UNIVERSITY OF LIVERPOOL

AN INVESTIGATION INTO THE GENETIC VARIATION OF HEPATITIS C VIRUS IN PATIENTS COINFECTED WITH HIV

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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ABSTRACT

Co-infection with HIV in patients with chronic HCV infection is a common occurrence, which accelerates disease progression and the rate of liver fibrosis. Current literature report conflicting results on the effect of HIV co-infection on HCV at the genetic level. This thesis sets out to further explore the genetic variation of HCV in this group of patients, and use new techniques which may impact upon the diagnosis and management of HCV.

In resource-limited settings such as sub-Saharan Africa, there is a paucity of data concerning rates of active HCV infection in patients with HIV infection. The limited infrastructure and financial restrictions are contributing factors in this. Consequently, cheaper and novel diagnostic procedures are required to promote testing in these important cohorts. A real-time PCR assay for use with pooled plasma and dried plasma spot specimens was developed to screen a large cohort of HIV-infected individuals from Ghana and assess its suitability for screening in such a setting. The prevalence of active HCV infection in this cohort was similar to previous estimates from blood donors but was much lower than estimates from serological tests, highlighting the potential risks in relying on these tests alone in this region. The diversity of strains found within this population agreed well with previous studies.

As the yield of HCV infections was low in Ghana, further studies were completed with the UK and European cohort. A deep sequencing approach was utilised to two effects. The first focussed on a specific notable polymorphism – Q80K in the NS3 gene – to determine whether deep sequencing would benefit the clinic in the detection of this polymorphism at low frequencies, which are below the threshold of detection by traditional population sequencing. The second use of deep sequencing aimed to determine the impact of HIV co-infection on the presence of resistance associated mutations occurring at baseline, studying the largest cohort to date.

The Q80K polymorphism was not observed in any sample as a minority variant, suggesting that the current clinical procedures for pre-treatment screening are suitable and provide sufficient sensitivity. Overall, resistance mutations were relatively common among patients and it was observed that, generally, there were no differences in the prevalences of resistance mutations between HCV mono-infected and HIV/HCV co-infected patients. This finding is in agreement with previous small-scale studies.

This work has direct clinical implications on the use of deep sequencing for screening patients for the presence of resistance mutations. Furthermore, it suggests that co-infected individuals are not at risk of further complications for the treatment of HCV.

DECLARATION

This thesis is the result of my own work. The material presented here has not been presented and is not being presented, either wholly or in part, for any other degree or qualification. Some of the technical procedures were carried out in collaboration with individuals at the institutions below or elsewhere and are indicated in the thesis.

The research work was carried out at the Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool

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ABBREVIATIONS

^{o}C	Degrees Centigrade
aa	Amino Acid
ALT	Alanine Aminotransferase
ART	Antiretroviral Therapy
AST	Aspartate Aminotransferase
bp	base pairs
cDNA	Complementary deoxyribonucleic acid
CGR	Centre for Genomics Research
CI	Confidence Interval
CoV	Coefficient of Variation
C_T	Cycle Threshold
DAA	Direct Acting Antiviral
DBS	Dried Blood Spot
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside Triphosphate
DPS	Dried Plasma Spot
FC	Fold-change
Gt	Genotype
HAART	Highly Active Antiretroviral Therapy
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus

IC	Internal Control
IQR	Interquartile Range
ISDR	Interferon-α Sensitivity Determining Region
IU	International Units
LMIC	Lower Middle Income Country
Mb	Megabase
min	Minutes
ml	Millilitre
NA	Not applicable/available
NANB	Non A, Non B
NAT	Nucleic Acid Amplification Technology
ng	nanograms
NGS	Next Generation Sequencing
nM	Nanomolar
NT	Not Tested
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDV	Phocine Distemper Virus
peg-IFN	pegylated-Interferon
PI	Protease Inhibitor
P/R	pegylated-Interferon/Ribavirin
PTH	Post Transfusion Hepatitis
RAM	Resistance Associated Mutation
RBV	Ribavirin

RdRp	RNA-dependent RNA Polymerase
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
s	Seconds
SD	Standard Deviation
SSA	sub-Saharan Africa
SVR	Sustained Virological Response
UK	United Kingdom
USA	United States of America
WHO	World Health Organisation
µl	Microlitre
µM	Micromolar

PUBLICATIONS ARISING FROM WORK PRESENTED IN THIS THESIS

King, S., Adjei-Asante, K., Appiah, L., Adinku, D., Beloukas, A., Atkins, M., Sarfo, S. F., Chadwick, D., Phillips, R. O. & Geretti, A. M. 2015. Antibody screening tests variably overestimate the prevalence of hepatitis C virus infection among HIV-infected adults in Ghana. *J Viral Hepat*, 22(5), 461-8.

Beloukas, A.*, King, S.*, Childs, K., Papadimitropoulos, A., Hopkins, M., Atkins, M., Agarwal, K., Nelson, M. & Geretti, A. M. 2015. Detection of the NS3 Q80K polymorphism by Sanger and deep sequencing in hepatitis C virus genotype 1a strains in the UK. *Clin Microbiol Infect*, 21(11), 1033-9.

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Chapter One

General Introduction

1. General Introduction

1.1. HCV

During the 1970s, it was observed that a non-A, non-B (NANB) hepatitis agent in contaminated blood supplies was responsible for cases of post-transfusion hepatitis (PTH), which continued to occur even after screening tests for hepatitis B surface antigen (HBsAg) became available (Feinstone et al., 1975). A NANB hepatitis agent was deemed to be responsible for such PTH cases after the exclusion of other infectious agents including hepatitis A virus, hepatitis B virus (HBV), Epstein-Barr virus and cytomegalovirus.

In 1989, the NANB hepatitis agent was successfully cloned and defined as hepatitis C virus (HCV) (Choo et al., 1989). HCV is a positive-sense, single stranded RNA virus belonging to the *Flaviviridae* family. Transmission routes include contact with infected blood or blood products, such as transfusion with contaminated blood and the sharing of needles among injecting drug users (IDUs). Current estimates place the number of HCV infections at approximately 130-150 million people worldwide, with an estimated 500,000 HCV-related mortalities annually (World Health Organisation, 2015).

1.2. HCV structure

Despite the recent advances in the development of a replicon system to culture HCV *in vitro*, the structure of the virion has not yet been fully elucidated. Virus particles are approximately 40-100nm in diameter (Catanese et al., 2013). The single-stranded RNA genome interacts with the core protein to form a nucleocapsid, which is surrounded by the lipid membrane into which the envelope glycoproteins are

anchored (Lindenbach, 2013). Two models have been proposed regarding the interaction of lipoproteins with the virus particles; the hybrid particle model and the two particle model. The hybrid model suggests that the particle comprises both a lipoprotein and a viral moiety (Andre et al., 2002). Alternatively, the two particle model proposes serum lipoproteins attach to virus particles through interactions with apolipoproteins and lipids in the virus particle envelope (Thomssen et al., 1992, Lindenbach, 2013).

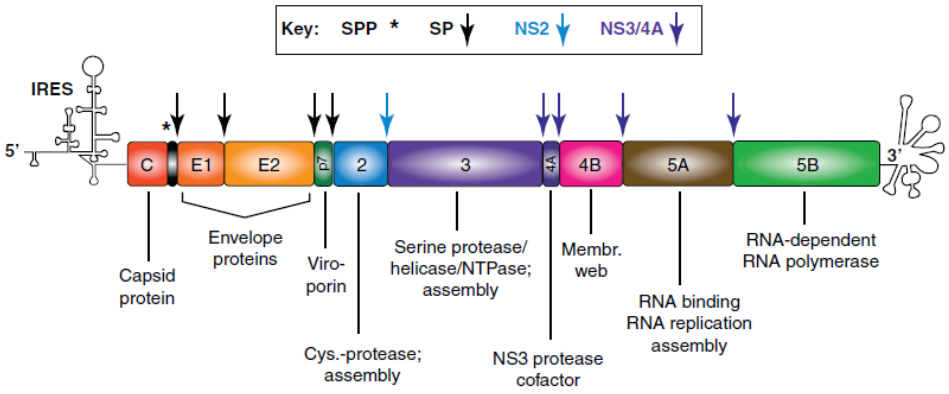

Figure 1.1. Taken from (Bartenschlager et al., 2011) Structure of the HCV genome and functions of the viral proteins. Proteases involved in the processing of the polyprotein are shown at the top and cleavage sites are indicated by the arrows.

1.3. HCV Genome

Through use of overlapping cDNA clones, the HCV genome was determined to consist of one continuous open reading frame (ORF) encoding a polyprotein 3011 amino acids in length (Choo et al., 1991). The single stranded RNA genome encodes a 5' non-coding region, the ORF encoding structural and non-structural proteins and a 3' non-coding region (Moradpour et al., 2007). The ORF is enzymatically cleaved

post-translationally to produce 10 proteins comprised of the structural (core, E1 and E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (Figure 1.1).

The core protein, encoded at the amino-terminal end of the polyprotein, encodes two domains responsible for promoting the formation of the nucleocapsid and interacting with lipid droplets (Boulant et al., 2005, Boulant et al., 2006). The core protein not only plays a structural role, but also interacts with cellular proteins to influence processes such as lipid metabolism and apoptosis (McLauchlan, 2000).

The E1 and E2 genes encode the envelope glycoproteins of the virion. The two proteins are expressed on the surface of the virus and are essential for binding to various receptors on the surface of the host cell, facilitating cell entry. The E2 gene encodes both hypervariable region (HVR) 1 and HVR 2; two of the most variable regions of the entire genome (Hijikata et al., 1991a). HVR1 is a target of neutralising antibodies and the heterogeneity found in this region allows the virus to evade the response of the immune system (Kato et al., 1993, van Doorn et al., 1995).

The seven remaining non-structural proteins are all involved in the replication of the genome and assembly of new virions, either through enzymatic activity or other roles.

P7 forms a transmembrane protein which plays a role in ion transfer and is also considered to be a viroporin; a protein involved in virus particle assembly and release from the host cell (Griffin et al., 2003).

The NS2 transmembrane protein inserts into the membrane of the ER. The C-terminal of the protein contains a cytosolic domain with cysteine protease activity which cleaves the NS2/3 junction (Grakoui et al., 1993b). *In vitro*, the NS2 protein

has also been shown to inhibit cell proliferation and arrest the cell cycle during S-phase in HeLa and Vero cells (Yang et al., 2006).

The NS3 protein is a multifunctional protein, encoding both a serine protease at the N-terminal and NTPase/RNA helicase at the C-terminal (Han et al., 1995, Kim et al., 1995). The RNA helicase of the C-terminus is involved in unwinding double stranded RNA in short sections at regular spacing, enabling the replication of the genome (Serebrov and Pyle, 2004). NS4A is a cofactor of the serine protease, which is responsible for the cleavage of the junctions between NS3/4A, 4A/4B, 4B/5A and 5A/5B (Bartenschlager et al., 1994). The NS3-4A serine protease has also been shown to cleave Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), thereby inhibiting the activation of NF-κB and IRF-3 and promoting the evasion of the innate immune system by the virus (Li et al., 2005). Three residues form the catalytic triad responsible for the protease activity; His-1083, Asp-1107 and Ser-1165 (Grakoui et al., 1993a). The interaction of NS3 with NS4A enables the activation of the active site of the protease and enhances the efficiency of cleavage (Kim et al., 1996).

The N-terminus of NS4A is involved in targeting NS3 to the membrane of the ER and also contains a transmembrane helix responsible for anchoring the complex to the ER (Wolk et al., 2000). In addition to its interactions with NS3, NS4A interacts with a region in the centre of NS5A to aid in the phosphorylation of NS5A (Kaneko et al., 1994).

NS4B is an integral membrane protein, which co-localises with the other non-structural proteins in the ER membrane (Hugle et al., 2001). It generates a membranous web by inducing changes in the ER, which serves the vital purpose of a

scaffold for the HCV replication complex as well as RNA binding activity important for the replication of the virus (Egger et al., 2002).

The NS5A protein comprises three domains and, although no enzymatic activity of the protein has been observed, it is vital for the replication of the virus. It is involved in the binding of RNA for replication (Huang et al., 2005) and is capable of down-regulating the translation of viral RNA, through interactions between amino acids and RNA (Hoffman et al., 2015a, Hoffman et al., 2015b). The protein is also responsible for binding to host Cyclophilin A (CypA), which is involved in the subcellular distribution of NS5A (Dujardin et al., 2015). Domain III of the protein is necessary for the assembly of virions (Appel et al., 2008). Alongside roles in replication and assembly, the protein also contains the interferon-α sensitivity-determining region (ISDR). This region of NS5A binds to the double-stranded RNA-activated protein kinase (PKR), the product of an interferon-α stimulated gene (ISG). PKR is an important cellular antiviral protein which inhibits the synthesis of proteins by activating eukaryotic initiation factor-2α (Gale et al., 1997, Gale et al., 1998). Through binding to PKR, ISDR inhibits its antiviral activity (Gale et al., 1997, Gale et al., 1998).

The NS5B protein encodes the RNA-dependent RNA polymerase (RdRP), essential for replication of the genome. The RdRP has a typical ‘right hand’ polymerase structure with thumb and finger domains enclosing the active site of the palm domain (Lesburg et al., 1999) and creating a channel for binding to a single stranded RNA template for translation (Penin et al., 2004).

Proteins which are vital to the replication of the virus make attractive therapeutic targets and the knowledge about the HCV genome has aided the development of

specifically targeted antiviral therapy for HCV (STAT-C) drugs, including protease and polymerase inhibitors (Manns et al., 2007).

1.4. Replication cycle of HCV

1.4.1. Cell entry

The entry of the virion into the host cell is a complex multistep process involving interactions between the viral envelope glycoproteins, E1 and E2, and numerous receptors on the surface of the target cell. Entry of the HCV virion is dependent on at least four cellular proteins, including CD81 (Pileri et al., 1998), scavenger receptor class B type I (SRB1) (Scarselli et al., 2002), claudin-1 (CLDN1) (Evans et al., 2007) and occludin (OCLN) (Ploss et al., 2009). SRB1 interacts with the E2 glycoprotein and lipoproteins mediating a post-binding event through increased exposure of the CD81 binding site on E2 (Scarselli et al., 2002, Dao Thi et al., 2012). Interaction of CD81 with E2 may prime the envelope glycoproteins for activation by the low pH experienced during entry (Sharma et al., 2011). This interaction also activates RHO GTPases allowing the lateral movement of HCV-CD81 complexes to cell-cell junctions and interaction of CD81 with CLDN1 (Brazzoli et al., 2008). The precise role of OCLN, a tight junction protein, in the process is currently unknown although it has been shown to be necessary in the late stages of virus entry (Benedicto et al., 2009, Sourisseau et al., 2013).

Endocytosis of the HCV particle into the host cell is mediated through a clathrin-dependent process (Blanchard et al., 2006) and fusion of the endocytosed virus takes place in early endosomes (Meertens et al., 2006, Coller et al., 2009). The multifactorial entry of HCV in the host cell is also influenced by the presence of host

restriction factors that protect cells from infection with virions. The restriction factor EW1-2wint blocks the vital interaction between CD81 and the envelope glycoproteins of HCV and may also block essential signalling pathways (Potel et al., 2013).

