.g., GT1a assigned as GT1b; Failed to subtype: Failure to assign to specific subtype, e.g., GT4a assigned as GT4. Rightly genotyped/subtyped denotes no discrepancy.

The performance of commercial assay for genotype 4 subtype classification was poor. All the sequences with subtypes 4b, 4c, 4h, 4k, 4m, 4t, and 4v, could not be identified to subtype level. For subtype 4d, 4f, and 4r, only 33%, 50%, and 9% frequencies respectively of these genotypes were rightly identified to subtype level. Furthermore, in two of the sequences harbouring GT 5 infection, just one was rightly subtyped as subtype 5a while the other strain could not be assigned to a specific subtype. Overall, there seems to be a high level of discordance between the commercial assays and NS5B sequence phylogenetic analyses. More importantly, 81/121 (67%) could not be accurately subtyped; either they could not be assigned a subtype, were mis-subtyped, or in the worse cases, were mis-

genotyped. Also, considering some of the endemic subtypes, only 2 (9%) of the 23 GT 4r were correctly identified to subtype level, whilst none of the GT1I was correctly typed.

4.3 DISCUSSION

Accurate genotyping is essential for epidemiological studies, clinical evaluation, management, and treatment of HCV infection. Although the use of pan-genotypic DAAs, in theory, precludes the use of HCV genotyping in a clinical setting, accurate genotyping/subtyping of HCV strains is relevant especially in this era of treatment failure [319, 473]. Commercial assays have contributed immensely to HCV genotyping since their introduction more than a decade ago [474]. The high HCV genetic variability of the genomic regions used in assay design confers some limitations on accurate genotyping, especially at subtype level [475]. NS5B sequencing and phylogenetic analysis is the gold standard for genotyping. However, it is tedious, laborious, and expensive and time-consuming to perform in the clinical setting [476]. The accuracy of the genotype assignment cannot be overemphasized. Since the classification of HCV into six genotypes by Simmonds in 1996 using sequence variability of the 222 base pairs of NS5B region, this sequence analysis has gained full acceptance [477]. The 19 isolates (genotypes 1? and 2?) that were not assigned to specific subtypes had a phylogenetic concordance between the NJ and ML NS5B phylogenetic analyses. It is worthy of note here to state that there is a disparity in Ethiopian reference strain (KY627918gt1cET) from Hundie *et al.*, 2017 study [478] and Madagascar reference isolate (DQ345622gt2kMG) from Razafindratsima *et al.*, 2007 study [479]; they were assigned to subtypes

1c and 2k respectively. In our study, as shown in figures 17 and 18, these strains did not cluster with other confirmed reference subtypes from GenBank. From these phylogenetic analyses, subtyping assignment errors may be contemplated. So far, only 13 subtypes have been described for GT1 (subtypes 1a to 1m), including a new strain reported in Equatorial Guinea (AJ851228GQ) [480].

Similarly, 13 of GT2 have been reported, based on full-length genomic sequences, in the Los Alamos HCV Databases. The phylogenetic diversity of these subtypes and the inability of some of them to segregate into clearly defined distinct clades or subtypes suggested extensive variability and heterogeneity. It implies they are endemic and possibly originated in Africa as previously reported [387, 481-483]. Furthermore, the online genotyping tools seem to vary in their concordance with phylogenetic analyses. HCVGLUE had the highest rate of concordance of 98%, followed by LANL (95%), HCVOxford (93%), Geno2pheno (92%), VIRP (86%), and NCBI (47%). The implication is that online genotyping tools need to be selected for use with caution, most especially in SSA, where wide sequence diversity of genotypes are prevalent. HCVGLUE and LANL appeared to perform better than other online tools. Also, using NS5B phylogenetic analyses, we have re-evaluated the results of 121 samples whose commercial genotype assay data were available. Overall, the percentage concordance was 31%, which was very low compared to a study carried out by Natalia Chueca 2016 *et al.*, where they recorded 89.4% in an Italian cohort [240]. The high success rate in their study could be explained by differences in the relative frequencies of potentially 'difficult-to-type' sequences, such as some subtypes within GT4. The prevalence of genotype 4 in this study was 44%

(53/121), and it is worthy of note that only 8% (4/53) were accurately assigned to a definite subtype. The performances of most commercial assays available in Western countries seem to be biased toward genotypes 1 and subtypes common in these regions. For example, in the study conducted by Yang *et al.*, 2014 where genotype 6 (n=57) formed a considerable percentage of clinical isolates, 10% of these isolates were either mis-genotyped or mis-subtyped [484].

Similarly, a study carried out in China in which three different commercial assays were compared with NS5B-based phylogenetic analysis, 11/21 GTs 6a samples were misclassified as subtype 1b [485]. Furthermore, studies from Western countries reported on the limitations of commercial assays, which necessitated an improvement in the design and introduction of new assays [243]. For example, Boucha *et al.*, 2007, tested 135 samples with LiPA (based on 5'UTR region) compared to a novel LiPA Versant HCV 2.0 assay (based on simultaneous detection of 5'UTR and core) for subtypes 1a and 1b. The LiPA assay utilising 5'UTR and core improved the characterization of the subtypes from 45.2% to 96.8% [486]. Another study carried out by Fernandez Caballero, reported a new Cobas HCV genotyping tool with a 98% success rate. However, the assay still has challenges in accurately assigning some 1a, 3a, and 4d subtypes [241].

4.4 CONCLUSION.

This study showed some limitations of the commercially available genotyping assays and online genotyping tools in assigning genotype to subtype level on SSA HCV infected patients. More importantly, endemic subtypes 4r and 1l, which studies have indicated harbour DAA resistance-

associated substitutions were poorly characterized [487, 488]. There is a need to improve on the existing commercial assays, and in the interim embrace, the NS5B sequence phylogenetic analyses in SSA where there is high HCV genetic diversity and endemic subtypes are more prevalent.

5 CHAPTER FIVE: PREVALENCE OF NS5A AND NS5B RESISTANCE-ASSOCIATED SUBSTITUTIONS IN HCV SSA INFECTED INDIVIDUALS

5.1 INTRODUCTION

SSA bears a substantial portion of the global burden of HCV infection with a prevalence of 3.0% [489]. Despite the high prevalence, almost all the countries in this sub-region have limited data on treatment and resistance profile [383, 397, 490]. Over the last three decades, huge successes have been recorded in the improvement of HCV treatment, most especially with the use of new DAAs globally [491, 492]. This success was achieved by the intensive efforts put into the evaluation and assessment of all oral DAAs combination therapy, safety, and efficacy both *in vivo* and *in vitro* [493-495]. Most clinical trials were carried out on patients from Western countries, which were infected mainly with genotypes 1 and or 3 [496, 497]. Treatment of HCV in sub-Saharan Africa is still primarily based on PEGylated interferon and ribavirin, which has a low SVR of about 40% and is genotype-dependent most effective against genotypes 2 and 3. This regimen has a propensity for the emergence of resistant strains [498, 499]. Adequate treatment of HCV infection in SSA has been quite challenging due to the lack of access to HCV health care facilities and the cost of more efficient DAAs therapy [500-502]. Of recent, some national stakeholders reviewed the current status of HCV in SSA in an attempt at providing a roadmap towards achieving the elimination of HCV by the year 2030 has stipulated by WHO [397]. They recommended regimens depending on genotypes, treatment history, and presence or absence of cirrhosis. However, an effective treatment regimen which is comparable to other treatment in

Western countries should be based on clinical trials, real-world evaluation, and *in vivo* analyses [503-506]. The recent study of HCV treatment in SSA using DAAs was the SHARED study where 300 participants with GT1 or 4 achieved 86.7% SVR12 after treatment with LDV/SOF [464]. This study excluded patients with decompensated cirrhosis, HBV coinfection, uncontrolled HIV infection, and hepatocellular carcinoma. Based on this, the authors suggested the use of this therapy in Rwanda, where GT4 is predominant. However, GT4 is heterogeneous. As was described in chapter four, several studies have shown that subtypes 4r often harboured RASs, which were less responsive and difficult to treat [488]. Consequently, more studies will need to be carried out to be able to have a more robust evidenced-based treatment decision. Furthermore, beyond SSA, a study in France showed that among the 537 who failed DAAs treatment between 2015-2018, 27 (22.3%) were subtype 4r [507]. This implies that GT 4 variants are spreading from sub-Saharan Africa to other parts of the world. If the WHO agenda in global eradication of HCV by the year 2030 is to be achieved, there should be a scale-up of accurate diagnosis, genotyping and resistance profiling of HCV irrespective of geographic area. All of this should be continuous, most especially in resource-limited regions such as SSA. In this chapter, we investigated the NS5A-NS5B Resistance-associated substitutions in HCV patients of SSA origin living in the UK. The NS5A and NS5B partial regions were successfully amplified, sequenced and RASs were analysed using the Geno2pheno software. These RASs were then compared with the known existing RASs.

5.2 RESULTS

5.2.1 NS5A and NS5B RAS Prevalence

Of the 135 samples that were amplified, sequenced, and subjected to Geno2pheno online tool for NS5B RASs analysis, only 13.3% (18) sequences carried NS5B RAS. Although, this could have been higher if the NS5B region amplified cover all the known RAS positions. Missing important sites e.g. 159 for SOF; 368, 414, 448, 663, 554, 556, 558, 559 for Dasabuvir. NS5B resistance mutations were present in 72% (13/18), 22% (4/18), and 6% (1/18) of GT 4r, GT 1b and unassigned GT 1 sequences respectively (Table 14). The NS5B RASs included S282R, V321I, C316N and C316H variants. Mutations C316N was observed in 25% (4/16) of sequence with genotype 1b and 7% (1/14) in sequence with unassigned genotype 1. Other mutations observed in subtype 4r sequences included S282R, V321I, and C316H, which were detected in 7%, 40%, and 40% respectively. Although mutation S282T which has been shown to cause a high level of resistance to sofosbuvir, was not detected in this cohort, these other mutations have been implicated with reduced susceptibility to sofosbuvir in some clinical trials [508, 509].

Table 14: The NS5B RASs detected in 18 of 135 studied sequences.

Patient Genotype	Number of Patients	Number of Resistance	Resistance Drugs	Resistance associated substitution		
				S282A/R/T (n ;%) n=2	V/C321I/N/H (n ;%) n=11	C316H/N (n ;%) n=14
GT1?	15	1	DSV	-	-	C316N(1;7)
GT1b	16	4	DSV	-	-	C316N(4;25)
GT4r	27	13	SOF	S282R(2;7)	V321I(11;40)	C316H(11;40)
Others	77	-		-	-	-
Total	135	18		2	11	16

Frequency and percentages of each RAS indicated in parenthesis. The drug resistance to the RAS are as follows: Sofosbuvir, SOF; Dasabuvir, DSV, Others are GTs 1e, 1g, 1l, 2? 2a,2c,3a,3h,4b,4c,4d,4f,4h,4k,4m,4t, and 5a.

In the case of NS5A RASs, 132/135 (98%) samples were successfully amplified, sequenced, and analysed. 42% (56/132) of samples harboured sequences containing resistance-associated substitutions (Table 15). There were considerable variations among the isolates in the distribution of resistance mutations. The codon 28 had the highest number of RASs (61%, 35/56) followed by codon: 30 (25%, 14/56), 31 (20%, 11/56), 93 (14%, 8/56), and 92 (2%, 1/56). An analysis of RASs distribution according to genotype/subtypes showed that 11 unassigned subtype GT1 carried L28M (5), M28T (1), Q30R (2), Q30S (1), L31M (4), Y93H (1) and Y93N (1) variants. Also, the GT1a sequence had one L31M variant.

Table 15: The NS5A RASs detected in 57 of 132 studied sequences.

Patient Genotype	Number Of patients	Number of Resistance (n=56;42%)	Resistance Drugs	Resistance associated substitution				
				M/L28/T/G V(n;%)	Q30/R/K E/H/L/Y/G T/D/I(n;%)	L31M/V /F/I(n;%)	A92K/T/ (n;%)	Y93H/N/ C/S/F/ L(n;%)
GT1	14	11	DCV,LDV, OMB	M(5;46), T(1;9)	R(2;20) S(1;10)	M(4;40)	-	H(1;10), N(1;10)
GT1a	13	1	-	-	-	M(1;50)	-	-
GT1b	16	6	-	M(1;17)	-	M(2;33)	T(1;17)	H(2;33)
GT1e	2	2	DCV,LDV, EBR	M(1;50)	R(1;50)	-	-	-
GT1g	1	1	-	-	Q(1;100)	-	-	-
GT1l	7	3	DCV,OMB	M(3;100)	R(3;100)	M(2;67)	--	-
GT3h	8	2	-	-	-	-	-	H(2;100)
GT4b	2	2	DCV,LDV, EBR	M(1;50)	S(1;50),	-	-	H(2;100)
GT4f	2	2	DCV,LDV, EBR	-	R(2;100)	-	-	-
GT4k	6	1	-	-	-	M(1;100)	-	-
GT4m	1	1	DCV,EBR, LDV	-	S(1;100)	-	-	-
GT4r	27	23	DCV,OMB	M(14;58), V(9;38)	S(1;4)	M(1;4)	-	-
GT4v	9	1	DCV,LDV	-	S(1;100)	-	-	-
Others	24	-						
Total	132	56		35	14	11	1	8

Frequency and percentages of each RAS indicated in parentheses. The drug resistance to the RAS are as follows: Daclatasvir, DCV; Elbasvir, EBR; Ledipasvir, LDV; Ombitasvir, OMB; Velpatasvir, VEL; Pibrentasvir, PIB, Others are GTs 2?, 2a, 2c, 3a, 4c, 4d, 4h, and 5a.

Out of 6 GT1b sequences, one had L28M, two L31M, one A92T, and two Y93H variants. GT1e sequences had one L28M, one Q30R variant while GT1g had one R30Q variant. For GT1l sequences, three had L28M, three Q30R and two had L31M. Likewise, two of the GT3h sequences had a Y93H variant. Furthermore, other RASs detected in GT4 subtypes included: L28M (n=1), L30S (n=1), and Y93H (n=2) for GT4b; L28M (n=14), L28V (n=9), Q30S (n=1) and L31M (n=1) for GT4r; Q30R (n=2) for GT4f; L31M (n=1) for GT4k; Q30S (n=1) for GT4m and Q30S (n=1) for GT4v. In contrast, sequences harbouring GTs 2, 2a, 2c, 3a, 4c, 4d, 4h and 5a carried

no RAS variants. Interestingly, some subtypes such as GTs 1?, 1b, 1e, 4b, and 4r carried multiple RASs comprising either NS5A or both NS5A and NS5B mutations.

5.2.2 The treatment outcome of 109 patients analyzed for RAS

The patients in this cohort were treated with either PEGylated interferon+Ribavirin±DAAs or PEGylated interferon+ Ribavirin. Out of 135 HCV, infected patients analyzed for RAS, 81% (109/135) of the patients had treatment outcome records. Forty-five percent responded to therapy, 33% did not respond while 20.2% responded, but relapsed after an initial response. The remaining 2% were either awaiting PCR results or were lost to follow up. (Figure 21). Furthermore, analysis of the 109 patients whose treatment outcome are available showed the presence of 41.3% (45/109) of RAS.

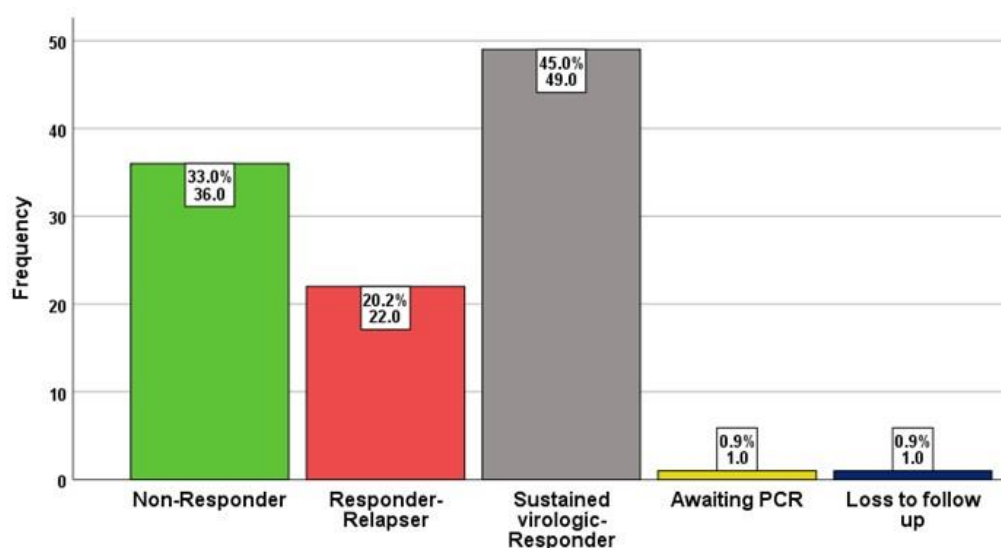


Figure 21: Frequency distribution of 109 Patients with Treatment outcome data.