1.4.2. RNA translation and replication

Upon internalisation into the cytoplasm, the positive sense RNA genome is translated. To initiate this process, the internal ribosome entry site (IRES) of the 5' non-coding region binds to the 40S ribosomal subunit and eukaryotic initiation factor 3 (eIF3) (Lindenbach and Rice, 2005). This ternary complex then associates with eIF2, GTP and initiator tRNA to produce a 48S complex before an 80S complex can be formed and translation begins to produce the viral polyprotein (Lindenbach and Rice, 2005). Proteases, both cellular and viral, are involved in processing the polyprotein into the ten proteins encoded by the genome. The core/E1, E1/E2 and E2/p7 junctions are cleaved by ER signal peptidases (Hijikata et al., 1991b). NS4A is then cleaved from its position by NS3 before forming a protease complex by associating with the N-terminus of NS3. This protease is responsible for the cleavage of junctions between NS4B/5A and NS5A/5B whilst the junction between NS2 and NS3 is cleaved by the NS2 cysteine protease (Bartenschlager et al., 1993, Grakoui et al., 1993b).

Prior to replication, HCV first induces rearrangements in the intracellular membranes to produce a membranous web, with which non-structural proteins can associate (Egger et al., 2002, Gosert et al., 2003). The translated proteins associate with the membranous web forming the replication machinery necessary for replicating the viral genome via a negative sense intermediate (Dubuisson and

Cosset, 2014). The positive sense viral genomes produced during replication serve three purposes: to produce new proteins, act as templates for RNA replication and are used in the production of infectious virions (Dubuisson and Cosset, 2014).

This process is dependent on the liver-specific microRNA 122, which stabilises the 5' end of the genome by recruiting Argonaute 2 (Shimakami et al., 2012). Subsequent degradation by the cellular exonuclease Xrn1 is reduced (Li et al., 2013).

1.4.3. Assembly and release

The exact mechanism through which virus particles are assembled is not yet clear but some of the roles played by the viral proteins have been determined (Figure 1.2). The core protein associates around lipid droplets (LDs) in the cytoplasm, rearranging their intracellular distribution and leading to their accumulation in the perinuclear region (Barba et al., 1997, Boulant et al., 2008). The NS5A protein is also recruited to LDs, interacting with the associated core protein through its C-terminal domain (Appel et al., 2008, Masaki et al., 2008). The NS2 protein is vital for the recruitment of the E1-E2 heterodimer from the ER and its interaction with the NS3/4A protease complex (Phan et al., 2009, Jirasko et al., 2010). This latter interaction is necessary for the recruitment of the core protein from LDs to the site of virion assembly (Counihan et al., 2011). The HCV RNA may also be packaged by the NS3/4A complex during assembly of virus particles (Counihan et al., 2011). The p7 protein forms ion channels capable of equilibriating the pH gradient within the secretory pathway of the cell (Wozniak et al., 2010), thereby protecting the virion from exposure to the low pH during the release of the particles from the infected cell (Wozniak et al., 2010). The production of infectious virions is closely linked to the VLDL assembly pathway (Gastaminza et al., 2008). *In vitro*, it was observed that the

intracellular virus particles had a higher density than that of the extracellular particles, leading to the assumption that the particles seem to acquire the lower density in a post-ER compartment during transit through the secretory pathway (Gastaminza et al., 2006).

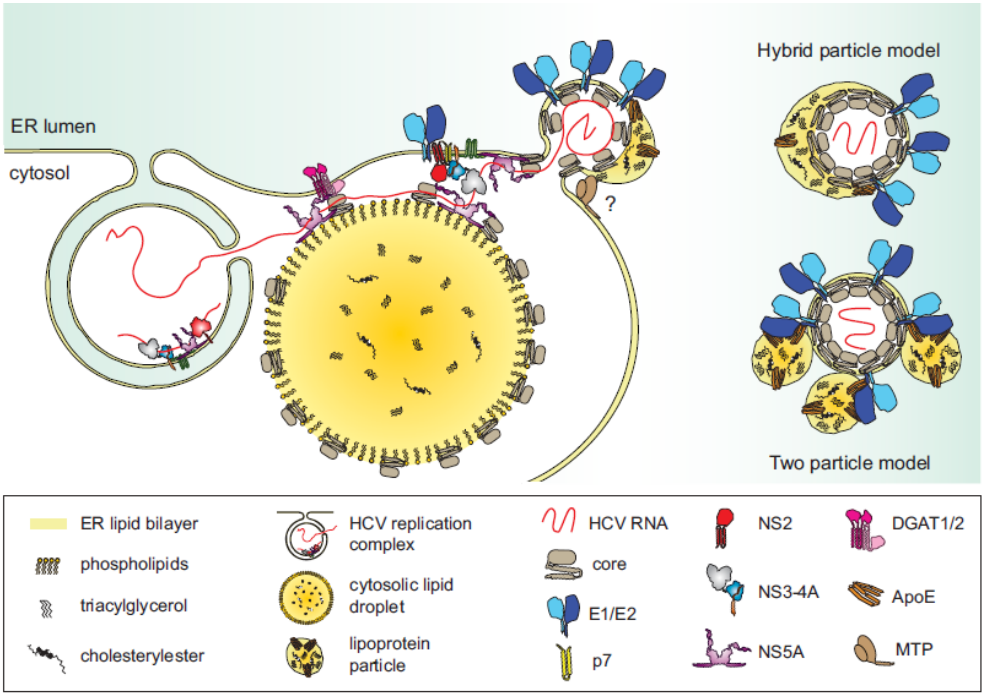

Figure 1.2. Taken from (Paul et al., 2014). Models of HCV virion production. The left side shows a hypothetical model of virion assembly. NS3 and NS5A aid in the transport of viral RNA to cytosolic lipid droplets. The re-recruitment of core protein to the ER is thought to trigger the formation of the nucleocapsid and budding into the ER lumen. Virions incorporate cellular lipoproteins, which either occurs during budding (hybrid particle model) or during egress via interaction between the virion and VLDL particles (two particle model). ER endoplasmic reticulum, E1/E2 envelope glycoprotein 1/2, NS non-structural, DGAT 1/2 Diglyceride acyltransferase 1/2, ApoE apolipoprotein E, MTP microsomal triglyceride transfer protein.

1.5. Immune responses against HCV

Both the innate and adaptive immune responses play a role in the outcome of HCV infection; either viral clearance or the evolution of the virus.

The innate immune response provides the initial defence against a viral infection such as HCV. The virus is recognised by various receptors including retinoic acid inducible gene-I (RIG-I) and toll-like receptors (TLRs), leading to the activation of transcription factors NF-κB and interferon regulatory factor 3 and 7, which bind to promoter regions of interferon genes (reviewed in Heim and Thimme, 2014). Interferons regulate the transcription of interferon stimulated genes (ISGs), the products of which are the main antiviral effectors, facilitating in the clearance of infected cells and the recruitment of immune cells to promote an adaptive response (reviewed in Yang and Zhu, 2015).

Natural killer (NK) cells play an important role in the innate immune response and can generally be divided into two subsets; i) $CD56^{bright}$ cells providing non-cytolytic responses, such as the release of IFN-γ and tumour necrosis factor-α (TNF-α), and the priming of T helper cell type I cells and ii) $CD56^{dim}$ cells capable of direct cytotoxicity (Ahlenstiel, 2013). The significance of the role that NK cells play in acute HCV infection is not currently known (reviewed in Rehermann, 2015). In chronic HCV infection, NK cells remain activated but their functions are skewed towards a reduction in cytokine levels and an increase in cytotoxic capacity, which may contribute to viral persistence and disease progression (Oliviero et al., 2009, Ahlenstiel et al., 2010).

HCV has evolved a range of mechanisms to evade the innate immune system and the selective pressures exerted by it. The NS3/4A protease is responsible for the cleavage of MAVS from intracellular membranes, thereby preventing the RIG-I

signalling pathway and the activation of interferons (Loo et al., 2006). Lower levels of IFN induction have been observed in the livers of patients with chronic HCV infection, confirming the role of MAVS cleavage *in vivo* (Bellecave et al., 2010). Other viral proteins, such as the core, are also involved in the regulation of the innate immune system. The core protein can prevent the nuclear accumulation of STAT1 to prevent the activation of ISGs as well as promoting expression of suppressor of cytokine signalling-3 (SOCS3), which is a negative regulator of the Jak/Stat pathway (Bode et al., 2003, Melen et al., 2004).

The impairment of NK cells by HCV is not well understood. *In vitro,* HCV virions have been shown to downregulate the production of IFN-γ by NK cells through the interaction of the HCV E2 protein with CD81 resulting in an environment with a cytokine profile more permissive to HCV infectivity (Crotta et al., 2010). The stabilisation of HLA-E through binding to a specific HCV core-derived epitope results in the inhibition of NK cell cytotoxicity (Nattermann et al., 2005).

The adaptive immune response comprises the humoral and cellular immune responses. The humoral immune response involves neutralising antibodies, the majority of which have been mapped against the E1 and E2 glycoproteins (reviewed in Edwards et al., 2012). These targets play a role in cell binding so neutralising antibodies may be able prevent the spread of virus to other cells. *In vitro*, several anti-E2 antibodies prevent infection at a post-attachment stage (Sabo et al., 2011).

The cellular immune response comprises the response by CD4 and CD8 T cells. The CD4 response is an important factor in determining whether a patient will spontaneously clear the virus or develop a chronic infection (Urbani et al., 2006). In cases of self-limited infection, CD4 cells produce Th1 cytokines which aid in sustaining cytotoxic T lymphocyte function as well as generating CD8 memory cells

(Urbani et al., 2006). However, virus-specific CD4 cell responses are rarely detected in chronic infection (Lucas et al., 2007).

The CD8 cells are the main effector cells involved in the clearance of the virus. HCV-specific CD8 cells appear in the blood approximately 6-8 weeks after infection and this is linked with a sharp decline in the viral load (Thimme et al., 2001). A study in chimpanzees demonstrated that after the antibody-mediated depletion of CD8 cells, the viral load remained high for a prolonged period, only declining upon the re-emergence of HCV-specific CD8 cells (Shoukry et al., 2003). CD8 T cells can act through both cytotoxic and non-cytolytic mechanisms in order to inhibit HCV replication although IFN-γ-mediated non-cytolytic mechanism is the primary effector for inhibition (Jo et al., 2009).

Previous studies have shown that the immune system provides a selective pressure for HCV and may be a driving force in its evolution in the host. One of the main factors in the persistence of an HCV infection is the appearance of viral escape mutations, which are not recognised by the immune system. Such mutations have been observed in multiple epitopes which are targets of neutralising antibodies (von Hahn et al., 2007). Some mutations in the target epitopes of neutralising antibodies act through an altered use of host-cell entry factors (Fofana et al., 2012).

There are three possible mutation sites within epitopes which may affect the CD8 T cell response; i) Mutations within the binding anchors of the epitope prevent presentation of the antigen to T cell, ii) Mutations within the contact residues of the T cell receptor region which impair recognition by epitope-specific CD8 T cells (Soderholm et al., 2006) and iii) Mutations in flanking regions of an epitope which can inhibit antigen presentation through the disruption of antigen processing by the proteasome (Seifert et al., 2004, Kimura et al., 2005). Amino acid mutations have

been observed in Class I-restricted epitopes from multiple HCV proteins, including NS3 and NS5B, in a longitudinal study examining the evolution of transmitted/founder viruses (Bull et al., 2015). The Q80K polymorphism lies within a Class I-restricted epitope (Ward et al., 2002), which may have played a role in the origins of this mutation.

Mutations in Class II-restricted epitopes can also aid in viral escape. An *in vitro* study of a specific $NS3_{358-375}$ Class II-restricted epitope found that mutations within this region reduced the T cell and IFN-γ responses compared to the original epitope (Cusick et al., 2011). Variants of a HVR1 epitope have also been shown to inhibit cytokine production (Frasca et al., 1999).

1.6. Genetic variability and resistance

HCV is currently classified into seven genotypes (shown by a number 1-7) and each genotype is subdivided into many different subtypes (represented by a lower case letter). There are considered to be over 80 different subtypes, demonstrating the vast diversity of HCV due to its rapid evolution (Argentini et al., 2009). At the nucleotide level, genotypes have a 31-33% difference whereas subtypes differ by 20-25% (Simmonds et al., 2005). Presently, the classification system of genotypes and subtypes is based on both sequence data and phylogenetic analysis of the NS5B region of the genome (Argentini et al., 2009).

There is also a large degree of heterogeneity at the intra-patient level of the virus. The term quasispecies describes a group of genetically linked variants that contains a dominant sequence alongside a range of other variants (Martell et al., 1992). HCV has several key features which contribute to its existence as a quasispecies. Firstly, it has a high rate of replication, with approximately 10^{12} virions produced daily, at a

rate of 50 infectious particles per hepatocyte per day (Neumann et al., 1998, Zeuzem et al., 1998).

Secondly, whereas the DNA polymerases of DNA-based viruses have a proofreading ability to minimise the risk of misincorporations, the RNA polymerase of HCV lacks such a mechanism. This results in an increased error rate, estimated at approximately $1.2x10^{-4}$ to $2.5x10^{-5}$ mutations per nucleotide per genome replication *in vivo* (Cuevas et al., 2009, Ribeiro et al., 2012). However, this mutation rate is likely to vary between regions of the genome. Through the use of statistical models, it has been demonstrated the majority of the non-structural genes, which are vital to the replication of the virus, mutated at a rate of $1x10^{-3}$ substitutions/site/year whereas the genome regions containing E1/E2 envelope proteins showed a higher mutation rate up to $6.9x10^{-3}$ substitutions/site/year (Gray et al., 2011).

The combination of these two factors plays a pivotal role in the diversity and evolution of the virus.

The incorporation of incorrect nucleotides during transcription leads to mutations in the virions produced. An estimated 8.7×10^{10} and 4.2×10^{9} virions produced each day contain a single or double nucleotide substitution respectively (Rong et al., 2010). There are 2.9×10^{4} and 4.1×10^{8} possible single and double mutants, respectively, and due to the large volume of virions produced in a host, each mutant is expected to be produced multiple times each day (Rong et al., 2010).

Mutations conferring resistance to antiviral drugs will be spontaneously generated during replication, without the need for the selective pressure of antiviral therapy. Mutations in proteins that are essential for the virus' replication generally confer a reduced fitness, especially if they lie within an active site. The A156V mutation of the NS3 gene increases the resistance of the virus to the NS3 protease inhibitor

simeprevir by 177-fold but also reduces the viral fitness by approximately 5-fold, impairing its ability to produce infectious virions (Lenz et al., 2010). If the replicative fitness of a mutant is negatively affected, then the mutant will remain at a low, background frequency until a selection pressure is applied which positively selects for the mutant i.e. antiviral therapy. A previous study determined that *in vitro* incubation with non-nucleoside inhibitors (NNIs) resulted in NNI-resistant variants present at a background level becoming predominant in the HCV quasispecies population (Le Pogam et al., 2008). *In vitro*, the emergence of resistant variants is also dependent on the concentration of drug administered (Verbinnen et al., 2010). One study observed the emergence of low-level resistance mutations upon exposure to a low concentration of the NS3 inhibitor TMC380765, which were replaced by variants harbouring a high-level resistance mutation when exposure to the drug was increased (Verbinnen et al., 2010).