The impact of genotype/subtypes on treatment was evaluated. There were considerable differences among genotypes/subtypes with regards to treatment outcomes. For the genotypes that did not respond to treatment, GT4t, GT4h had the highest rates of treatment failure at 100%, followed by GT4k (60.0%), GT4r (46.2%), GT1l (43.0%), GT1? (42.0%), GT1a (42.0%), and GT1b (29.0%). However, quite a number of genotypes/subtypes responded to treatment initially but later relapsed. These included the following: GT 2? (50.0%), GT 4v (29.0%), GT1l (29.0%), GT 1? (25.0%), GT4r (23.0%), and GT1b (21.0%). In contrast, isolates that achieved sustained virological response to treatment had genotypes GT4b (100%), GT4f (100%), GT5a (100%), GT2c (100%), GT1e (100%), GT1g (100%), GT3h (75.0%), GT4v (57.0%), GT3a (50.0%), and GT4k (40.0%) respectively.

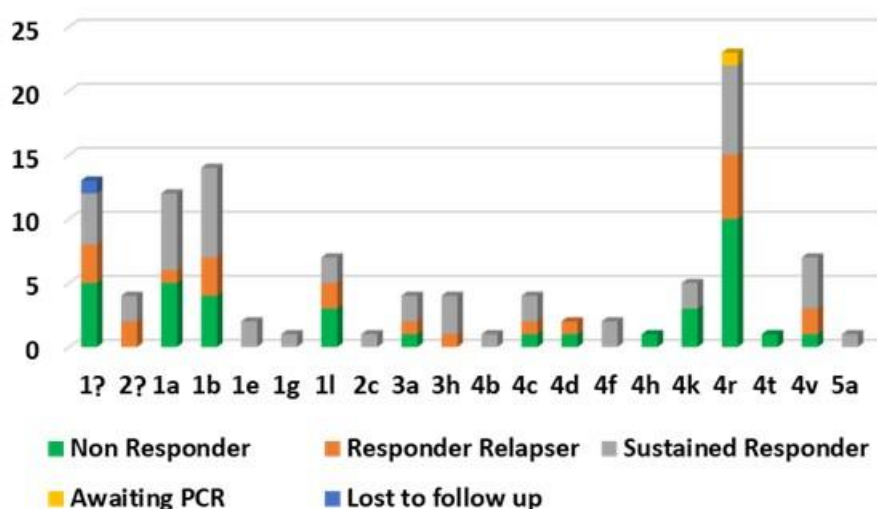


Figure 22: Impact of Genotypes/Subtypes on treatment outcome.

5.3 DISCUSSION

In this study, we used population Sanger sequencing to analyze NS5B (135) and NS5A (132) sequences from patients of Sub Saharan African origin to determine the prevalence of RASs. The success of any PCR amplification depends on viral loads of the samples, careful and proper selection of primers for the PCR assays, among other relevant factors [510, 511]. The initial PCR amplification of the NS5A segment yielded 48% (63/135) PCR products using pan-genotypic primers. The use of genotype-specific reverse primer sets with the pan-genotypic forward primer improved the success rate to 98% (132/135). Although NS3, NS5A, and NS5B inhibitors form the DAAs backbone commonly use in the treatment of HCV infection, studies have shown that most treatment failure is due to the presence of NS5A RAS [512, 513]. The major S282T mutation that is responsible for the high level of resistance to sofosbuvir is rare and was not found in this study [321]. However, variants (S282C/R) of this mutation do occur at varying frequencies in different populations. They reduce susceptibility to sofosbuvir and other NS5B inhibitors [514]. The variant S282R was observed in 7% (2/27) of subtype 4r sequences in our study. Though this variant seems relatively scarce, Ji *et al.* 2015, identified it in 9 out of 127 Canadian cohorts; a study in which they also provided modelling data to support S282R's potential association with resistance [509]. Furthermore, in the case of the C316N variant, 4 of the 16 sequences with genotype 1b and one of the 14 unassigned subtype genotype 1 had this variant. Variant C316N is relatively common among subtype 1b. In a study carried out by Alves *et*

et al., 2013, they reported a 36% (119/329) prevalence of this mutation in the sequence data from the Los Alamos database [515]. However, in some real-world studies, prevalence has been shown to be much higher; C316N prevalence was 43% in subtype 1b present in an Italian cohort [516], and 100% in a Chinese study [302]. Other clinically relevant NS5B RASs detected in genotype 4r infected patients were C316H and V321I at 40 % (11/27) prevalence each. A recent study conducted by Stalin Raj *et al.*, 2017 reported resistance to nucleotide analogues NS5B polymerase inhibitors by V321I RAS in 10 out of 18 treatment naïve patients and 16 patients with the C316N/Y/H variants were resistant to non-nucleoside inhibitors [517]. The prevalence of NS5B RAS could have been higher as NS5B region amplified does not cover all RAS positions missing important sites such as 159 for sofosbuvir; 368, 414, 448, 553, 554, 556, 558, 559 for Dasabuvir [518].

The NS5A inhibitors target and bind to the first domain of NS5A proteins impairing its phosphorylation and dimerization [519]. This effect impedes the replication complex formation, virion assembly, and release, which are critical to the production of infectious virion [520]. Unlike NS5B protein inhibitors, which have a high barrier to resistance [521], NS5A inhibitors exhibit a low to the medium barrier to resistance [343].

Studies have shown variation in the seven major amino acid positions associated with the failure of NS5A protease inhibitor therapy. The positions are 24, 28, 30, 31, 58, 92 and 93, however amino acid substitution conferring treatment failure depends greatly on HCV genotype/subtypes [331, 341]. The implication of this is that the clinical relevance of individual RASs depends on the type of HCV genotype/subtypes and the DAAs under

consideration [522]. In this study, L28M, L31M, and 93H frequencies were 17%, 33%, and 33%, respectively, in patients with genotype 1b infection. These mutations can occur naturally in treatment naïve individuals and can also be found in treatment-experienced patients with treatment failure [314, 523]. They confer varying levels of resistance to NS5A inhibitors. In a study involving the characterization of HCV GT1 resistance from a multi-dose clinical trial, L31M mutation was observed to confer >30 fold reduction in daclatasvir susceptibility in an *in vitro* analysis [524]. Furthermore, variants L28M and Y93H vary considerably in prevalence among genotype 1 infected patients, and the presence of single or coexistence of these variants has a negative implication on resistance profile. Two contemporary studies reported that 2% of GT1 HCV patient harbouring the mutation L28M had low-level resistance to daclatasvir/ledipasvir/ombitasvir [525], while another study demonstrated a high level of resistance to daclatasvir in the presence of Y93H mutant [526]. M28T, in the context of GT1a background, has been shown to confer >800 fold level of resistance to ombitasvir [527], and also high-level resistance to daclatasvir [526]. A recent study showed that this RAS confers resistance to pretty much all NS5A inhibitors except PIB in GT1a [528]. 38% M28V and 9% M28T were observed in GT4r and GT1? respectively. The M28V variant has been observed both in baseline NS5A RAS and treatment-emergent RAS conferring high level of resistance in GT3 and GT4 [332, 529]. Furthermore, in the case of variant Q30R that has been found in 20% of GT1?, 50% of GT1e, and 100% of GT1l, this variant has been reported to confer high-level resistance in GT1a *in vitro* to daclatasvir [530], and was also found to be selected by elbasvir treatment [531]. Also, Q30R mutation has been detected in 93.2% of treatment naïve

GT6a infected patients, but its impact in RASs has not been determined [302].