The genetic barrier to resistance is an important factor in the emergence of resistant variants. The genetic barrier is the number of mutations required to negate the antiviral activity of a drug and the difficulty in generating these specific mutations (Gotte, 2012). A resistant variant requiring a single transition has a lower barrier to resistance than those requiring a transversion or more than one transition (Powdrill et al., 2011). The differences between genotypes can impact upon the barrier to resistance. One prominent example of this is the substitution of arginine to lysine at position 155 of NS3. Subtype 1a requires a single transition (AGG to AAG) whilst both a transition and transversion (CGG to AAG) is necessary for the same amino acid substitution in subtype 1b (Powdrill et al., 2011).

DAAs with a low barrier to resistance can still be used to effectively treat infection when used with other drugs in a combination therapy regimen. Alternatively,

increasing the level of the antiviral to that of the variants' IC_{90} value can also overcome a low genetic barrier (Lim and Gallay, 2014). The opposite also applies in cases where antiviral drugs with high barriers to resistance are used in low concentrations allowing resistance to emerge (Lim and Gallay, 2014).

In vivo, the impact of low frequency resistant variants has not been fully elucidated although small scale studies have begun to make progress into this area (Akuta et al., 2013, Akuta et al., 2014b, Kosaka et al., 2015). From these studies, variants present at baseline at a frequency ≤1% do not appear to have an impact on treatment responses. Instead, they remain at background levels and treatment failure in patients seems to be a consequence of *de novo* mutations absent at baseline which emerge in response to the antiviral treatment. In a very limited number of patients (n=4) undergoing treatment with simeprevir plus sofosbuvir, the presence of minority variants, at frequencies ranging from 1.3%-13.0%, did not impact upon the treatment response (Fevery et al., 2015).

Cessation of antiviral therapy leads to resistant mutants reverting to a low frequency as the wild-type strain with a higher replicative fitness outgrows them (Sarrazin et al., 2007). This process has been observed to begin as soon as 7 days after the end of therapy and takes place over several months (Sarrazin et al., 2007). Resistant variants have been observed persisting at varying degrees, according to the gene in which they exist and their replicative capacity. One study observed protease inhibitor resistant variants persisting at frequencies up to 9% for a period of up to 4 years after the discontinuation of treatment (Susser et al., 2011). Another found differences in persistence between RAMs; the R155K variant was found in 25% of patients at week 48 post-treatment in contrast to only 2% of patients harbouring D168V at the same time point (Krishnan et al., 2015b). Regarding NS5A variants, persistence of RAMs

at high and low frequencies have both been reported. In a proportion of patients at 6 months post-treatment, resistant variants still dominated in the population at frequencies up to 100% (Wang et al., 2013). Another study found that 94.4% of patients still harboured a RAM in NS5A, detectable by deep sequencing, 96 weeks post-treatment, although the frequencies had declined over this period (dvory-Sobol et al., 2015). Upon the continuation of replication, a dominant variant can continue to accumulate mutations which may compensate for the reduction in viral fitness or increase resistance to other antiviral drugs. This is one of the factors likely to influence the rate at which resistant variants are outgrown by the wild-type virus.

1.7. Natural history

The acute stage of HCV infection presents with mild non-specific clinical symptoms such as lethargy in only a small proportion of patients, with many infections remaining asymptomatic in the acute stage (Orland et al., 2001). The acute illness refers to the first 6 months of infection, characterised by the detection of virus in the blood, rise in the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and development of anti-HCV antibodies (Hajarizadeh et al., 2013).

In approximately 25% of acute infections, the virus is spontaneously cleared although this is dependent on several host and viral factors. Infection with genotype (Gt) 1 increases the likelihood of spontaneous clearance (Harris et al., 2007) as does low quasispecies diversity (Ray et al., 1999). Polymorphisms at the IL28B site of the host genome have a strong influence on viral clearance; patients with a CC allele have an increased likelihood of naturally clearing the infection compared to those with a CT or TT allele (Tillmann et al., 2010).

Patients who do not spontaneously clear HCV during the acute stage of infection go on to develop chronic HCV infection. This is often asymptomatic and remains undiagnosed until later complications of the infection arise. Generally, the infection progresses slowly to cause cirrhosis in 16% of patients over a period of up to 20 years (Thein et al., 2008a). In some patients, cirrhosis eventually leads to hepatocellular carcinoma (HCC) and death.

The increased risk of fibrosis progression during chronic HCV infection is influenced by numerous host and behavioural factors. Age is a significant factor, with a positive correlation between the rate of fibrosis progression and the age at which a patient is infected (Minola et al., 2002).

1.8. Epidemiology

The global estimate of HCV infection is approximately 130-150 million people, accounting for ~2% of the total world population (World Health Organisation, 2015). The prevalence between countries varies widely, with rates >10% reported in few countries, including Egypt (Guerra et al., 2012). Despite relatively low prevalence rates in many Asian countries, the large populations contribute to the high number of infections found in these countries, which comprise approximately 50% of the global infections (Lavanchy, 2011). However, the estimates in developing regions vary widely, in part due to limited surveillance and differences in testing strategies employed by studies.

Alongside the varying prevalence between different countries, the primary transmission routes also tend to differ. In northern European countries such as the UK and Norway, injecting drug users (IDUs) account for approximately 90% and 67% of infections, respectively (Cornberg et al., 2011). In contrast to this, iatrogenic

exposure is considered to be the main risk factor in developing countries such as Egypt (Paez Jimenez et al., 2010).

The distribution of HCV genotypes and subtypes varies throughout the world (Figure 1.3). Genotypes 1, 2 and 3 are prevalent throughout the world with 1 and 3 making up the majority of cases in Europe and the USA. Endemic strains are typically classified as those subtypes which are geographically restricted, displaying a high degree of heterogeneity and long-term persistence in the region (Pybus et al., 2007). Genotypes 2 and 6 are endemic in West Africa and Southeast Asia, respectively, each displaying a large degree of local genetic variation (Markov et al., 2009, Pybus et al., 2009). The high divergence of strains found in these regions may indicate a long-term presence of HCV infections, allowing the virus time to evolve into a range of different subtypes (Pybus et al., 2001).

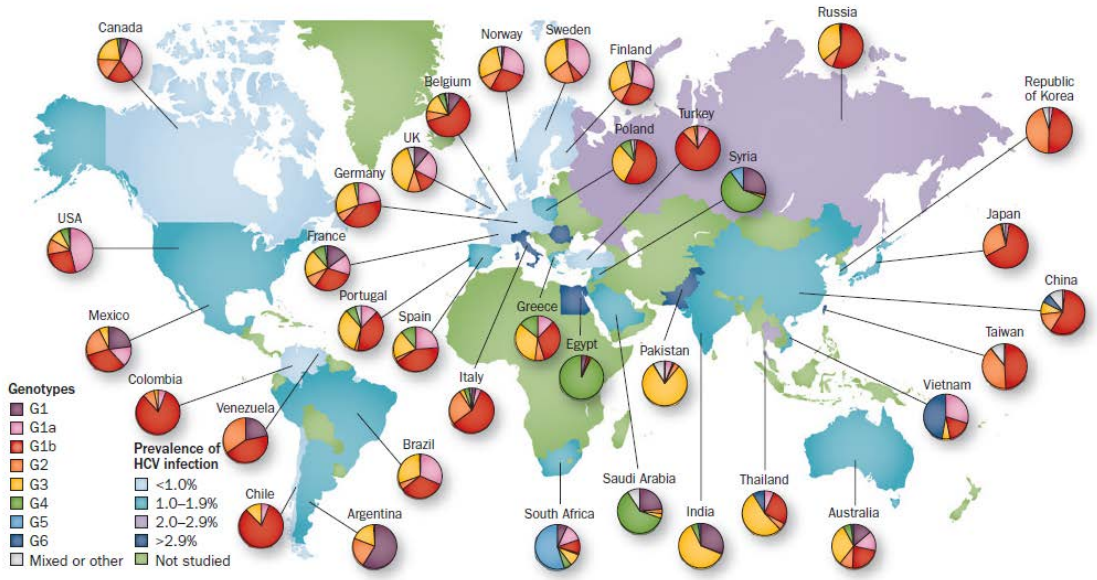

Figure 1.3. Taken from (Hajarizadeh et al., 2013) Estimated prevalence and genotype distribution of HCV throughout the world.

Both the prevalence and genotype distribution show temporal changes in pattern. Global movement and immigration of people has been determined as a significant factor in the distribution of specific genotypes (Savvas et al., 2005, Markov et al., 2012). One of the largest historical occurrences to influence this distribution is the movement of millions of people from Africa to the Americas during the trans-Atlantic slave trade (Markov et al., 2009). Movements of Gt2 lineages from West Africa to Suriname have been estimated to have occurred at the time of these major trans-Atlantic slave movements (Markov et al., 2012). In Greece, 15% of infections are Gt4, which is one of the dominant genotypes of North and Central Africa (Savvas et al., 2005). This distribution is likely to be caused by the return of communities from these regions into Greece, bringing with them this genotype, where it has subsequently remained amongst the population (Savvas et al., 2005).

1.9. Co-infection with HIV

It is estimated there are approximately 35 million HIV infections globally (UNAIDS, 2014a) and the shared transmission routes between viruses mean that persons at high risk of HIV are also likely to be at risk of infection with HCV (Sulkowski, 2008). In high risk groups such as IDUs, prevalence rates of HCV co-infection have been observed at >90% in HIV-infected individuals (Garten et al., 2004). Consequently, the CDC recommends routine HCV testing for HIV-infected patients (Centers for Disease Control and Prevention, 2013).

An almost synergistic effect exists between the two viruses in the context of transmission. The risk of HCV infection through vertical and perinatal transmission is doubled in HIV-infected mothers (Thomas et al., 1998, Mast et al., 2005) as well

as a higher risk of HCV transmission from a co-infected individual following percutaneous exposure (De Carli et al., 2003).

Co-infection with HIV has several notable effects on the natural history of HCV infection including a marked increase in HCV RNA load which has been demonstrated in several different cohorts, including IDUs and haemophiliac patients who have received transfusions (Eyster et al., 1994, Beld et al., 1998). Co-infected patients are less likely to spontaneous clear the acute HCV infection (Thomas et al., 2000), probably due to the diminished T-cell responses, which play an important role in the prevention of a chronic infection (Claassen et al., 2013).

Infection with HIV has a profound effect on both the innate and adaptive immune systems and the combined influence of both viruses during co-infection is complex.

Untreated HIV infection can alter the subset distribution of NK cells, skewing the population towards a high proportion of $CD56^{-}/CD16^{+}$ cells which show a reduction in both cytolytic activity and the production of cytokines required for the modulation of other immune effectors (Mavilio et al., 2003, Mavilio et al., 2005). This change may decrease the pressure placed on HCV by the innate immune system, and therefore increase the risk of the infection becoming persistent.

HIV also leads to the impairment and death of CD4 cells, which may affect the adaptive immune response against HCV. A previous study observed a decrease in levels of antibodies specific to non-structural proteins after HIV infection, with this decrease more evident in patients with a low CD4 count (Netski et al., 2007). Another study observed a similar trend in anti-HCV antibodies specific to E1 and E2 glycoproteins (Bailey et al., 2015). This trend may affect the evolution of HCV, with less pressure from the immune system leading to a decline in the quasispecies variation. The CD4 count of HIV/HCV co-infected patients has also been shown to

affect the HCV-specific CD8 response, with such responses only present in 30% of subjects with a CD4 count of <500 cells/mm^3 (Kim et al., 2005).

The advent of highly active antiretroviral therapy (HAART) has dramatically decreased HIV-related mortality in recent years. However, this has had the knock-on effect of HCV co-infection now emerging as a leading cause of disease and death among HIV-positive patients (Rotman and Liang, 2009). The effects of HCV are exacerbated by HIV co-infection, markedly accelerating the progression of liver fibrosis and increasing the likelihood of HCV-related morbidity and mortality (Rotman and Liang, 2009). Co-infected patients are at an increased risk of cirrhosis (Thein et al., 2008b), at an earlier stage compared to mono-infected patients (Benhamou et al., 1999). The effects of HIV on HCV quasispecies and drug resistance have been poorly studied, with no consensus determined between the studies (Sherman et al., 1996, Shuhart et al., 2006, Lopez-Labrador et al., 2007, Tanaka et al., 2007, Netski et al., 2008).

1.10. Diagnosis

Diagnosis of an active HCV infection is completed through the use of multiple tests. The CDC recommends a testing algorithm of a serological assay for the detection of anti-HCV antibodies or HCV antigens, followed by a nucleic acid amplification test (NAT) to confirm the presence of a current infection (Centers for Disease Control and Prevention, 2013). Upon a negative result by NAT, an immunoblot assay can be used as a tertiary test to confirm a resolved past infection.

During the initial acute phase of HCV infection, there is a period of approximately 7-8 weeks before the development of anti-HCV antibodies by the immune system of the host (Bowen and Walker, 2005). Prior to this seroconversion, infection can be

diagnosed by assays that directly target the virus, typically nucleic acid amplification technology (NAT) for the detection of HCV RNA (Centers for Disease Control and Prevention, 2013). Real-time PCR is the most common NAT employed to detect HCV RNA in routine diagnostic settings. In addition, quantitative real-time PCR measures the HCV RNA load and plays an important role in the clinic by guiding antiviral therapy and measuring its efficacy. Commercial real-time PCR assays are available in kit format from several manufacturers, although these are often expensive to use. On the other hand, serological assays represent a relatively cheap diagnostic test, requiring little infrastructure to complete. These features make serological assays a prime candidate for initial tests, especially in developing regions. However, some reports from studies in African populations have found these tests to have a high rate of false-positive results (Chasela et al., 2012, Mullis et al., 2013, King et al., 2015).

1.11. Treatment: Drug classes, mechanism of action and resistance pathways

1.11.1. Treatment outcomes

Unlike HIV and HBV, HCV does not integrate into the genome of the host or have a DNA intermediate. Consequently, infection with HCV can be cleared with the use of antiviral therapy. The aim of treatment is the eradication of the virus from the patient, which then prevents further complications of chronic infection including cirrhosis, hepatocellular carcinoma and death (European Association for the Study of the Liver, 2015). There are several possible outcomes of therapy at timepoints during and after treatment. During therapy, a rapid virological response (RVR) is the

decrease in HCV RNA to undetectable levels at week 4 of treatment (Wedemeyer et al., 2012). The achievement of an RVR has been observed as a strong predictor of the patient achieving sustained virological response (SVR) in trials of treatment with a protease inhibitor (PI) plus pegylated interferon and ribavirin (P/R) (Jacobson et al., 2011). Despite suppression of the virus during early therapy, some patients experience a re-emergence of HCV RNA in a phenomenon known as virologic breakthrough. Patients may also fail to suppress the levels of HCV RNA by ≥ 2 $\log_{10}$ during the first 12 weeks of therapy. This is known as a null-response.

An SVR is defined as undetectable HCV RNA at either 12 or 24 weeks after the cessation of treatment (Wedemeyer et al., 2012). The alternative outcome to an SVR is a virologic relapse, in which the levels of HCV RNA return to pre-treatment level in a patient who showed undetectable RNA at the end of treatment.