Similarly, variant R30Q was observed in only one patient harbouring GT1g; this variant was reported by Kliemann *et al.* 2016 with a prevalence of 5.95% in GT1b and 22% prevalence in GT1a alongside other NS5A variants (L28M, L31M, Q54H, A92T, and Y93H) [532]. However, the impact of this mutation in the context of GT1g has not been shown. A92T variant seems to be relatively scarce; in our study, its prevalence was 17% in GT1b sequences while its prevalence was 12% along with other NS5A variants in GT1b patients reported in 100 HCV mono-infected DAAs-naïve patients [330]. Other variants observed at position 93 in unassigned GT1 subtype sequences were Y93N and Y93H. These variants were present in 10% of GT1? sequences, while GT1b infected patients had a 33% prevalence of only the Y93H variant. Variant Y93N has been shown to confer a high level of resistance to daclatasvir in an *in vitro* (GT1b) replicon system [526], whereas Y93H variant was said to be a pan-genotypic variant that presents in almost all HCV genotypes and confers resistance to all NS5A inhibitors in GT1a,GT1b (except PIB) ,GT3 and GT4 [528]. Regarding NS5A specific RASs for genotype 4 HCV infection, limited data are available. Meanwhile, our data revealed the prevalence of 50% each of variants L28M, Q30S, and 100% of Y93H in GT4b; 100% of Q30S in GT4m, 58% of L28M, 38% of L28V in GT4r and 100% of Q30S in GT4v. Cuypers *et al.* 2015 evaluated the significance of drug resistance and genetic diversity of HCV using HCV sequenced deposited in the Los Alamos database and reported a 10.7% prevalence of both the L28M and Q30S variants associated with resistance to daclatasvir [533]. However, in a real-life cohort study conducted in Italy

on an HCV infected DAAs naïve patient (n=1445), 3.8% of GT4 patients harboured triple variants (28V+30S+93H) [534]. This variant was associated with a >100-fold decrease in sensitivity to elbasvir. In another study, a 4 fold loss to elbasvir NS5A inhibitor in the presence of the L30S variant from Replicon bearing GT4 clinical isolates was reported [535].

Overall, the outcome of therapy in the 109 cohorts was in line with both NS5A and NS5B RASs observed. Most of the treatment failures were experienced by patients infected with HCV GTs 1 and 4, more importantly, GT4r and GT1l subtypes. Some of these patients harboured two or more RASs, which can be predicted to confer increased resistance to DAAs. Specifically, a recent study reported two of four genotypes 4r infected patients harbouring 3 NS5A RASs (L28M+L30R+L31M) and 1 NS5B RAS (V321I); did not achieve SVR at 12 weeks of treatment with LDV/SOF [536]. Furthermore, the presence of multiple NS5A RASs or co-existence of NS5A and NS5B RASs associated with virologic failure to SOF/LDV have been well documented [537, 538].

5.4 CONCLUSION

This study has shown a high prevalence of both NS5A (43%) and NS5B (13%) RASs in this cohort of 135 SSA patients. Since there is increasing evidence that subtypes harbouring RAS have the potential of spreading from one geographic region to the other, effort should be made to focus more attention in SSA towards accurate diagnosis, treatment, and prevention.

6 CHAPTER SIX: The use of dried serum spot for genotyping and NS5B RAS analysis in HCV infected patients in Sao Paolo, Brazil.

6.1 INTRODUCTION

In Brazil, an estimated 1.5% of the general population is infected with HCV, and the Northeast region had the highest prevalence [539]. The predominant mode of HCV transmission is intravenous drug use [540]. However, other sources related to blood exposure, such as blood donation, tattooing, cuticle scissors, and crack cocaine have been implicated as vehicles of dissemination [541-543]. Genotype distribution is consistent across most studies with genotype 1 having the highest prevalence, followed by genotypes 3 [544-546].

Genotyping of HCV using direct blood/serum/plasma samples in resource-poor countries continues to be a huge problem most especially when the procedure cannot be carried out immediately following specimen collection and if the facilities for carrying out tests and analyses are not readily available at the sample collection sites. In most cases, it requires storage, mailing/shipping specimens to distance testing sites (laboratories). These specimens need refrigeration to prevent RNA degradation [547]. DBS technology has played a significant role in circumventing this problem. The alternative use of DBS for blood /plasma collection first started after 1960, when Dr. Robert Guthrie used it to screen phenylketonuria in newborns [548]. Since then, DBS has been popular in screening most blood-borne pathogens such as HIV, HBV, and HCV [549, 550]. DBS is a bio sampling technique where whole blood samples are blotted and dried on the filter paper while dried plasma spot (DPS) indicates blotted plasmas on the filter

paper. Similarly, dried serum spot (DSS) depicts serums blotted and air-dried on filter paper. There has been a good correlation in the titres obtained in these techniques with the conventional plasma samples when used in some RNA viruses (HAV and HIV) detection and quantification [551-553]. In most industrialized countries, DBS has been put to use at the point of care, community screening of HCV, detection of HCV antibody, RNA detection, viral load assessment, and genotype determination [554, 555]. Some of the key advantages are: non-invasiveness, requires no phlebotomist, and causes little or no discomfort following blood collection from the fingertips [556]. Studies have shown that it is useful and relevant for screening high-risk patients such as IVDU that have collapsed veins [557]. The essential usefulness of DBS is its easy transportation, relatively stable with regards to RNA degradation at room temperature, and it does not require a cold chain to be transported to the point of testing [558]. Studies revealed a good correlation between HCV RNA detection in the plasma and DBS using various modified commercial assays [559].

Consequently, HCV RNA recovery is as good in DBS and comparable with the blood/plasma. Also, inter-assay and intra-assay variability, different storage temperature, and length of the period of storage on the stability of HCV RNA in DBS have been evaluated, and no significant variations detected [560]. Furthermore, there is evidence that HCV RNA in DBS can be detected at levels >1000 IU/mL and the limit of detection is between 178 to 1779 IU/mL [561]; Although this is higher than the detection limit of 45 IU/mL for plasma [29]. Nevertheless, it has been a valuable tool in the surveillance, diagnosis, treatment, and monitoring of HCV infection [562-564]. Different methods have been reported in the literature for the

recovery and elution of HCV RNA from DBS [565, 566]. However, there is no standard protocol for the HCV RNA extraction procedure from the DBS specimen [567, 568]. There are fewer studies reported with the use of DSS for molecular studies of HCV [549]. In this study, we chose Brazil as one of the LMICs, where facilities for genotyping and resistance testing are limited. Hopefully, this method can be replicated in other LMICs, for example, sub-Saharan Africa. We used DBS Cards to collect serum samples from 80 Brazilian HCV-infected patients and carried out a molecular study in the UK. We estimated the duration of processing time, including the analysis of samples. The HCV RNA extracted from these samples were sequenced and genotyped using our optimized in-house RNA elution/extraction protocol here in the UK.

6.2 RESULTS.

6.2.1 Epidemiological Characteristics of the Study Subjects

In this study, we amplified and sequenced 66(83%) PCR products from eighty DSS obtained from the participants. The un-amplifiable samples might be due to poor handling of the sample in the collection sites since all the samples were of high viral loads. As shown in figure 23, there is no difference in the HCV RNA viral load between the PCR positive and PCR negative patients ($p=0.630$, Mann Whitney test=424, $N_1=66$, $N_2=14$, two-tailed) The viral loads varied from 3.04×10^4 and 5.39×10^7 IU/mL, with 1.5% <60,000IU/mL, 1.5% 60,000-80,000IU/mL and 95.5% >80,000IU/mL. The median viral load of the patient was 718,631IU/mL. The 66 Brazilian patients were recruited from six cities consisting of six specialized centres (SAE SJRP-Specialized service, CADIP-Infectious and

Parasitic Infectious Diseases Centre, AMHV-Municipal Outpatient Clinic of Viral Hepatitis, HEC-Emilio Carlos Hospital, SAE-VOTU-Specialized Service and SANTA FE-Specialized Service) in Sao Paulo, Brazil. The samples obtained varied considerably from one centre to another. Thirteen of 66 (19.7%) blood samples were collected from Catandura, 11/66 (16.7%) from Fernandopolis, 9/66 (13.6%) from Jales Sae, 20/66 (30.3%) from Sao Jose Do Rio Presto, 11/66 (16.7%) from Voltuporanga, and 2/66 (3.0%) from Santa Fe do Sul. (Figure 24).