Table 1.1. Currently licensed and discontinued direct-acting antivirals for the treatment of HCV

Class	Target	DAAs
Peptidomimetic linear ketoamide inhibitor	NS3 protease	Boceprevir Telaprevir Paritaprevir
Macrocyclic inhibitor	NS3 protease	Simeprevir
NS5A inhibitor	NS5A	Daclatasvir Ledipasvir Ombitasvir
Nucleotide inhibitor	NS5B polymerase	Sofosbuvir
Non-nucleoside inhibitor	NS5B polymerase	Dasabuvir
CYP3A inhibitor	Pharmacologic booster for paritaprevir	Ritonavir

1.11.2. Pegylated-interferon and ribavirin

Up until 2011 and the introduction of direct acting antivirals (DAAs) (Table 1.1), the gold standard treatment for HCV infection was a combination of pegylated-interferon (peg-IFN) and ribavirin (RBV). This treatment had a low response rate of approximately 50% (Manns et al., 2001) although both host and viral factors affected the efficacy of this regimen. Host factors such as African-American ethnicity reduced the efficacy of this treatment (Jeffers et al., 2004) although one of the greatest influences was polymorphisms at the IL28B locus (Ge et al., 2009, Suppiah

et al., 2009). The genotype of HCV also affected the outcome of treatment; Gt1 being more difficult to treat than other genotypes (McHutchison et al., 1998).

Peg-IFN and RBV decrease the viral load of HCV through a variety of different mechanisms, although they are not virus specific. RBV is a guanosine nucleoside analogue that inhibits the replication of HCV. One of the derivatives from the phosphorylation of RBV is its triphosphate form, RTP. *In vitro*, high concentrations of RBV results in the blocking of RNA elongation through the misincorporation of this molecule opposite cytosine or uridine in the viral RNA chain (Maag et al., 2001). For clinical use, RBV is used at much lower concentrations so it is likely that this mechanism only has a small effect on the replication of HCV (Feld and Hoofnagle, 2005). Another proposed mechanism of RBV is that it acts as a viral mutagen and this pushes the virus towards the 'error catastrophe'; the virus carries so many mutations that its replicative capacity is completely diminished (Crotty et al., 2000, Crotty et al., 2001).

Interferon-alpha acts by binding to IFN cell-surface-receptors, which subsequently activate the Janus-activated kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2) (Beadling et al., 1994). These kinases phosphorylate the signal transducer and activator of transcription proteins 1 and 2 (STAT1 and STAT2), leading to the formation of IFN-stimulated gene factor 3 (ISGF-3) which binds to the promoter regions IFN-stimulated genes (ISGs) and activates their transcription (Kessler et al., 1988, Levy et al., 1988, Beadling et al., 1994, reviewed in Sen, 2001). Functions of ISGs include apoptosis and the degradation of proteins (de Veer et al., 2001).

1.11.3. NS3 protease inhibitors

Four PIs – boceprevir, telaprevir, simeprevir and paritaprevir – have been licensed for the clinical treatment of chronic HCV infection. PIs can be divided into two classes; linear and macrocyclic inhibitors. The introduction of boceprevir and telaprevir to the clinic increased SVR rates to 70% in treatment-naïve cohorts (Jacobson et al., 2011, Poordad et al., 2011). However, an increase in side effects was also reported alongside the higher response rates. These included skin rashes and anaemia in telaprevir and boceprevir, respectively, leading to the discontinuation of treatment in 7-11% of patients (Jacobson et al., 2011, Poordad et al., 2011). The risk of virological failure was high when these first-generation PIs were used as a monotherapy or in dual combination with peg-IFN only (Hezode et al., 2009). For this reason, combination therapy with P/R or another class of DAA was recommended. A lead-in phase of P/R before the initiation of boceprevir was shown to reduce the subsequent risk of treatment failure and resistance (Kwo et al., 2010). These two first-generation PIs are soon to be discontinued, with simeprevir and paritaprevir acting as their replacements in the treatment of HCV. Simeprevir is used with P/R or in combination with sofosbuvir while paritaprevir (boosted with ritonavir) is used in combination with ombitasvir ± dasabuvir (European Association for the Study of the Liver, 2015). These treatments show improved SVR rates (>80%) compared to first-generation PIs, with lower treatment discontinuation rates, typically <3% (Feld et al., 2014, Jacobson et al., 2014, Manns et al., 2014).

Protease inhibitors compete with the substrate of the NS3 protease, inhibiting the post-translational cleavage of the polyprotein and thereby terminating replication (Raney et al., 2010).

Resistance mutations to PIs have been well established, with many of the mutations identified conferring resistance to both linear and macrocyclic inhibitors. Sites of resistance lie within the inhibitor envelope (the van der Waals surface boundary of the inhibitor) and can directly interact with it (Raney et al., 2010). A non-synonymous mutation at such a site would negatively affect the binding of the inhibitor, decreasing the virus' susceptibility to the drug. The position of these resistance sites lie outside the substrate envelope and catalytic site of the protease maintaining the enzymatic activity of the protease without completely eliminating the replicative fitness of the virus (Raney et al., 2010).

1.11.4. NS5A inhibitors

Three NS5A inhibitors have been licensed for the treatment of HCV infection; daclatasvir (in combination with sofosbuvir), ledipasvir (in combination with sofosbuvir) and ombitasvir (in combination with paritaprevir and dasabuvir). Treatment with daclatasvir plus P/R resulted in relatively low SVR rates of ~60% in treatment-naïve Gt1 patients (Hezode et al., 2015). In comparison, the addition of sofosbuvir to daclatasvir therapy resulted in SVR rates of >90% (Sulkowski et al., 2014). Ledipasvir in combination with sofosbuvir also lead to SVR rates of >90% in Gt1 infected patients who were treatment-naïve or previously treated with a DAA and/or P/R (Afdhal et al., 2014a, Afdhal et al., 2014b).

NS5A has no known enzymatic activity, which makes it difficult to understand its mechanism of action. Upon administration of daclatasvir, the rapid early decline of the virus observed *in vivo* can be attributed to the drug having mechanisms which block both the synthesis of viral RNA and the assembly or secretion of infectious virions (Guedj et al., 2013). The phosphorylation state of NS5A affects its

interaction with other proteins, regulating its function (Evans et al., 2004), and daclatasvir has been shown to block hyperphosphorylation of NS5A (Qiu et al., 2011). Ledipasvir and daclatasvir have been shown to directly bind to the NS5A protein (Ascher et al., 2014, Kwon et al., 2015). Several other hypotheses have been proposed to explain the mechanism of action, including altering the localisation of NS5A to the surface of lipid droplets (Qiu et al., 2011, Targett-Adams et al., 2011).

While NS5A inhibitors display extremely high potency against HCV, they also have a low barrier to resistance and the resistant variants have a high replicative capacity (Fridell et al., 2011b). Consequently, NS5A inhibitors are all used as part of combination therapy with other DAAs rather than alone with P/R (European Association for the Study of the Liver, 2015). The known sites of resistance mutations are located in domain I of the protein. The Y93H mutation significantly reduces the binding affinity of the NS5A molecule to ledipasvir, reducing its efficacy (Kwon et al., 2015). As is the case with NS3 RAMs, NS5A mutations are responsible for cross-resistance to more than one inhibitor.

1.11.5. NS5B polymerase inhibitors

The polymerase inhibitors sofosbuvir and dasabuvir have been licensed for clinical use; sofosbuvir in combination with the ledipasvir (NS5A inhibitor) and dasabuvir in combination with paritaprevir (PI), ombitasvir (NS5A inhibitor).

In clinical trials of combination therapies of the aforementioned drugs, SVR rates were over 94% in both treatment-naïve and previously treated patients with Gt1 infections (Afdhal et al., 2014a, Afdhal et al., 2014b, Feld et al., 2014, Zeuzem et al., 2014). When sofosbuvir was used in conjunction with P/R, 90% of Gt1, treatment-naïve patients achieved SVR_{12} (Lawitz et al., 2013).

NS5B inhibitors can be classified into two classes; nucleoside inhibitors (NIs) and non-nucleoside inhibitors (NNIs). NIs act as a substrate of the viral polymerase and, after phosphorylation to their triphosphate derivative, are incorporated into the elongating chain of RNA causing premature termination (Migliaccio et al., 2003). NIs target the conserved active site of the polymerase, displaying pangenotypic activity both *in vitro* (Lam et al., 2012) and *in vivo* (Lawitz et al., 2013, Pockros et al., 2013).

The viral polymerase contains four sites to which allosteric site NNIs can bind including the palm domain near the active site (palm I) and the outer surface of the thumb domain (thumb II) (Najera, 2013). Through this binding, the conformational changes to polymerase which are necessary for replication are blocked, thereby inhibiting the synthesis of RNA (Biswal et al., 2005).

The S282T mutation lies close to the active site of the polymerase, conferring resistance to both sofosbuvir and mericitabine. S282T results in a reduced affinity of the polymerase for the antiviral and an ability to extend the RNA chain which has been terminated by the NI (Migliaccio et al., 2003). This mutation is rarely seen at baseline in patients, reflecting the negative influence it has on the replicative capacity of the virus (Franco et al., 2013, Paolucci et al., 2013).

The site to which the allosteric NNI binds determines the mutations which confer resistance to it. An NNI which binds to the thumb domain is not affected by those mutations which confer resistance to an NNI that binds to the palm domain and vice versa.

No cross-resistance between NIs and allosteric NNIs is known to exist (Lam et al., 2012). The distinct region that each inhibitor binds to allows two polymerase inhibitors to be used in combination, without the risk of cross-resistance.

1.11.6. Combination therapy

Although the SVR rates have increased dramatically with the advent and licensing of new DAAs, it is clear that these drugs still have a limited effect when used as monotherapy or in combination with P/R.

The highest rates of SVR have been observed in clinical trials of a combination of two or more DAAs. Combination therapy for the treatment of HCV combines two or more DAAs from different classes i.e. an NS5A inhibitor such as ledipasvir with an NS5B polymerase inhibitor such as sofosbuvir. Such treatment options also offer a viable treatment option to every class of patient including those patients who are ineligible or intolerant to P/R therapy (Suzuki et al., 2013). The combination of multiple drugs of the same class, such as PIs, is limited because of the extensive cross-resistance observed.

The genetic barrier to resistance is increased when multiple DAAs with different targets are used, decreasing the risk of virological escape and treatment failure. This approach is used in HAART for the treatment of HIV to increase the likelihood of suppressing the virus. Through the use of multiple drugs, a wide range of the existing variants will be eliminated before drug resistant mutants can emerge. Synergistic interactions between DAAs have been observed *in vitro*, enhancing the antiviral activity of each drug (Grunberger et al., 2008, Wyles et al., 2008). Despite the increased genetic barrier, treatment failure and virological breakthrough have still been observed in combination therapies with two DAAs. Variants harbouring NS3 and NS5A RAMs were observed in patients experiencing virologic breakthrough when treated with daclatasvir and asunaprevir (Karino et al., 2013). These breakthroughs occurred in patients whose trough exposures to the drugs were below the IC_{90} (Karino et al., 2013).

1.12. Detection of minor variants

Before the initiation of therapy, genotypic testing for resistance mutations may be performed. This is completed by traditional Sanger sequencing which will detect mutants present at a frequency of ≥10-20%. The frequency at which drug resistant variants are present before treatment initiation is likely to fall below this threshold meaning that more sensitive methods such as allele-specific PCR, cloning or deep sequencing are required.

Allele specific PCR employs the use of primers specific to drug resistance sites in the viral genome, enabling the identification of variants present at less than 1%. The main disadvantage to this approach is the *a priori* selection of variants, eliminating the possibility of identifying novel mutations (Halfon and Locarnini, 2011).

Clonal analysis represents another option. Although this method enables the identification of mutations at all sites of the examined region, the sensitivity of the assay is directly correlated to the number of clones required (Halfon and Locarnini, 2011). This labour intensive approach is clearly unsuitable for a high-throughput clinical environment.

Next generation sequencing (NGS) represents a viable alternative to traditional sequencing methods for the detection of low-frequency variants. Currently, there are multiple platforms available, each offering different advantages and the associated shortcomings (Quail et al., 2012). Although the initial outlay for the equipment is high, NGS is a cost-effective method per amount of data generated, with a cost as low as US$0.5 per Mb (Loman et al., 2012).

Research Aims

1. Develop and/or optimise assays for i) screening pools of plasma and dried plasma spot specimens for the presence of HCV RNA and ii) genotyping HCV RNA positive specimens using two regions of the genome (detailed in chapter three).
2. Determine the prevalence of active HCV infection in an HIV-positive cohort from Kumasi, Ghana and perform phylogenetic analyses to assess the diversity of the strains found in this region (detailed in chapter four).
3. Investigate the prevalence of the Q80K polymorphism in a UK population and determine whether traditional Sanger sequencing is sufficient for the routine screening of samples for Q80K (detailed in chapter five).
4. Perform deep sequencing on samples from HCV mono-infected and HIV/HCV co-infected samples naïve to all anti-HCV therapy, in order to assess the impact of HIV co-infection on the prevalence of resistance associated mutations (detailed in chapter six).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Patient cohorts

2.1.1. Kumasi HIV-positive cohort

Plasma samples were collected from HIV-infected patients attending the HIV clinic at the Komfo Anokye Teaching Hospital in Kumasi, Ghana between 2010 and 2012. The study was approved by the Committee on Human Research Publications and Ethics at the Kwame Nkrumah University of Science and Technology of Kumasi, Ghana (reference CHRPE/143/10). A copy of the approval letter can be found in the Appendix.

2.1.2. European cohort

Plasma samples from HCV mono-infected and HIV/HCV co-infected patients stored at sites throughout the UK (Royal Liverpool Hospital in Liverpool, Chelsea & Westminster Hospital in London, King's College Hospital in London, and Charing Cross Hospital in London) and Europe (Hospital Carlos III in Madrid, Spain and University Hospital in Siena, Italy) were collected. The study was approved by the South Berkshire Regional Ethics Committee (reference 12/SC/0346). Surplus samples obtained from hospitals did not require patient consent as, detailed by section 1(9) of the Human Tissue Act 2004, there is no legal requirement for consent to store or use the tissue for research. All patient identifiable information was removed before the samples were made available to the study. Each sample was assigned a unique study ID. A copy of the approval letter can be found in the Appendix.

2.2. Sample storage

Plasma samples were stored in 1ml aliquots at -80^{o}C until needed.

2.3. Dried plasma spot preparation

Dried plasma spots (DPS) were prepared by spotting 50µl plasma onto each of five 12mm discs on a Protein saver 903 card (Whatman, Maidstone, UK). DPS were dried at room temperature for a minimum and maximum of 12 hours and 18 hours, respectively, before being placed into individual sealable plastic bags with silica desiccant sachets. DPS were stored at -80^{o}C until needed. Upon being taken out of the freezer, DPS were left at room temperature for 30 minutes before being removed from bags to prevent the formation of condensation.

2.4. Extraction using easyMAG automated extraction platform prior to real-time PCR

Frozen plasma samples were thawed at room temperature and vortexed to ensure homogenisation. Prior to extraction, Phocine Distemper Virus (PDV) was added to 1ml of plasma as an internal control (IC). Samples were then incubated at 56^{o}C for 30 minutes and 100^{o}C for 30 minutes (allowing a ramping time of 10 minutes between temperatures) with 2ml Nuclisens lysis buffer (bioMérieux, Boxtel, Netherlands) containing 40µl Proteinase K (Qiagen) before being transferred to the EasyMAG® automated extractor (bioMérieux). Extraction was completed using the Specific B protocol 2.0.1 with the elution volume set at 60µl, following manufacturer's instructions. A negative control of water was included on each extraction run. For DPS specimens, two 6mm punches were eluted in 3ml Nuclisens

lysis buffer containing 40µl Proteinase K (Qiagen) and PDV for 2 hours with gentle agitation on a roller-mixer before being extracted as detailed above.