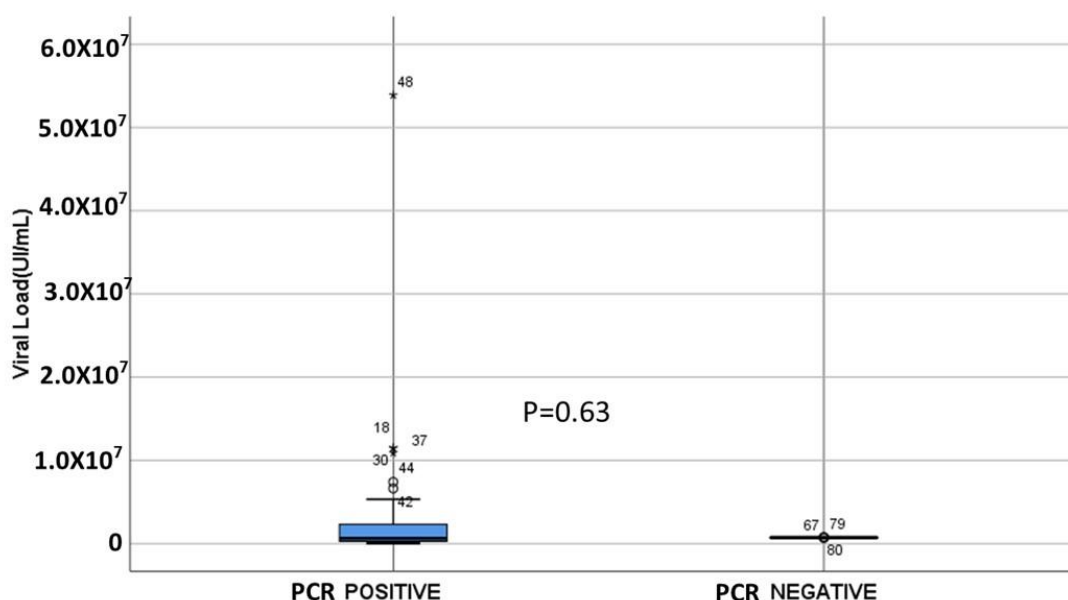


Figure 23: RNA viral load in PCR positive and PCR-negative Brazilian HCV patients; displaying the impact of viral load on PCR outcome.

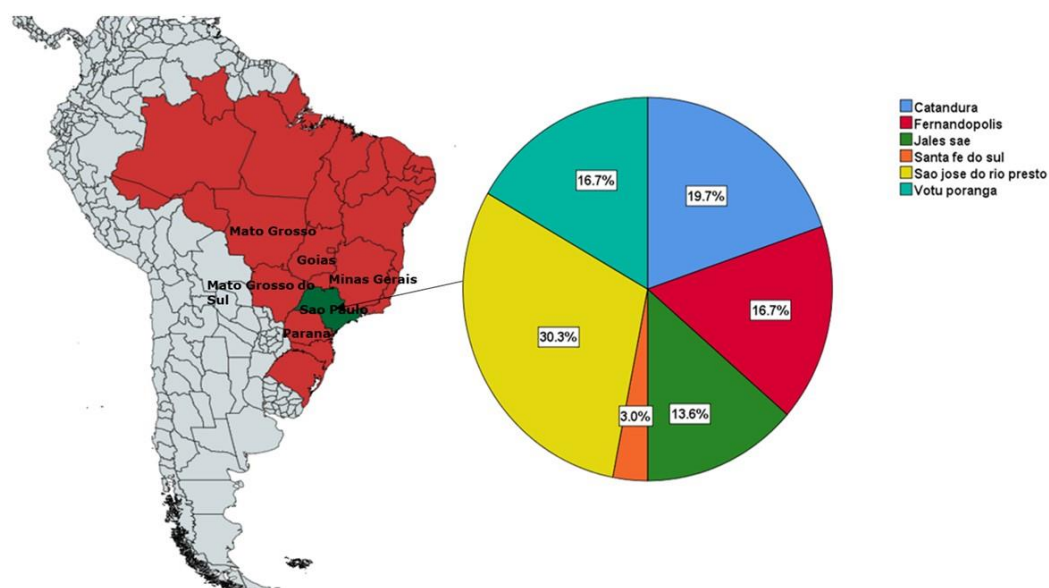


Figure 24: Frequency distribution of DSS among the six cities sample collection sites.

Thirty-five (53%) of the participants were males, 31 (47%) were females, and the ages ranged between 30 and 80 years. Ten tested positive for HIV infection, while 2 were HIV/HBV co-infected. Thirteen (20%) had an infection through the parenteral route, 8 (12%) had a history of blood transfusion, 7 (11%) a history of sexual contact, 9 (13%) implicated in intravenous/inhaling drug use, 4 (6%) surgical procedures and 4 (6%) traced the infection to cutting materials/ tattooing whilst 21 (32%) had no identified risk factor. See table 16 for further demographic details.

Table 16: Demographic Characteristics of the study samples

Variable	Categories	Number (%) [*]
Gender	Male	35(53)
	Female	31(47)
Suspected route of infection	Blood Product	8(12)
	Intravenous drug use/inhalation	9(14)
	Sexual	7(11)
	Parenteral	13(20)
	Surgical Procedure	4(6)
	Tattooing/cutting materials	4(6)
	Unknown	21(32)
Viral Loads IU/mL	<60X10 ³	1(1.5)
	60X10 ³ -80X10 ³	2(3.0)
	>80X10 ³	63(95.5)
Co-infection	HIV	10(15.2)
	HIV/HBV	2(3.0)
	No-coinfection	48(72.7)
	Unknown	6(9.1)

^{*}Frequency precede the percentage distribution in parentheses.

6.2.2 Genotype Assignment and prevalence

The genotype prediction was based on the four online tools and validated with the phylogenetic analysis of the partial NS5B sequences from the isolates. Interestingly, there was 100% concordance in the genotypic assignment of all the isolates with the four online genotyping tools. Similarly, these samples have high sequence similarity ($\geq 91\%$) to the reference strains used in each of the tools (Table 17). Also, there was no discrepancy in the phylogenetic analysis of all the 66 sequences when evaluated with confirmed full-length reference sequences from the GeneBank and LANL. The sample sequences were supported by high

bootstrap values ($\geq 85\%$). The phylogenetic trees of subtypes were constructed separately to assist visualization (Figure 25).

Table 17: Four online tools and phylogenetic analysis used for the prediction of subtype assignment of the study samples

Patient id	Phylogenetic Genotype Prediction	LANL	CometHCV	VIPR DATABASE	GENO2PHENO
1	1b	1b(96%)	1b(100%)	1b(96%)	1b(94.95%)
2	3a	3a(95%)	3a(100%)	3a(95%)	3a(93.62%)
3	1b	1b(96%)	1b(100%)	1b(96%)	1b(95.12%)
4	1a	1a(98%)	1a(100%)	1a(98%)	1a(97.83%)
5	1a	1a(97%)	1a(100%)	1a(97%)	1a(96.27%)
6	3a	3a(95%)	3a(100%)	3a(95%)	3a(93.5%)
7	1a	1a(97%)	1a(100%)	1a(97%)	1a(96.53%)
8	1a	1a(97%)	1a(100%)	1a(97%)	1a(96.47%)
9	1a	1a(97%)	1a(100%)	1a(96%)	1a(96.26%)
10	1a	1a(98%)	1a(100%)	1a(98%)	1a(97.29%)
11	1a	1a(98%)	1a(100%)	1a(97%)	1a(96.28%)
12	1a	1a(99%)	1a(100%)	1a(98%)	1a(97.87%)
13	1b	1b(97%)	1b(100%)	1b(97%)	1b(94.41%)
14	1b	1b(97%)	1b(100%)	1b(96%)	1b(94.68%)
15	3a	3a(96%)	3a(100%)	3a(97%)	3a(95.66%)
16	3a	3a(97%)	3a(100%)	3a(96%)	3a(94.93%)
17	3a	3a(95%)	3a(100%)	3a(95%)	3a(94.84%)
18	1a	1a(98%)	1a(100%)	1a(98%)	1a(96.8%)
19	3a	3a(95%)	3a(100%)	3a(96%)	3a(94.95%)
20	1a	1a(96%)	1a(100%)	1a(97%)	1a(95.93%)
21	1b	1b(97%)	1b(100%)	1b(96%)	1b(94.13%)
22	1a	1a(97%)	1a(100%)	1a(97%)	1a(96.53%)
23	1b	1b(97%)	1a(100%)	1b(97%)	1b(94.67%)
24	1a	1a(98%)	1a(100%)	1a(98%)	1a(97.01%)
25	1a	1a(99%)	1a(100%)	1a(98%)	1a(98.13%)
26	1b	1b(97%)	1b(100%)	1b(97%)	1b(94.93%)
27	1b	1b(98%)	1b(100%)	1b(97%)	1b(95.48%)
28	1a	1a(98%)	1a(100%)	1a(97%)	1a(96.81%)
29	1b	1b(97%)	1b(100%)	1b(96%)	1b(95.48%)
30	3a	3a(97%)	3a(100%)	3a(96%)	3a(95.2%)
31	1a	1a(98%)	1a(100%)	1a(97%)	1a(97.61%)
32	1a	1a(97%)	1a(100%)	1a(97%)	1a(95.48%)

33	1a	1a(98%)	1a(100%)	1a(97%)	1a(96.54%)
34	1a	1a(98%)	1a(100%)	1a(98%)	1a(96.81%)
35	1b	1b(95%)	1b(100%)	1b(96%)	1b(92.55%)
36	1b	1b(98%)	1b(100%)	1b(98%)	1b(94.68%)
37	1a	1a(97%)	1a(100%)	1a(96%)	1a(95.73%)
38	3a	3a(97%)	3a(100%)	3a(96%)	3a(95.48%)
39	3a	3a(97%)	3a(100%)	3a(97%)	3a(96.81%)
40	2c	2c(94%)	2c(100%)	2c(92%)	2c(91.64%)
41	1a	1a(97%)	1a(100%)	1a(96%)	1a(95.21%)
42	3a	3a(95%)	3a(100%)	3a(95%)	3a(93.88%)
43	2a	2a(99%)	2a(100%)	2a(98%)	2a(98.94%)
44	1a	1a(97%)	1a(100%)	1a(96%)	1a(96.53%)
45	1b	1b(95%)	1b(100%)	1b(96%)	1b(93.88%)
46	1a	1a(98%)	1a(100%)	1a(98%)	1a(96.8%)
47	3a	3a(96%)	3a(100%)	3a(96%)	3a(94.68%)
48	1a	1a(98%)	1a(100%)	1a(97%)	1a(96.28%)
49	1a	1a(98%)	1a(100%)	1a(97%)	1a(95.67%)
50	1b	1b(98%)	1b(100%)	1b(97%)	1b(94.12%)
51	1b	1b(97%)	1b(100%)	1b(96%)	1b(94.12%)
52	1b	1b(96%)	1b(100%)	1b(96%)	1b(92.26%)
53	1b	1b(95%)	1b(100%)	1b(95%)	1b(91.64%)
54	1a	1a(98%)	1a(100%)	1a(97%)	1a(97.52%)
55	1a	1a(98%)	1a(100%)	1a(98%)	1a(96.28%)
56	1a	1a(98%)	1a(100%)	1a(98%)	1a(97.21%)
57	1b	1b(96%)	1b(100%)	1b(96%)	1b(93.19%)
58	1a	1a(99%)	1a(100%)	1a(98%)	1a(96.59%)
59	1a	1a(97%)	1a(100%)	1a(96%)	1a(94.41%)
60	1a	1a(98%)	1a(100%)	1a(97%)	1a(95.67%)
61	1b	1b(98%)	1b(100%)	1b(97%)	1b(96.59%)
62	1a	1a(98%)	1a(100%)	1a(97%)	1a(95.05%)
63	1a	1a(97%)	1a(100%)	1a(96%)	1a(94.41%)
64	1a	1a(99%)	1a(100%)	1a(98%)	1a(96.46%)
65	3a	3a(98%)	3a(100%)	3a(97%)	3a(95.89%)
66	1a	1a(98%)	1a(100%)	1a(97%)	1a(96.28%)

Percentages following the subtype assignments correspond to the percentage hit with the previous reference genotype predictions by each subtyping tool.

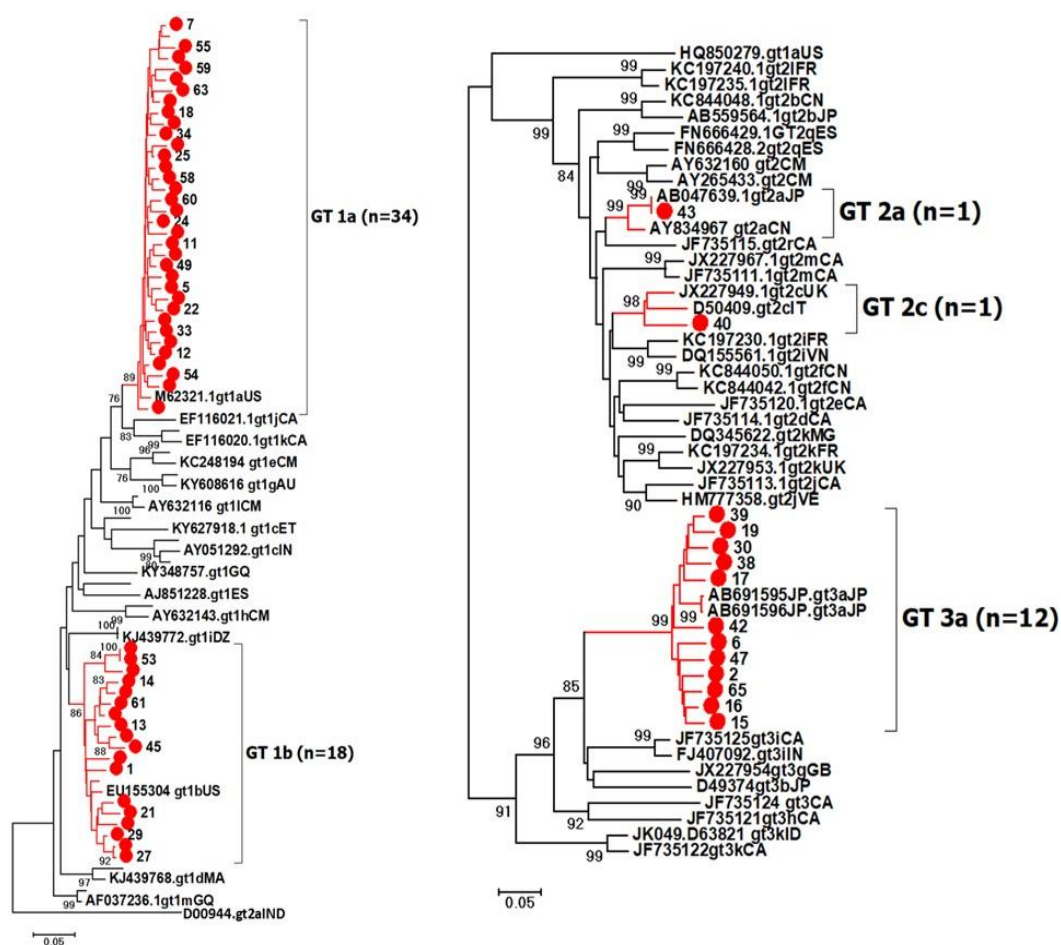


Figure 25: Phylogenetic tree of 66 NS5B sequences from HCV patients of Brazilian origin. Phylogenetic trees were constructed by the Neighbour-joining method. Subtypes 1a, 1b, and 2a, 2c, 3a were constructed on separate trees, respectively. Sample sequences were designated with red dots, while the references are indicated with their accession numbers, subtypes, and country of origin.

More importantly, all the sequences could be assigned at the subtype level. Subtype 1a predominated with 51.5% (34/66) frequency, followed by GT1b 27.3% (n=18); GT3a 18.2% (n=12); GT2a (1) and GT2c (1) 1.5% each (Figure 26).