Table 2.1. Primers used for the detection of HCV by real-time PCR

Primer/Probe Name	Target	Primer/Probe Sequence	Position
HCV Fwd	5'-UTR of HCV	5'-GTC TAG CCA TGG CGT TAG TA-3'	77-96[a]
HCV Rev	5'-UTR of HCV	5'-GTA CTC ACC GGT TCC GC-3'	150-166[a]
HCV Probe	5'-UTR of HCV	FAM-CCC TCC CGG GAG AGC CAT AGT G-TAMRA	124-145[a]
PDV Fwd	Haemagglutinin of PDV	5'-CGG GTG CCT TTT ACA AGA AC-3'	43-62[b]
PDV Rev	Haemagglutinin of PDV	5'-TTC TTT CCT CAA CCT CGT CC-3'	101-120[b]
PDV Probe	Haemagglutinin of PDV	JOE-ATG CAA GGG CCA ATT CTT CCA AGT T-BHQ1	64-88[b]

[a] Numbered according to H77 genome (accession number NC_004102)

[b] Numbered according to PDV accession number Z36979

2.5. Real-time PCR assay

HCV RNA was detected by a real-time PCR assay targeting a conserved 90bp sequence of the HCV 5'-UTR region (Daniel et al., 2008). The IC reaction targeted a 79bp region of the PDV haemagglutinin gene (van Doornum et al., 2007). Amplification was performed by the ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) using 20µl of extract in a 50µl final reaction volume containing 25µl 2X reaction mix, 1µl SuperScript® III/Platinum® Taq mix enzyme, and 200nM of

forward and reverse HCV and PDV primers and 100nM probes. Primer and probe sequences are listed in Table 2.1. The thermal cycling conditions were 50ºC for 15 min, 95ºC for 2 min, followed by 45 cycles of 95ºC for 15 s and 60ºC for 32 s.

2.6. Genotyping

2.6.1. Extraction using Qiagen Viral RNA Mini kit

Plasma samples were extracted following manufacturer's instructions. DPS specimens were extracted following the manufacturer's instructions, with an increased primary incubation step of 30 minutes in buffer AL instead of 10 minutes.

2.6.2. Amplification of the core region

A 403bp fragment of the core region of the HCV genome was amplified using previously described primers (Mellor et al., 1995). Reverse transcription (RT)-PCR was carried out on 15µl of extracted RNA (total reaction volume, 50µl) using the one-step RT-PCR kit (Qiagen). Other constituents were 4U of RNasin ribonuclease inhibitor (Promega, Madison, WI, USA) and 400nM of each primer (f288 and r751). The thermal cycling conditions of the RT-PCR were: reverse transcription at 50ºC for 30 min, denaturation at 95ºC for 15 min, then 35 cycles at 94ºC for 30 s, 50ºC for 30 s, 72ºC for 1 min, and a final elongation step at 72ºC for 10 min. The nested PCR was performed on 5µl of PCR product (total reaction volume, 50µl) using HotStarTaq DNA polymerase kit (Qiagen), with 400nM of each primer (f321 and r724). The following conditions were used: 95ºC for 15 min, then 35 cycles of 94ºC for 30 s, 50ºC for 30 s, 72ºC for 1 min, and a final stage at 72ºC for 10 min. Primer sequences are listed in Table 2.2.

2.6.3. Amplification of the NS5B region

A 380bp region of the NS5B gene was amplified using a modified version of a previously described method (Sandres-Sauné et al., 2003).

An RT-PCR was carried out on 10µl of extracted RNA (final reaction volume, 50µl) using the one-step RT-PCR kit (Qiagen), 400nM of each primer (Pr3 and Pr4) and 4U of RNasin ribonuclease inhibitor (Promega). The cycling conditions were: reverse transcription at 50^{o}C for 30 min, initial denaturation at 95^{o}C for 15 min followed by 5 cycles of 93^{o}C for 30 s, 60^{o}C for 45 s and 72^{o}C for 1 min, then 35 cycles of 93^{o}C for 30 s, 60^{o}C (0.3^{o}C drop per cycle) for 45 s and 72^{o}C for 1 min with a final elongation step of 72^{o}C for 5 min.

The hemi-nested PCR was carried out on 5µl first round PCR product in a final volume of 100µl using the Qiagen HotStarTaq DNA Polymerase kit (Qiagen). 200nM of Pr3 and Pr5 were used with the following cycling conditions: initial denaturation at 95^{o}C for 15 min followed by 35 cycles of 94^{o}C for 30 s, 55^{o}C for 30 s, 72^{o}C for 1 min and a final elongation step of 72^{o}C for 10 min. Primers are listed in Table 2.2.

Table 2.2. Primers used for the genotyping of samples

Primer/Probe Name	Target	Orientation	Primer/Probe Sequence	Position
Pr3	NS5B	Sense	5'-TAT GAY ACC CGC TGY TTT GAC TC-3'	8256-8278[a]
Pr4	NS5B	Sense	5'-GCN GAR TAY CTV GTC ATA GCC TC-3'	8622-8644[a]
Pr5	NS5B	Antisense	5'-GCT AGT CAT AGC CTC CGT-3'	8619-8636[a]
f288	Core	Sense	5'-ACT GCC TGA TAG GGT GCT TGC GAG-3'	288-311[a]
r751	Core	Antisense	5'-ATG TAC CCC ATG AGG TCG GC-3'	732-751[a]
f321	Core	Sense	5'-AGG TCT CGT AGA CCG TGC ATC ATG-3'	321-344[a]
r724	Core	Antisense	5'-CAY GTR AGG GTA TCG ATG AC-3'	705-724[a]

[a] Numbered according to H77 genome (accession number NC_004102)

2.7. NS3 amplification prior to Sanger sequencing

2.7.1. Extraction using easyMAG automated extractor

Plasma samples were removed from the -80ºC freezer and allowed to thaw at room temperature before being vortexed vigorously to ensure homogenisation. 100µl of sample was used as input into the EasyMAG® automated extractor (bioMérieux). Samples were extracted using the onboard lysis step of 10 minutes and generic

protocol v2.0.1 and the nucleic acid eluted into 60µl. The eluate was used in the subsequent RT-PCR immediately.

2.7.2. Amplification of NS3 prior to Sanger sequencing

cDNA synthesis was performed with the Qiagen OneStep RT-PCR Kit (Qiagen) with 400nM of each primer (f3278 and r4032) under the following cycling conditions: 50ºC for 30 min, 95ºC for 15 min, 5 cycles at 93ºC for 30s, 60ºC for 45s, 72ºC for 1 min, 35 cycles at 93ºC for 30s, 60ºC for 45s (0.3ºC drop each cycle), 72ºC for 1 min, and a final step at 72ºC for 5 min. The first round product was subjected to nested PCR using the Qiagen HotStarTaq Master Mix Kit (Qiagen) with 200nM of each primer (f3307 and r4014) under the following cycling conditions: 95ºC for 15 min, 35 cycles at 94ºC for 30s, 55ºC for 30s, 72ºC for 1 min and a final stage at 72ºC for 10 min. Primers are listed in Table 2.3.

2.8. Agarose gel electrophoresis

Agarose gels were made at a final concentration of 1% (w/v) using 1.5g of agarose (Bioline, London, UK) in 150ml of 1x TBE. SYBRSafe gel stain (Invitrogen) was added to the solution, at a ratio of 1µl to 10ml agarose solution. Gels were run in an electrophoresis tank containing 0.5x TBE running buffer for 45 minutes at 120V before being visualised under UV light.

Table 2.3. Primers used for the amplification of NS3 and NS5A genes prior to sequencing for the detection of resistance associated mutations

Primer/Probe Name	Target	Orientation	Primer/Probe Sequence	Position
f3278	NS3	Sense	5'-GGA GAC CAA GMT CAT CAC STG G-3'	3278-3299[a]
r4032	NS3	Antisense	5'-GCT CTT RCC GCT GCC RGT GGG-3'	4032-4052[a]
f3307	NS3	Sense	5'-ACA CCG CSG CGT GYG GKG ACA T-3'	3307-3328[a]
r4014	NS3	Antisense	5'-GGR GCR TGY AGR TGG GCC AC-3'	4014-4033[a]
F6057	NS5A Gt1a	Sense	5'-GTT GGC CCG GGC GAG G-3'	6057-6072[a]
1a_Rev_Out	NS5A Gt1a	Antisense	5'-GTC CAG GWR TAR GAC ATY GAG CA-3'	7599-7621[a]
F6084	NS5A Gt1b	Sense	5'-TGG ATG AAC CGG CTG ATA G-3'	6084-6102[a]
1b_Rev_Out	NS5A Gt1b	Antisense	5'-GAC CAR GAC CCG TCR CTG AGR T-3'	7537-7558[a]
F6129	NS5A Gt1	Sense	5'-TCC CCC ACG CAC TAY GTG-3'	6129-6146[a]
1a_Rev_In	NS5A Gt1a	Antisense	5'-GAG CAR CAC ACG ACR TCY TC-3'	7584-7603[a]
1b_Rev_In	NS5A Gt1b	Antisense	5'-GGC ATG GAG GAR TAY GAC-3'	7490-7507[a]

[a] Numbered according to H77 genome (accession number NC_004102)

2.9. Purification using Qiagen QIAquick PCR purification kit

Prior to cycle sequencing, second round PCR products were purified using the QIAquick PCR purification kit (Qiagen), following manufacturer's instructions. The kit uses spin columns and several wash steps and the purified amplicons were eluted in 50µl elution buffer.

2.10. Cycle sequencing

The forward and reverse primers from the nested PCR round alongside gene-specific primers were used for sequencing of amplicons (Table 2.4). A mix containing BigDye ready reaction mix, nuclease-free water and one primer was prepared. 8µl of amplicon was pipette mixed to the cycle sequencing mastermix. Cycle sequencing was performed with the following cycling conditions: 25 cycles of 96^{o}C for 10s, 50^{o}C for 5s and 60^{o}C for 4 min.

2.11. Purification of cycle sequencing products

Products were purified immediately after the cycle sequencing reaction finished. Sodium acetate (2µl per well) and molecular biology grade 100% ethanol (50µl per well) were mixed, added to each well and pipette mixed 3-5 times. The plate was sealed and centrifuged at 2000xg for 20 minutes at room temperature before the supernatant was drained by inverting the plate and centrifuging at 150xg for 1 minute at room temperature. 150µl of freshly made 70% ethanol was added to the wells and the plate centrifuged at 2000xg for 5 minutes before draining the supernatant by inverting the plate and centrifuging at 150xg for 2 minutes. An incubation time of 30 minutes at room temperature was used to ensure residual

ethanol had evaporated. The resulting DNA pellets were resuspended in 10µl of Hi-Di formamide and analysed on an ABI Prism 3730 Genetic Analyser (Applied Biosystems®).

Table 2.4. Cycle sequencing primers prior to Sanger population sequencing

Primer/Probe Name	Target	Orientation	Primer/Probe Sequence	Position
f3307	NS3	Sense	5'-ACA CCG CSG CGT GYG GKG ACA T-3'	3307-3328[a]
r4014	NS3	Antisense	5'-GGR GCR TGY AGR TGG GCC AC-3'	4014-4033[a]
1a_r3710	NS3 Gt1a	Antisense	5'-GCA GGG TGT CAA TGA GCG GG-3'	3691-3710[a]
1a_f3757	NS3 Gt1a	Sense	5'-TCA TTC CCG TGC GCC-3'	3757-3771[a]
1b_f3757	NS3 Gt1b	Sense	5'-TCA TYC CGG TGC GCC-3'	3757-3771[a]
1b_r3771	NS3 Gt1b	Antisense	5'-GGC GCA CCG GAA TGA-3'	3757-3771[a]

[a] Numbered according to H77 genome (accession number NC_004102)

2.12. Sequence analysis

Sequences were aligned against a reference sequence (accession number: NC_004102) in Seqscape v2.7 and manually checked for basecalling errors.

2.13. Phylogenetic Analysis

Sequences were aligned using Clustal W and then checked manually. Phylogenetic analyses were performed using the PHYLIP software package v3.69 (Felsenstein, 1989). Distance matrices were produced by DNADIST (using Kimura-2 parameter setting) which were analysed in NEIGHBOR, using neighbor-joining setting. Bootstrapping (1000 replicates) was performed by SEQBOOT and CONSENSE. Branch lengths on the consensus tree were reconstructed using the original sequences and Dnaml, with the consensus tree as the user-defined tree. Bootstrap values ≥80% were considered significant.

The bootstrapping process involves the replacement of *n* number of nucleotides in each sequence, with these new sequences being used to generate a phylogenetic tree (Felsenstein, 1985). Each new tree is compared against the original inferred tree and a bootstrap value produced which represents the percentage of trees in which the examined clade was observed. This bootstrap value is used to assess the confidence levels of the clades inferred by a phylogenetic tree, allowing one to make judgements about how well supported a phylogenetic relationship is.

2.14. Plasmid control for next generation sequencing

Two plasmids, each encoding a full length NS3 region in a pCR Topo vector were obtained from Andrea Rosi at the University of Siena, Italy. One plasmid was classified as a ‘wild-type’ sequence. The other encoded an identical sequence, with the exception of 5 single nucleotide polymorphisms at the site of 5 known sites associated with drug resistance: V36A, T54S, V55A, R155K and A156S. These plasmids served two purposes in the study; i) to determine the levels of error incorporated into sequences through a combination of PCR and Illumina MiSeq

sequencing errors, and ii) to ensure variants present at a set frequency in the population could be observed.

2.15. Extraction using Qiasymphony SP prior to next generation sequencing

Plasma samples were extracted using the Qiasymphony SP automated robot (Qiagen). 200µl plasma was added to 450µl 1x PBS and extracted using the DSP Virus/Pathogen Midi kit and CellFree_500 protocol v4 into a final elution volume of 60µl (in AVE buffer). A negative control of 1x PBS was included on each run. Extracts were used in the reverse transcription reaction immediately.

2.16. Reverse transcription

This step was developed in conjunction with Andrea Rosi at University of Siena, Italy. 20µl extract of each sample was heated at 70ºC for 5 min before being immediately placed on ice for a further 5 min to prevent the formation of secondary structure in the RNA. The RT reaction mix containing 5X reaction buffer, 10U RNasin, $MgCl_2$, dNTPs, 50ng random primers and ImProm-II RT (Promega) was added to the extract and pipette mixed 5 times. The following cycling conditions were used; 25ºC for 10min, 42ºC for 60min, 50ºC for 30min, 55ºC for 30min and a final step of 70ºC for 15min.

2.17. Amplification of NS3 prior to next generation sequencing

A partial region (727bp) of the NS3 gene was amplified using nested PCR. First round PCR was carried out using 5µl cDNA from the RT in a total volume of 50µl,

consisting 10µl reaction buffer, 10µl GC enhancer, 200nM forward and reverse primers (f3278 and r4032), 100µM dNTPs, 1U of Q5 polymerase and 21.5µl nuclease-free water. Cycling conditions were; initial denaturation at 98ºC for 2min, followed by 25 cycles of 98ºC for 10s, 62ºC for 20s, 72ºC for 30s and a final elongation step of 72ºC for 2min.

Nested PCR was carried out under the same cycling conditions, with an additional 5 cycles for a total of 30. Primers f3307 and r4014 were used (Table 2.3).

2.18. Amplification of NS5A prior to next generation sequencing

Near full length NS5A gene (1474bp and 1378bp for Gt1a and Gt1b, respectively) was amplified using nested PCR. First round PCR was carried out using 5µl cDNA from the RT in a total volume of 50µl, consisting 10µl reaction buffer, 10µl GC enhancer, 200nM forward and reverse primers (f6057/f6084 and 1a_Rev_Out/1b_Rev_Out), 100µM dNTPs, 1U of Q5 polymerase and 21.5µl nuclease-free water. Cycling conditions were; initial denaturation at 98ºC for 2min, followed by 25 cycles of 98ºC for 10s, 62ºC (61ºC for Gt1b) for 30s, 72ºC for 1min and a final elongation step of 72ºC for 2min.

Nested PCR was carried out under the same conditions, but with an annealing temperature of 62ºC regardless of subtype and an additional 5 cycles for a total of 30. The primers f6129 and 1a_Rev_In/1b_Rev_In were used (Table 2.3). The primers were adapted from previously published sequences (McCormick et al., 2015, Paolucci et al., 2013).

2.19. Purification of NS3 and NS5A amplicons using AMPure XP

PCR products for next generation sequencing were purified using AMPure XP magnetic beads, as per manufacturer's instructions. AMPure XP beads were mixed with PCR product at a 1.8x concentration. A 5 minute drying step was incorporated after the second ethanol wash, to ensure the evaporation of any residual ethanol. Purified products were eluted in 40µl of nuclease-free water.

2.20. Quantification of DNA

The concentration of DNA was determined using the Quant-iT dsDNA HS assay kit (Invitrogen) on a Qubit 2.0 fluorometer, following manufacturer's instructions.

2.21. Library preparation using Nextera XT

NS3 and NS5A amplicons from each patient were pooled together in equimolar concentrations and diluted to a final concentration of 0.2ng/µl in nuclease-free water. These pooled samples were the input into the Nextera XT sample preparation kit (Illumina, San Diego, CA, USA). Manufacturer's instructions were followed throughout. Briefly, the protocol involved the 'tagmentation' of input DNA to produce fragmented regions of the DNA with adapter sequences at each end. The tagmented DNA underwent a PCR program of 12 cycles to amplify the DNA with dual-indexed primers. AMPure XP beads at a concentration of 0.5x were used to purify the products, before the samples were normalised, denatured to single stranded DNA and pooled into one library.

The pooled amplicon library was sent to the Centre for Genomics Research at the University of Liverpool, where sequencing was performed on an Illumina MiSeq (Illumina) using v2 reagents.

2.22. Analysis of next generation sequencing data

Preliminary processing of the sequence files from the Illumina MiSeq runs was conducted at the CGR. The raw FASTQ files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1 with the 3' end of any reads which matched the adapter sequence for ≥3bp being trimmed (Martin, 2011). The reads were further trimmed using Sickle version 1.200 with a minimum window quality score of 20 (Joshi and Fass, 2011). Reads shorter than 10bp after trimming were removed.

The trimmed reads were analysed using the variant detection pipeline VirVarSeq (Verbist et al., 2015). The VirVarSeq pipeline utilises several steps to call variants at a codon level from reads obtained from Illumina next generation sequencing platforms. Briefly, the reads are aligned to a reference genome (H77 for Gt1a and HCV-J for Gt1b). Using this alignment, a consensus sequence for the sample is determined and the reads are realigned against this to increase the accuracy of mapping. Finally, a codon table is constructed, which utilises both the quality of the run and quality of individual bases to filter poor quality bases and reduce the number of false positives.

Chapter Three

Optimisation of techniques for the detection and genotyping of HCV

Part of the work presented in this chapter has been published in the article:

King, S., Adjei-Asante, K., Appiah, L., Adinku, D., Beloukas, A., Atkins, M., Sarfo, S. F., Chadwick, D., Phillips, R. O. & Geretti, A. M. 2015. Antibody screening tests variably overestimate the prevalence of hepatitis C virus infection among HIV-infected adults in Ghana. *J Viral Hepat*, 22(5), 461-8.

3. Optimisation of techniques for the detection and genotyping of HCV

Abstract

Background Testing for HCV RNA is not routinely available in resource-limited settings such as sub-Saharan Africa (SSA). Cheaper and novel HCV diagnostic approaches are required to improve access to HCV testing in such areas. The aims of this chapter were to i) optimise the use of pooled plasma and dried plasma spot (DPS) specimens for the detection of HCV RNA ii) Optimise the use of DPS for the sequencing of HCV for genotyping purposes.

Methods The WHO HCV RNA International Standard was used to produce dilution series' of plasma and DPS samples positive for HCV RNA, enabling the determination of the sensitivity of the real-time PCR protocol using single and pooled specimens. Sequencing protocols targeting the core and NS5B region of HCV were tested on extracts from clinical plasma and DPS specimens.

Results The real-time PCR protocol was found to have a sensitivity of 110 IU/ml and 2500 IU/ml for single plasma and DPS specimens, respectively. Sensitivity was reduced to 550 IU/ml and 5000 IU/ml when specimens were pooled in groups of five. The genotypes and subtypes determined by phylogenetic analyses of the sequencing products matched those provided by the clinic and did not differ between the two testing matrices.

Conclusion Pooling plasma and dried plasma spots represents a viable diagnostic strategy for resource-limited settings, reducing cost and workload whilst still retaining sensitivity. The recovery of nucleic acid from DPS for use in sequencing is also possible.

3.1. Introduction

During the initial acute phase of HCV infection, there is a period of approximately 7-8 weeks before the development of anti-HCV antibodies by the immune system of the host (Bowen and Walker, 2005). Prior to seroconversion, infection can be diagnosed by assays that directly target the virus, typically nucleic acid amplification technology (NAT) for the detection of HCV RNA (Centers for Disease Control and Prevention, 2013). In subjects that have already developed anti-HCV antibodies, HCV RNA testing is employed to confirm the presence of a current infection (Centers for Disease Control and Prevention, 2013). Real-time PCR is the most common NAT employed to detect HCV RNA in routine diagnostic settings. In addition, quantitative real-time PCR measures the HCV RNA load and plays an important role in the clinic by guiding antiviral therapy and measuring its efficacy. Commercial real-time PCR assays are available in kit format from several manufacturers, although these are often expensive to use.

Estimates of the global distribution of HCV place a large – albeit geographically variable – proportion of chronic infections in resource-limited settings in Africa and Asia (World Health Organisation, 2015). Testing for HCV RNA is not routinely available in sub-Saharan Africa (SSA), reflecting limited infrastructure for molecular testing and financial restrictions. Cheaper HCV diagnostic algorithms are required to improve HCV ascertainment in these settings. Furthermore, novel diagnostic approaches are required to promote access to HCV testing among difficult-to-reach populations in developed regions (Hope et al., 2011).

Dried blood spots (DBS) and dried plasma spots (DPS) have been used to screen populations for a range of infectious diseases, including HIV and HCV (Parker and Cubitt, 1999, Waterboer et al., 2012). They offer certain advantages over serum or plasma for testing, including nucleic acid stability at room temperature, facilitating the shipment of samples to centralised testing facilities (Bennett et al., 2012). Pooling samples for testing represents another cost- and labour-saving strategy to screen a large number of samples, especially where prevalence of infection is low. The pooling strategy has been successfully applied to plasma samples (Smith et al., 2009). A few studies have employed pooled DBS or DPS for the detection of HIV (Lakshmi et al., 2011, Pannus et al., 2013, van Zyl et al., 2011).

The HCV RNA genome is extremely variable (Elena and Sanjuan, 2005). HCV is currently divided into seven genotypes, with each of these further categorised into subtypes. The HCV genotype guides clinicians to the most appropriate therapy; even the subtype influences the efficacy of treatment, as seen in the case of subtypes 1a and 1b (European Association for the Study of the Liver, 2015). Sequencing of the HCV genome can be used to assign the genotype and subtype, and to identify mutations associated with drug resistance. It also provides precious epidemiological information, including the identification of transmission networks and novel strains (Sandres-Sauné et al., 2003).

Specific regions of the HCV genome are conserved due to their essential role in virus infectivity and replication, allowing pan-genotype primers to be designed for the purposes of HCV RNA amplification. One of these is the core region, which forms the viral nucleocapsid and rearranges the distribution of lipid droplets that are

essential for the formation of infectious virions (Boulant et al., 2005, Boulant et al., 2006). Another conserved region is NS5B, which encodes the RNA-dependent RNA polymerase enzyme, responsible for the replication of the HCV genome (Lindenbach and Rice, 2005). Although conserved, these two genes also retain sufficient diversity to allow the accurate identification of HCV genotypes and subtypes.

The aim of the studies presented in this chapter was to optimise the use of individual and pooled plasma and DPS for the detection of HCV RNA by real-time PCR, whereby pooling was explored as a strategy to reduce labour and costs, while still maintaining accuracy. A further aim was to optimise the recovery of HCV RNA from DPS to allow reliable sequencing for genotyping and molecular epidemiology investigations.

3.2. Methods and Results

3.2.1. Optimisation of the real-time PCR assay with plasma

The first step was the comparison of two extraction protocols using the NucliSens easyMAG automated extractor (bioMérieux, Boxtel, Netherlands). For this purpose, 20 replicates of the 4th World Health Organisation (WHO) International Standard for HCV RNA (06/102, NIBSC, Potters Bar, UK; HCV genotype 1a) in basematrix (SeraCare, Milford, MA, USA) were tested, each at a concentration of 100 IU/ml. Each 1ml replicate was mixed with 2ml Nuclisens lysis buffer (bioMérieux) containing 40µl Proteinase K (Qiagen, Hilden, Germany) and Phocine Distemper Virus (PDV) as an internal control (IC) prior to incubation. The first protocol utilised a single incubation at 56^{o}C for 30 minutes. The second protocol employed a second incubation at 100^{o}C for 30 minutes immediately following the first. The easyMAG Specific B 2.0.1 setting was used, with the elution volume set at 60µl. The real-time PCR conditions were those described in Chapter 2.5. The extraction protocol employing a single incubation step yielded a HCV RNA detection rate of 10/20 (50%). The addition of a second incubation step improved the HCV RNA detection rate to 19/20 (95%). The second protocol was therefore adopted for further experiments.

The second step was the evaluation of the sensitivity of the real-time PCR assay. For this purpose, 40 replicates of the WHO HCV RNA Standard in basematrix (SeraCare) were tested at concentrations of 10 IU/ml, 50 IU/ml, 100 IU/ml, and 110 IU/ml. The HCV RNA detection rate (1 ml input) ranged from 12/40 (30%) at 10 IU/ml to 40/40 (100%) at 110 IU/ml (Figure 3.1).

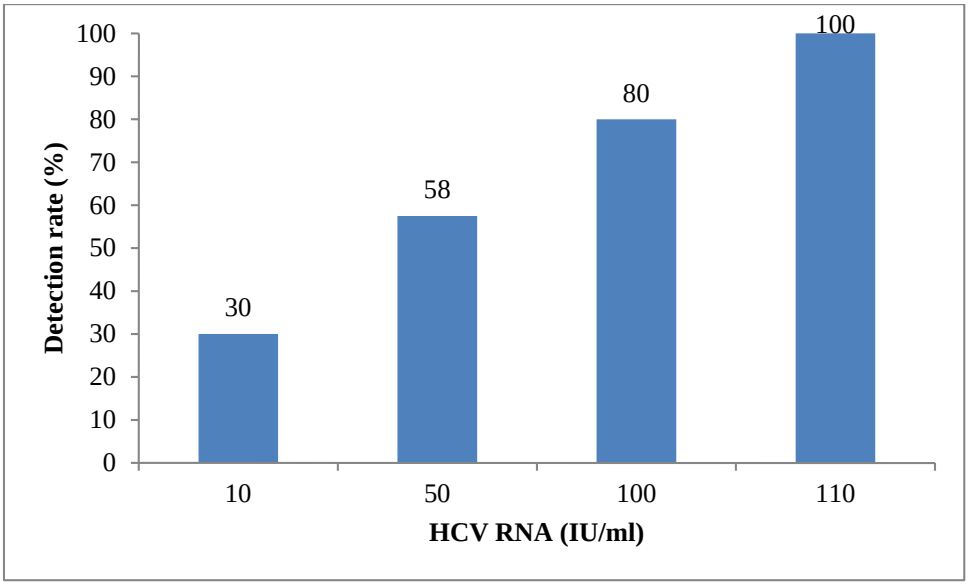

Figure 3.1. HCV RNA detection rate using serial dilutions of the WHO International Standard in basematrix. Each dilution underwent nucleic acid extraction (1ml input, two incubation steps), followed by real-time PCR. Numbers represent the percentage of positive tests in each series of 40 replicates.

3.2.2. Optimisation of HCV RNA detection in DPS

Dried plasma spots (DPS) were prepared by spotting 50µl of plasma onto each of five 12mm discs on a Protein saver 903 card (Whatman, Maidstone, UK). DPS were dried at room temperature for a minimum and maximum of 12 hours and 18 hours, respectively, before being placed into individual sealable plastic bags with silica desiccant sachets. DPS were then stored at -80^{o}C until needed. Upon being taken out of the freezer, DPS were left at room temperature for 30 minutes before being removed from bags to prevent the formation of condensation.

A dilution series of DPS was prepared in duplicate with WHO HCV RNA Standard at concentrations ranging from 100,000 IU/ml to 100 IU/ml. Prior to testing, the DPS underwent elution in an appropriate medium to allow the release of HCV RNA from the paper. Two different elution protocols were assessed for the recovery of HCV RNA from DPS, adapting approaches used in previous studies (Ayele et al., 2007, Andreotti et al., 2010, Tuaillon et al., 2010, Bennett et al., 2012, Ross et al., 2013, Shepherd et al., 2013). In the first protocol, two 6mm DPS punches per sample were eluted in 1ml 1xPBS with 0.5% Tween for 2 hours at room temperature with gentle agitation on a roller-mixer. In the second protocol, two 6mm DPS punches per sample were eluted in 3ml Nuclisens lysis buffer (bioMérieux) for 2 hours at room temperature with gentle agitation on a roller mixer. Both elution media contained 40µl Proteinase K (Qiagen) and PDV as IC. The eluted samples were then tested for HCV RNA by the real-time PCR assay. After elution in PBS/Tween, no HCV RNA was detected at any of the concentrations tested, including the highest viral load of 100,000 IU/ml. In contrast, elution in Nuclisens lysis buffer resulted in HCV RNA detection at viral loads down to 1000 IU/ml. The lysis buffer protocol was therefore adopted for further experiments.

Assay sensitivity was verified by testing DPS prepared with the WHO HCV RNA Standard in basematrix (Seracare) at concentrations ranging from 500 to 2500 IU/ml. DPS were prepared with 50µl of the appropriate dilution and two 6mm punches per specimen were eluted and extracted according to the method optimised above. Thirty replicates of each dilution were tested. The HCV RNA detection rates were 30/30 (100%) at 2500 IU/ml, 29/30 (97%) at 2000 IU/ml, 21/30 (70%) at 1000 IU/ml, and (9/30) 30% at 500 IU/ml (Figure 3.2).