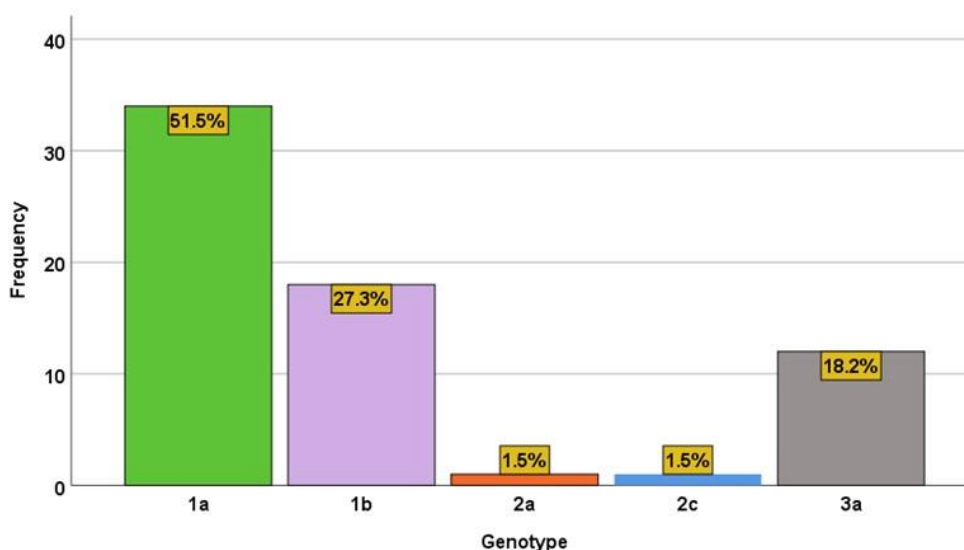


Figure 26: Subtypes distribution of the study samples.

Chi-square test showed that HCV subtypes were not significantly associated with gender ($p=0.757$) and suspected route of infection ($p=0.765$). However, there was a significant association between subtypes and age group ($p=0.042$). Subtype 1a has the highest prevalence of 41.2% (14/34) in the age group 40-49 years, followed by age group 50-59 years, with a 23.5% (8/34) prevalence. Subtype 1b was predominant (7/18 ; 38.9%) in the age group 60-69 years, while subtype 3a has 33.3% (4/12) and 25.0% (3/12) prevalence in the age group 40-49 years and 50-59 years, respectively (Figure 27).

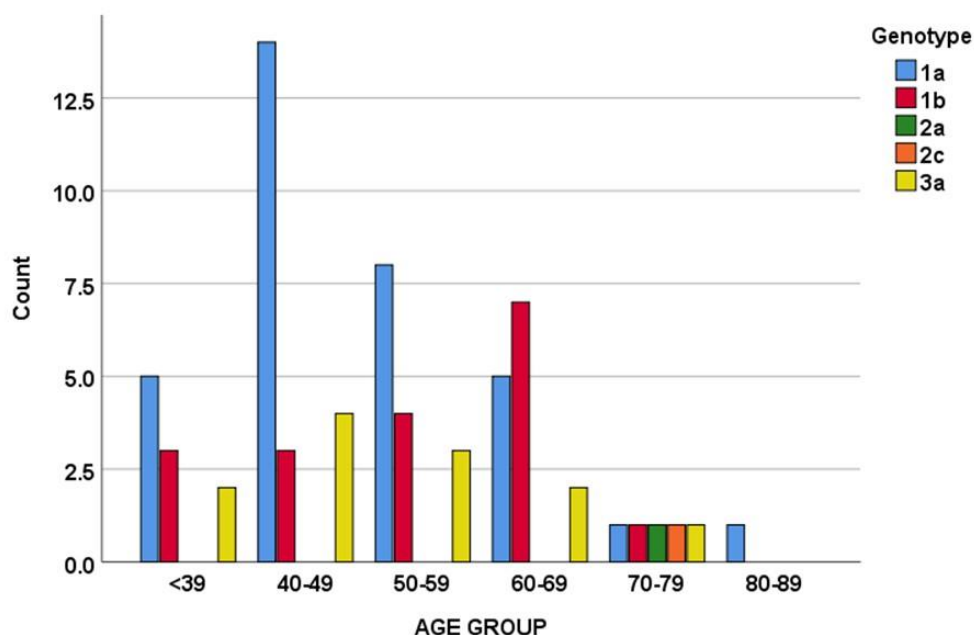


Figure 27: Distribution of HCV subtypes by age group.

6.2.3 Resistance association Prediction

Out of 66 samples successfully sequenced and analyzed with the geno2pheno RAS tool, only 8% (5/66), which belong to GT1b, were identified to harbour NS5B-specific RAS. Analysis of the 18 sequences (subtype 1b) showed 17% (3) and 11% (2) prevalence of C316N and V321I variants respectively. However, no RAS were detected in the other subtypes. (Table 18)

Table 18: Prevalence of RAS in the NS5B coding region of the study samples

Patient Genotype	Number of Patients	Number of resistance	Resistance Drugs	Resistance associated substitution		
				S282A/T(n;%)	C316N/H(n;%)	V321I/L(n;%)
1a	34	-	SOF	-	-	
1b	18	5		-	C316N(3/18;17)	V321I(2/18;11)
2a	1	-		-	-	
2c	1	-			-	
3a	12	-		-	-	
Total	66	5		-	3	2

NS5B gene of the study samples was evaluated for RAS by subjecting the sequences to the Geno2Pheno software tool.

6.3 DISCUSSION

DBS technique for HCV diagnosis and genotyping in industrialized countries, has led to improved diagnoses [569]. Presently, in the era of the high prevalence of RAS and associated treatment failure, the use of DBS seems logical as an alternative technique to proffer solutions to the problem associated with serum/plasma transportation for testing most especially in LMIC [570]. In this study, I recovered HCV RNA, amplified, sequenced, and performed in-silico phylogenetic analysis from six health facilities within one week of DSS samples being generated in various health centres in Brazil. The success rate of HCV RNA recovery and genotyping was 83% (66/80) from the DSS. Studies on genotyping using DSS are limited, and most studies on DBS are based on HCV antigen/antibody detection, HCV RNA detection and viral load determination using commercial assays [571]. Studies have shown a high concordance in terms of genotyping outcome between DBS and plasma [559, 572]. Previous studies have shown a good

correlation between results obtained from plasma and DBS samples using HCV core and NS5B sequence phylogenetic analysis [573]. Our result is similar to Ndiaye *et al.*, who reported an 84% success rate of HCV detection, and genotyping of intravenous drug users in Senegal using DBS [574]; two commercial assays (HCV genotype 2.0 assay, LIPA, and Abbott Real-time Genotype II assay) were used for genotyping. Similarly, Soulier *et al.* reported an 84.5% success rate genotyping using the NS5B region of 262 HCV samples collected with the DBS technique [568]. Higher success rates of up to 100% have also been reported [575].

Sao Paulo is one of the most populated cities in Brazil, with an estimated HCV prevalence of about 1.8% [576]. It contributes about 67% of the population, covering the south-eastern area where the samples were collected [577]. In Brazil, there is a wide variation in the frequencies of HCV genotypes/subtypes from one region to another [578]. One study carried out recently showed the highest prevalence of genotype 1, followed by genotype 3, while genotypes 2, 4, and 5 were in the minority [579]. In this study, genotype 1a had the highest proportion of 51.5%, followed by GT1b (27.3%), GT3a (18.2%), with a smaller frequency of 1.5% each for GT2a and GT2c. These data are similar to those reported by Romano *et al.* in 2010, using NS5B gene sequence analysis of HCV in samples from 591 Sao Paulo HCV infected patients. They reported subtype frequencies of 48.7%, 51.3%, 24.2%, 2.1% and 1.9% among genotypes 1a, 1b, 3a, 2b and 2c respectively [429]. Similarly, a study recently carried out by de Souza Guimare in 2018, supported the predominance of genotypes 1 and 3 (GT1a, 19.2%; GT1b, 34.6%; GT2, 11.5%; gt3, 34.6%) in south-eastern Brazil [580]. In contrast, Zarife *et al.*, reported a predominance of GT3 in

Salvador, North-eastern Brazil, in about 1308 serum collected from 30 sentinel areas [581]. The variation in the HCV subtypes among the six sample collection centre was not significant ($p=0.151$). Risk factors for HCV acquisition in our study are similar to the previous studies. Parenteral transmission of HCV in this study was 19.7% compared with the previous studies, which found 12% [582], and 25% [583] transmission rates. Furthermore, the proportion of intravenous drug use/inhalation in our study was 13% which is slightly lower compared with the study carried out by Nishiya *et al.* 2015 (17.5%) in the same region (Sao Paolo) [584]. The high frequency may be connected with increased consumption of illicit drugs and socio-behavioural pattern, which is becoming more and more popular [585]. However, the use of non-disposable syringes for the administration of vitamins among athletes has been identified as a cause of the increase in parenteral route transmission [586]. The risk of HCV transmission via blood (12.1%) in our study was relatively high compared with one previous study [587]. However, it seems to agree with a 16.7% prevalence of the study in multi-transfused patients in Brazil [588]. Such a high frequency is not expected since HCV screening of blood donors using various anti-HCV antibodies assays, and nucleic acid testing has reduced the transmission rate via transfusion [589, 590]. Also, a sexual transmission which is an inefficient route of transmission of HCV in the general population has a high frequency of 11% in our study. The high proportion of sexual transmitted HCV has been adduced to the presence of mucosal trauma, ulcerative genital diseases [591], and the practice of MSM [592, 593].