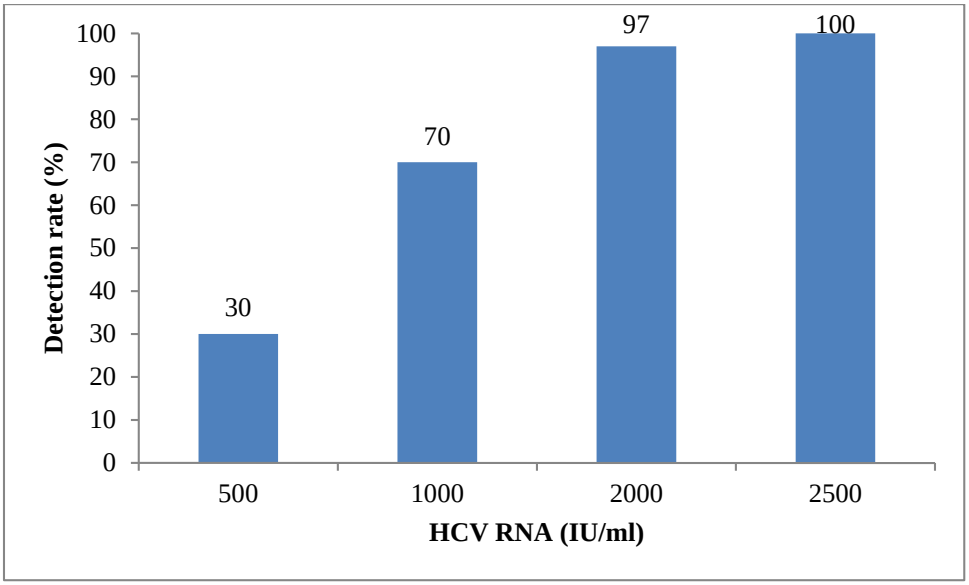

Figure 3.2. HCV RNA detection rate in individual dried plasma spots prepared with standard in serial dilutions. Input was 50µl per disc and two 6mm punches from each disc were tested. Numbers represent the percentage of positive tests in each series of 30 replicates.

Inter-assay and intra-assay variation was calculated using DPS prepared with 10,000 IU/ml of the WHO HCV RNA Standard in basematrix (Seracare) and tested in five replicates in three independent experiments. The C_T values showed an intra-assay coefficient of variation (CoV) ranging from 0.8% to 1.4% across the three experiments (Table 3.1), whilst the mean inter-assay CoV from three experiments was 1.0% (Table 3.2).

Table 3.1. Intra-assay variation of the real-time PCR protocol when using DPS. Numbers given for each replicate are the C_T values. CoV = Coefficient of variation

Assay Number	Replicate					Mean C_T value	Standard Deviation	CoV (%)
	1	2	3	4	5			
1	37.5	37.7	37.9	37.7	38.4	37.9	0.34	0.9
2	39.3	38.6	38.2	38.0	39.0	38.6	0.54	1.4
3	37.9	37.6	38.0	38.4	38.1	38.0	0.29	0.8

Table 3.2. Inter-assay variation of the real-time PCR protocol when using DPS. Numbers given for each replicate are the C_T values. CoV = Coefficient of Variation

Assay Number			Mean C_T Value	Standard Deviation	CoV (%)
1	2	3			
37.9	38.6	38.0	38.2	0.38	1.0

3.2.3. Optimisation of pooling strategy

Based upon the earlier finding of a lower limit of detection of 110 IU/ml with a single plasma specimen, it was assumed that a lower limit of detection of 550 IU/ml would be obtained by pooling five plasma specimens of 200µl each, which was considered acceptable for screening an untreated HCV positive population. In order to test this assumption, mock plasma pools were prepared and tested in triplicate,

each comprising one HCV RNA positive specimen and four negative specimens. HCV negative plasma was spiked with the WHO HCV RNA Standard at concentrations of 500 IU/ml, 550 IU/ml, 1000 IU/ml, 5000 IU/ml and 10000 IU/ml, and each spiked sample was combined with equal volumes of four negative plasma specimens to make up mock pools of 1 ml each. In triplicate testing, HCV RNA was detected in all pools containing a positive sample with HCV RNA concentration ≥550 IU/ml, and in 2 of 3 pools containing the 500 IU/ml sample (Table 3.3).

Table 3.3. Sensitivity of the real-time PCR assay for the detection of HCV RNA in pools of five specimens. NT=not tested

Viral load of positive sample in pool (IU/ml)	Detection rate in plasma pools (%)	Detection rate in DPS pools (%)
500	2/3 (67)	NT
550	3/3 (100)	NT
1000	3/3 (100)	NT
5000	3/3 (100)	3/3 (100)
10000	3/3 (100)	3/3 (100)

To determine the number of DPS samples that could be pooled together without a detrimental effect on assay sensitivity, mock DPS pools comprising one positive specimen and a further one to four negative specimens were prepared. The positive DPS specimen was prepared using HCV RNA negative plasma spiked with the WHO HCV RNA Standard at a concentration of 10,000 IU/ml. Two 6mm punches from each specimen were used per pool. Each of the pools was detected as HCV RNA positive, with little variation in the C_T values between the pool containing two specimens and the pool containing five specimens. Five DPS specimens were

deemed to be a suitable number of samples to pool together to reduce the cost whilst still maintaining an appropriate level of sensitivity.

To verify the assay sensitivity of pooled DPS specimens, mock pools comprising five samples were tested in triplicate. Pools consisted of one WHO International Standard at 5000 IU/ml or 10000 IU/ml and four negative specimens and were eluted and extracted according to the method optimised above. The pool containing one positive DPS prepared with 5000 IU/ml international standard and four negative specimens was HCV RNA positive in all three replicates (Table 3.3), providing a suitable sensitivity for screening a treatment-naïve population.

3.2.4. Genotyping by sequencing optimisation

Clinical plasma samples positive for HCV RNA genotypes 1a, 2b, 3a, and 4d were obtained from the Royal Liverpool Hospital, UK. DPS samples were prepared with 50µl of plasma as described above. Two methods were tested for the extraction of plasma and DPS specimens prior to amplification for NS5B genotyping. One method was to use the two-step extraction protocol described above for the real-time PCR assay. The other extraction method used the Qiagen Viral RNA Mini kit (Qiagen), following manufacturer's instructions. For DPS extraction with the Qiagen Viral RNA Mini kit, the initial incubation step with AL lysis buffer was increased to 30 minutes. For DPS, two 6mm punches were extracted for each specimen. Interestingly, the NS5B region was not amplified from either plasma or DPS when following the extraction protocol used for the real-time PCR assay. The extracts from the Qiagen kit were successfully amplified for both plasma and DPS

specimens, so this method was chosen for the future genotyping of clinical specimens.

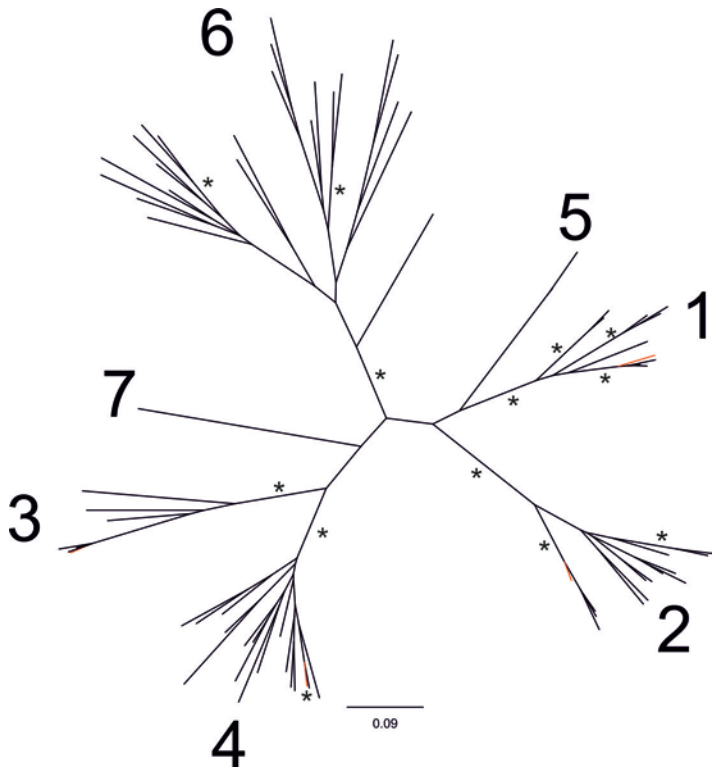

Figure 3.3. Phylogenetic tree displaying the relationship between the NS5B sequences of test samples (highlighted red) and genotype reference sequences. The number shown alongside clusters represents the genotype. Bootstrap values ≥80% are shown.

The protocols for the amplification and Sanger sequencing of core and NS5B were those described in Chapter 2. Sequences obtained from plasma samples were aligned against reference sequences from the Los Alamos Database and phylogenetically analysed (pipeline in Chapter 2.13).

The genotypes/subtypes determined through the NS5B (Figure 3.3) and core sequencing from plasma samples were the same.

HCV NS5B sequences obtained from DPS were compared to those recovered from plasma samples. The assigned genotypes and subtypes with plasma and DPS were identical to each other and to those provided by the reference diagnostic laboratory.

When comparing plasma and DPS, upon alignment, the sequences showed identities of 99.7% to 100%. The differences observed were always mixed bases versus a single base e.g. C/T versus T, although these differences were rare occurring a maximum of one time per alignment.

3.3. Discussion

The data presented in this chapter relate to the optimisation of a labour- and cost-effective methodology for large volume HCV screening. We first focused on the optimisation of the in-house real-time PCR assay for HCV RNA detection. Using plasma, we found that the efficiency of the extraction process was markedly increased when a second incubation step of 100^{o}C was added to the protocol. This may be due to the fact that a higher temperature relaxes the secondary structure of the HCV RNA target region, thereby increasing the binding capabilities of primers in the subsequent PCR reaction. A similar effect was previously seen when testing plasma for HBV DNA. Prior to easyMAG extraction, an incubation of 80^{o}C for 10 minutes improved detection rate compared to a 37^{o}C incubation period for 30 minutes (Gobbers et al., 2001).

We then evaluated the performance of the real-time PCR assay with DPS. When using DPS or DBS, the nucleic acid must be eluted from the filter paper prior to extraction. Although elution in PBS and Tween has been previously used to elute HCV RNA from DBS, this approach resulted in no amplification from any of the samples tested despite a relatively high HCV RNA load. This was in contrast with the excellent yield of elution in the Nuclisens lysis buffer. It should be noted that previous studies (Ross et al., 2013, Shepherd et al., 2013) incubated DBS in PBS overnight before the extraction process, differing from the 2 hours tested in this study. This shortened incubation time may be the key determining factor in the lack of detection. However, we were interested in a reduced incubation time to facilitate use in resource-limited settings where power interruptions during the agitation step may be an issue. At the shortened incubation time, the PBS may not be a strong

enough agent to elute sufficient viral nucleic acid material from the filter paper. The previous studies used PBS as an eluting agent for DBS rather than the DPS used in this study. One possibility is also that the difference in composition between blood and plasma drives the difference in efficacies between the eluting agents.

Real-time detection of HIV-1 RNA from DPS has been documented (Cassol et al., 1997, Brambilla et al., 2003, Ayele et al., 2007, Rottinghaus et al., 2012). In contrast, there is a paucity of data available on HCV RNA testing using DPS. Whilst some studies have observed strong correlations between HIV viral load measurements in plasma and DPS (Cassol et al., 1997, Brambilla et al., 2003, Ayele et al., 2007), there is also evidence that suggests DPS show greater variability and underestimate the viral load of samples, showing a significantly lower mean HIV viral load than plasma samples (Rottinghaus et al., 2012). Many studies on DBS for the detection of HCV only used the technique for a qualitative assessment (Solmone et al., 2002, De Crignis et al., 2010, Bennett et al., 2012, Santos et al., 2012), as we have done in this study. This does not affect the assay's performance at diagnosing an active infection.

The lower limit of detection with single plasma samples was found to be 110 IU/ml while individual DPS specimens showed a lower limit of detection of 2500 IU/ml with an input of two 6mm punches. This lower sensitivity is in concordance with previous studies employing DBS for the detection of HCV RNA, which observed limits of detection of 250 IU/ml to 4830-24160 IU/ml (Bennett et al., 2012, Rottinghaus et al., 2012). The difference in sensitivity between plasma and DPS is easily explained by the much lower input into the reaction when using DPS. A DPS sample with a viral load of 100,000 IU/ml would contain only 5000 IU, as the input

into each of the DPS circles is 50µl. In addition to this, only two 6mm punches are used in the extraction. Assuming an even distribution of viral particles over the area of the dried spot, each 6mm punch would hold approximately 1250 IU, yielding a total input of around 2500 IU in each reaction. This lower sensitivity is in concordance with previous studies observing DBS, with researchers concluding the limited sample size obtained from DBS and less efficient nucleic acid extraction were the contributing factors (Bennett et al., 2012, Santos et al., 2012). An increase in the number of DPS input into the reaction may counteract this effect. However, it would also increase the number of DPS to be collected, negating the benefit that only a small sample volume is required for DPS.

A high sensitivity of detection was maintained when five samples were pooled together for extraction and testing. There was a dilution effect when pooling plasma samples, due to a five-fold reduction in input per sample. However, DPS pools did not experience such a phenomenon. An explanation for this is that under the set conditions, the lysis buffer effectively eluted available nucleic acid from the DPS without saturation effects leading to a loss of sensitivity.

The topic of HCV RNA stability in DBS and DPS at room temperature has been studied relatively little, given the importance that this plays in the role of this testing matrix. It should be noted that the term room temperature is non-specific and would vary widely from country to country. The studies which have explored this have found conflicting results although the lack of information regarding environmental conditions such as humidity and specific temperatures between the studies make it more difficult to draw firm conclusions. Two studies found a decrease in RNA; one

observed a ten-fold decrease after a period of 4 weeks (Abe and Konomi, 1998) and another determined a three-fold decrease after 6 days (Tuaillon et al., 2010). These reductions in HCV RNA would have to be taken into consideration when planning the testing procedure. While it is important to ensure optimal sample preservation, for diagnostic purposes, any degradation may not be as detrimental in an untreated patient cohort given the likely high viral loads. Two further studies found no significant decrease in RNA levels of DBS stored at room temperature and tested intermittently over one year and 11 months, respectively (Solmone et al., 2002, Bennett et al., 2012). The DPS used in this study were stored at -80^{o}C until required so the stability of the RNA should not be an issue, giving accurate results during the optimisation. In resource-limited settings, immediate transportation of specimens may not be possible. Alongside this, room temperature in SSA is likely to be significantly higher than the European countries in which the above studies took place. Consequently, in order to ensure the preservation of samples, short-term cold storage of samples at -20^{o}C may be required until shipment to a centralised facility could be arranged. However, the effect of freeze-thaw on DPS samples should first be investigated further.

This real-time PCR assay was developed to screen a population of HIV-infected individuals from Ghana. A previous study from blood donors in Ghana identified a median viral load of 2.5 x 10^5 IU/ml (range 3.3 × 10^3 to 9.0 × 10^6 IU/ml) (n=20) (Candotti et al., 2003). Another study states that the majority of untreated patients have viral loads above 1000 IU/ml (Tuaillon et al., 2010). Both of these figures suggest that plasma and DPS, either single specimens or pools, are suitable testing matrices for this assay and for the initial diagnosis of the infection.