There is a high frequency of subtypes 1a and 3a in the age group 40-59 years, which is similar to the previous study and corroborates the

relationship between late detection and the asymptomatic nature of HCV infection [594]. Also subtypes 1a and 3a were more transmitted via intravenous drug use and parenteral than by any other route, and this is in agreement with a history of HCV transmission in Brazil [595], and European countries [596]. The rate of HCV co-infection (either coinfection with HIV or HIV/HBV co-infection) in this study is 18.2% (12/66). This result was consistent with the report of a previous study in Brazil in which the frequency ranged between 3.3% in serum samples to 82.4% in patients injecting drugs [597]. However, no significant association between coinfection and route of infection was found ($p=0.226$).

The overall prevalence of RASs in this study was 7.6% (5/66), and this was restricted to GT1b. There is limited data on RASs among Brazilian HCV patients. A recent study conducted by Tavares *et al.* in 2018 in Sao Paulo reported the prevalence of NS5B RASs to be 8.4% with more RASs detected in HCV GT1a (42.9%) than GT1b (32.4%) [598]. This was in contrast with our study, where the only RASs detected were in GT1b with an 8% frequency. Regarding specific NS5B RASs, a 17% rate of C316N was detected and was in agreement with 7.1% -16.3% reported by Noble *et al.* 2017 [599]. In another study reported by Peres da Silva in Brazil, 3.85 % of the HCV infected treatment naïve patients had a single C316N variant, while 25% presented with the double L159F-C316N variants [600]. NS5B C316N variant has been detected in HCV patients that relapsed following LDV/SOF therapy in real-world practice [601]. Furthermore, the NS5B V321I variant that was detected in 11% of patients in our study appears to be a rare NS5B variant. However, 2.2% of GT1b patients harbouring this RASs among the HCV infected patients from the Netherlands were reported

to potentially associated with reduced response to sofosbuvir along with other NS5B variants [517]. Moreover, its role in viral replication and drug resistance has been established and described [602].

6.4 CONCLUSION

In this study, we genotyped and performed NS5B RASs analyses within one week of sample collection from Brazil. More importantly, HCV strains were identified to subtype levels. Thus, the DSS technique is a valuable tool in genotyping and can be used for treatment and follow up of HCV patients under therapy. It has promise for hard-to-reach areas in the world and can be of significant value in surveillance and epidemiological studies of HCV infection in low and medium resource countries. Moreover, DSS will be useful for the accomplishment of the WHO objective of the global eradication of HCV by the year 2030.

7 CHAPTER SEVEN:

7.1 FINAL DISCUSSION AND FUTURE PERSPECTIVES/DIRECTION

WHO has the responsibility for HCV infection surveillance, prevention, control and has put a target of its eradication in 2030. The SVR of the DAAs in use today have shown efficacy and proved that HCV infection total cure is very near. However, the high cost of DAAs is prohibitory even in high-income countries, so more for LMICs [603, 604]. The cost of treatment of HCV infection depends on the type of DAAs, genotype/subtypes, treatment history, presence or absence of liver damage, duration of therapy, and country of treatment [605-607]. For instance, Harvoni costs about \$48,000 and \$12,000 for a 12-week course in Malaysia and Chile, respectively, while the cost in the United States is \$94,500 [608]. In the case of vosevi therapy in the United Kingdom, the cost of treatment for the 8-week course is £29,884, and that of 12-week is £44,826 [609]. Although the NHS usually pays less than this cost because they give a confidential discount. Shortened treatment duration has been proposed to reduce the cost [610]. A study carried out on direct-acting-antiviral treatment in GT1 non-cirrhotic treatment naïve chronic HCV patient using a probabilistic decision tree and a Markov model. Three unstratified shortened treatment durations (4, 6 and 8 weeks) were compared against the standard 12-week treatment and the study showed that 8 (56% probability of cost-effective than the 6 weeks, which showed 25%) week treatment duration had an expected incremental net monetary benefit of £7737 as against the standard 12-week treatment per 1000 patients. Nonetheless, the use of generic drugs is much more

cost-effective; and if this can enjoy a full range coverage, it will go a long way in effective treatment compliance [605].

The HCV genome is genetically diverse [611], and this diversity has a significant impact on the diagnostic and genotyping assays since most of these assays were designed based on HCV sequences detected in industrialized countries [224]. Also, treatment choices have been primarily based on clinical trials performed in these Western countries. However, there are increasing reports from some HCV subtypes considered rare (based on studies performed in Western countries) that is, in fact, endemic in parts of Africa. Importantly some of these endemic subtypes have also been shown to harbour RAS [488, 536]. In this study, we identified 339 HCV patients of African origin living in the United Kingdom from HCV Research UK cohorts of more than 11,800 patients. Within this cohort, 135 patients were identified as being of SSA origin, HCV RNA positive, and whose main risk factor for HCV acquisition was being born abroad [612]. Through amplification, sequencing, and phylogenetic or online- analysis of a fragment of the NS5B gene, I was able to assess the HCV genotype and subtype distribution in individuals of sub-Saharan African origin living in the United Kingdom. One of the significant outcomes of this analysis was the realization that commercial assays routinely used within the UK to assign subtype were performing poorly compared to this NS5B phylogenetic or online-tool-based gold-standard. In the diagnostic setting, phylogeny would be the best, but in reality, an online tool would be easier, and HCVGLUE (98%) and LANL (95%) performed the best. It implies that these genotyping tools are suitable for predicting genotype/subtype assignment in a clinical specimen obtained from patients of African origin. Interestingly,

in Brazilian cohorts, all the four online tools have 100% concordance with the phylogenetic analysis; indicating that any of these genotyping tools is suitable for HCV patients of Brazilian origin. In contrast, the commercial assay performed poorly and this seem to be biased to Western HCV infection where HCV diversity is not as strong as HCV sequences found in Africa.

Also, we equally used population Sanger sequencing to determine the prevalence of NS5A and NS5B RAS. Initially, the pan-genotype primer set for NS5A hit 50% rate; however, with the use of genotype-specific reverse primer sets, this was increased to 100% as in the case of NS5B amplification. Overall, 13.3% (18/135) of NS5B RAS and 42% (56/132) of NS5A RAS were detected in this study. Subtype 4r has the highest frequency of NS5A RAS (85.2%, 23/27) and NS5B RAS (48%, 13/27), respectively. However, the analysis of NS5B RAS, which is restricted to 338bp fragment did not cover the mostly described NS5B RAS has mentioned in chapter five. Also, amplification and sequence analysis of NS3 fragment covering the first 237 codons with Q80K mutation known to confer resistance to Simeprevir would have been a great opportunity if there were more time and resources. Another limitation of the sub-Saharan African study is the lack of data that reflects/suggests the precise source of HCV infection. Overall, while each gene gives some information on subtypes and RAS, both are needed to provide insight to inform appropriate treatment regimens.

Furthermore, the DSS success rate (83%, 66/80) in this study corroborate with other previous studies [557, 575], and this has proved efficient in HCV genotyping and RAS determination. On the final note, DSS techniques, HCV genotyping, RAS determination approach utilized in this study can be

established in LMICs such as SSA with high HCV burden and diversity. The challenges involved in clinical diagnosis and treatment of HCV in Africa is enormous. The sequelae and prognosis of chronic HCV infection generally cannot be over-emphasized. More studies on the genetic diversity of HCV in SSA needed to be carried out to understand better and get a more accurate insight of HCV in SSA. This information will have a more significant impact on WHO in formulating accurate and evidence-based policy in the treatment and eradication of HCV. Future work will be more on molecular epidemiological study and characterization of HCV in SSA. This will provide a more robust understanding of HCV and also help in attaining a global HCV eradication.

8 CHAPTER EIGHT: REFERENCES

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