A previous study (Lakshmi et al., 2011) extracted DBS separately before pooling the extracts for the real-time PCR assay whereas our study both extracted and amplified pools of DPS. This strategy reduces the extraction costs, an important consideration for resource-limited areas. The use of pooled samples in diagnostic testing for HCV, as used in this study, has the potential to reduce costs by up to five-fold. A previous study demonstrated that $345,000 per year could be saved through the use of pooled DBS samples in HIV viral load testing in Malawi (Pannus et al., 2013). The exact saving from the tests used here would be dependent on the prevalence of HCV in the population undergoing screening. A cohort with a high percentage of HCV RNA positive samples would not reduce costs if real-time PCR was used as the initial test. Appropriate use of serological screening may first reduce the number of samples to be tested by pooled real-time PCR by eliminating antibody-negative samples.

Although real-time PCR represents a sensitive and specific test for the diagnosis of active HCV infection, the specialised equipment required does present an obstacle in resource limited settings. Loop-mediated isothermal amplification (LAMP) is a recent development, which overcomes this obstacle (Notomi et al., 2000). It employs a constant temperature and a positive result can be identified by either a change in turbidity or a fluorescence visible to the naked eye (reviewed in Mori and Notomi, 2009), negating the requirement for specialised equipment. These features make it a suitable candidate for routine clinical diagnostics in resource limited settings. Previous studies have explored the use of this technique for the detection of HCV (Wang et al., 2011, Yang et al., 2011, Kargar et al., 2012). LAMP uses multiple primers with amplification only occurring upon the binding of all primers to the

target regions, making it highly specific (Notomi et al., 2000). However, this may present a problem in sub-Saharan Africa where there is a large degree of variation in HCV subtypes (Jeannel et al., 1998, Candotti et al., 2003, Markov et al., 2009). Variation in one of the target regions would restrict amplification and thereby give a false negative result.

The methods employed for detection of HCV each have their own advantages and disadvantages. Immunoassays targeting anti-HCV antibodies are relatively cheap and require little infrastructure, making them a suitable diagnostic tool for sub-Saharan Africa. However, they rely on the presence of anti-HCV antibodies, which do not appear until approximately 7-8 weeks after infection (Bowen and Walker, 2005). In some African cohorts, their specificity has also been questioned with high rates of false positive results (Chasela et al., 2012, Mullis et al., 2013). Such tests also cannot distinguish between an active and resolved infection, meaning that further diagnostic procedures are required following a positive antibody test in order to confirm infection. Rapid screening assays also target anti-HCV antibodies but can use a much smaller sample size i.e. finger stick blood specimens making them a suitable candidate for hard-to-reach populations. Similar sensitivities and specificities between rapid tests and immunoassays have been observed although HIV-positive patients were more likely to have a false result when tested with a rapid test (Smith et al., 2011).

Nucleic acid amplification techniques remain the most reliable procedure for the diagnosis of an active HCV infection. As their target is the nucleic acid of the virus, they can provide a diagnosis from a very early time during infection, before

antibodies have appeared (Centers for Disease Control and Prevention, 2013). However, these tests are often expensive and require specialised equipment.

Non-specific tests such as the measurement ALT levels provide an indication of liver damage although this is not necessarily due to HCV or even infection with a virus. However, as these tests are relatively cheap, the identification of patients with an elevated ALT may aid in reducing the number of patients targeted for HCV screening with further tests. A previous study examining patients with a single elevated ALT result observed an anti-HCV prevalence of 8.4% (Smith and Yartel, 2014). However, an estimated 30%-50% of patients infected with HCV demonstrate persistently normal ALT levels (Alberti et al., 2002, Puoti, 2003), meaning they would not be identified for screening.

Unfortunately, there does not seem to be a definitive method of diagnosis which meets all of the requirements for a resource-limited setting such as SSA, especially given the genetic variation observed in this region. However, the combination of more than one method alongside the careful interpretation of results may provide the most reliable results.

NS5B sequences of 329bp in length provide sufficient distance between samples when phylogenetically analysed to allow the interpretation of 99.8% of strains (Sandres-Sauné et al., 2003). Consequently, the NS5B sequencing protocol used here, which produces an amplicon with a maximum length of 380bp is more than sufficient for subsequent phylogenetic analysis to determine genotype and subtype. The use of a sequencing protocol provides researchers with an electropherogram which can be manually inspected, if a mixed infection is suspected. Previous studies have successfully sequenced HIV-1 RNA recovered from DPS for the purposes of

genotyping and monitoring drug resistance (Dachraoui et al., 2008). Genotyping HCV from DBS using sequencing has been established (Solmone et al., 2002, Tuaillon et al., 2010) but, thus far, DPS have not been used for this purpose. Previous studies have demonstrated the ability to obtain a sufficient quantity of HCV RNA from DBS for amplification and sequencing and a strong concordance between the HCV genotypes obtained from the sequencing of plasma and DBS samples has been shown (Solmone et al., 2002, Tuaillon et al., 2010). This study has demonstrated that HCV RNA of sufficient quality can be recovered from DPS for sequencing. Thus, the method has the potential for use in determining the molecular epidemiology of HCV infections.

Many of the commercial assays employ either fluorescent oligonucleotide probes to bind to specific region of the genome or reverse hybridisation technology. These tests are able to differentiate between genotypes but are often not able to distinguish a subtype level. Previous studies comparing genotyping by sequencing to commercial assays have found good concordance between the two methods with regards to the determination of genotypes. However, at the subtype level of HCV, discordance levels were as high as 47.9% (Nakatani et al., 2011). Another study found that HCV subtypes were only assigned to 47.2% of samples when using a commercial line probe assay compared to 95.8% when sequencing was used (Avo et al., 2013). Misclassification or non-assignment of subtype by commercial assay represents a problem in resource-limited settings such as SSA where previous work has shown a wide genome diversity among isolates (Ruggieri et al., 1996, Candotti et al., 2003, Ndjomou et al., 2003, Markov et al., 2009), displaying possible new subtypes. In such cases, commercial assays would fail to determine the subtypes of

the isolates, making the epidemiological study and identification of possible transmission networks impossible. Mixed infections may prove more difficult to genotype successfully as the more dominant of the two strains present will account for a higher proportion of the sequenced amplicons (Sandres-Sauné et al., 2003). However, commercial genotyping assays also encounter similar problems in mixed infections (Sandres-Sauné et al., 2003). The cost of commercial assays versus in-house sequencing also affects the uptake in resource-limited areas. The sequencing of PCR products has been shown to be three times cheaper than some commercial genotyping assays and also has the benefit of providing a large amount of information to the researcher (Lam et al., 2010).

In conclusion, this work identified and optimised two techniques which are important tools in the monitoring of HCV infection at both an individual and population level. The transfer of these techniques for use on DPS specimens represents an important step in reducing costs while still maintaining a high degree of sensitivity, allowing resource-limited settings to adopt the strategies in an effort to gain a definitive measure of the burden and epidemiology of HCV.

Chapter Four

Prevalence and molecular epidemiology of HCV infection in a Ghanaian HIV-positive cohort

Part of the work presented in this chapter has been published in the following article: King, S., Adjei-Asante, K., Appiah, L., Adinku, D., Beloukas, A., Atkins, M., Sarfo, S. F., Chadwick, D., Phillips, R. O. & Geretti, A. M. 2015. Antibody screening tests variably overestimate the prevalence of hepatitis C virus infection among HIV-infected adults in Ghana. *J Viral Hepat*, 22(5), 461-8.

4. Prevalence and molecular epidemiology of HCV infection in a Ghanaian HIV-positive cohort

Abstract

Background HIV co-infection with HCV has been poorly studied in Ghana and the reliability of current HCV seroprevalence estimates remains uncertain. The study aim was to determine HCV RNA prevalence in HIV-infected subjects receiving care in Kumasi, and assess the diversity of the circulating strains.

Methods From a population of 1520 HIV-infected adults undergoing hepatitis B surface antigen (HBsAg) testing, all HBsAg positive subjects (n=236) and a random subset of HBsAg negative subject (n=172) were screened for HCV RNA using pooled plasma. A further 875 HBsAg negative patients were tested for HCV RNA using pooled dried plasma spots. HCV RNA positive samples were genotyped by core and NS5B sequencing.

Results HCV RNA prevalence was 0.70% (95% confidence interval 0.24-1.16%) and 1.27% vs. 0.57% in HBsAg positive vs. HBsAg-negative subjects, respectively. Of the HCV positive subjects available for questionnaire, surgical procedures and blood transfusion were reported as risk factors for HCV infection. Circulating HCV strains were predominantly genotype 2 (subtypes 2c/r/j, 2l, 2q/l, unassigned) and a small minority of genotype 1 (subtype unassigned).

Conclusions The prevalence of HCV RNA was substantially lower than previous estimates using serological assays. A subset of co-infected subjects carried a HCV genotype 1 strain unique to West and Central Africa.

4.1. Introduction

HCV infection is a common co-infection in Western HIV-positive cohorts, reflecting shared transmission routes, predominantly injecting drug use (Kim et al., 2013). Sub-Saharan African (SSA) countries are thought to account for 32 million of the estimated 150 million cases of chronic HCV worldwide (Karoney and Siika, 2013, World Health Organisation, 2015). However, there have been few studies on HCV infection in SSA and the figures from previous studies vary depending on the testing methods used. As access to HCV RNA testing is limited in SSA, the majority of HCV prevalence studies have typically relied solely on antibody screening tests with few surveys employing the use of supplementary antibody testing or HCV RNA testing (Madhava et al., 2002, Agwale et al., 2004, Laurent et al., 2010, Mohd Hanafiah et al., 2013). A systematic review of 35 studies indicated a mean HCV seroprevalence of 7% among 9029 HIV-infected adults in SSA (Barth et al., 2010). However, there has been a wide variety in the results, with estimates ranging from 1.6% to 6% in the general population (Karoney and Siika, 2013) and up to 22% among HIV-positive patients (Barth et al., 2010). This disparity between studies is likely to reflect, at least in part, the variable performance of serological assays in the African context (Seremba et al., 2010, Mullis et al., 2013).

Ghana is a SSA country in West Africa with an estimated 250000 individuals infected with HIV in 2014, comprising a majority of HIV-1 and a subset of HIV-2 infections (UNAIDS, 2014b). Recent WHO guidelines recommend HCV serology testing for high-risk groups, which includes HIV-infected patients (World Health Organisation, 2014). Despite this, testing for HCV infection is not commonly undertaken in Ghana. A study published in the late 90s reported that 8% of 182 HIV-

positive subjects tested positive for HCV antibody (Brandful et al., 1999). More recently, HCV seroprevalence was nearly 4% among 138 HIV-positive adults that in 2007 were receiving care in the capital city of Accra (Sagoe et al., 2012). If proven to be accurate, the high estimates presented in these studies would indicate that without appropriate intervention and management strategies, a high burden of HCV co-infection threatens the long-term success of HAART programmes in the region. Large surveys are urgently required to determine the true burden of chronic HCV infection in Ghana and the rest of sub-Saharan Africa, and the associated risk factors.

The use of dried blood spots (DBS) for sample collection offers a modality for epidemiological surveys with the advantage of nucleic acid stability at room temperature (Solmone et al., 2002, Tuaillon et al., 2010, Bennett et al., 2012) and reduced biohazard risk, facilitating storage and transport to centralised molecular testing facilities. Whereas whole blood collection for DBS preparation is highly suitable for epidemiological surveys, there are circumstances where there may be access to plasma and dried plasma spots (DPS) may be used instead. These include circumstances when EDTA blood is processed locally for other tests such as HIV-1 RNA load monitoring during antiretroviral therapy, or when local plasma repositories exist that would require expensive shipment on dry ice to preserve sample viability during transport to molecular testing facilities.

One added advantage of DBS and DPS usage is that it also allows recovery of sufficient nucleic acid for sequencing and phylogenetics. The distribution of HCV genotypes and subtypes varies worldwide, with clear differences between regions. Western Europe and the USA have high proportions of genotypes 1 and 3 whereas in

Africa, genotypes 1, 2 and 4 are more commonplace (Simmonds et al., 2005). Previous investigations in Ghana and West Africa identified a dominance of genotype 2 HCV strains (Jeannel et al., 1998, Candotti et al., 2003, Markov et al., 2009), in comparison to a high proportion of genotype 4 in Central Africa (Ndjomou et al., 2003). Molecular characterisation of HCV enables the identification of possible transmission networks, thus aiding management and prevention strategies.

In order to obtain more definitive evidence on the rates of HIV/HCV co-infection in Ghana, the work outlined in this chapter investigated HCV RNA prevalence in a large HIV-positive cohort receiving care in Kumasi, the second largest city of Ghana using the techniques optimised in the previous chapter. Molecular analysis was used in a bid to understand the diversity of the circulating strains.

4.2. Materials and Methods

4.2.1. Study population

From a total of 1520 HIV-infected adults that attended the outpatient service of the Komfo Anokye Teaching Hospital in Kumasi between 2010 and 2012, all HBsAg positive subjects (n=236) and a random subset of HBsAg negative subjects (n=172) had plasma stored locally at -80^{o}C before transport on dry ice to the United Kingdom for HCV testing. A further 875 DPS were prepared locally from HIV-positive/HBsAg-negative patients using plasma collected for HIV-1 RNA testing and stored at -80^{o}C until use. DPS were prepared according to the protocol detailed earlier (Chapter 2.3) and shipped to the UK at room temperature, before storage at -80^{o}C. Retrospectively, subjects with confirmed HCV infection were invited to complete a structured questionnaire about risk factors for acquiring HCV. The study conformed to the standards of the Helsinki Declaration and was approved by the Committee on Human Research Publications and Ethics at the Kwame Nkrumah University of Science and Technology of Kumasi, Ghana (reference CHRPE/143/10). Written consent was obtained from participants.

4.2.2. HCV RNA detection

Plasma samples were initially screened for HCV RNA by pooling five samples (200µl each) into a single 1 ml specimen, followed by individual screening of the samples from RNA positive pools. Basematrix (SeraCare, Milford, MA, USA) was added to pools containing fewer than five specimens to maintain a total volume of 1ml. DPS specimens were initially screened for HCV RNA in pools of five

specimens prepared by combining two 6mm punches of each specimen. DPS from positive pools were then screened individually using two 6mm punches.

Nucleic acid elution, extraction and real-time PCR were performed as detailed in Chapter 2.

4.2.3. Sequencing of HCV core and NS5B regions

Nucleic acid extraction, amplification and sequencing were performed as described in Chapter 2. The primers for each region targeted conserved regions of the genome, allowing multiple genotypes to be amplified. Sequence electropherograms were manually inspected for quality, before undergoing phylogenetic analysis and being submitted to GenBank. The GenBank accession numbers of nucleotide sequences analysed in the study are KJ642622 to KJ642630 (core region) and KJ642631 to KJ642638 (NS5B region).

4.2.4. Phylogenetic and statistical analysis

HCV RNA prevalence in HBsAg positive vs. negative patients was compared by Fisher's exact test. Statistical analysis was performed using SPSS version 21 (IBM Corporation, Armonk, NY, USA). Sequences were aligned against reference sequences (genotypes 1-7) from the Los Alamos database for the purposes of genotyping. For subtyping, sequences were aligned against reference sequences from the appropriate genotype from throughout the world. The pipeline for phylogenetic analyses is described in Chapter 2.13. Assessment of sequence identity by pairwise alignment was performed using the EMBOSS Needle Nucleotide Alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html). The sequences were trimmed to the same size before pairwise alignment.

4.3. Results

4.3.1. Prevalence of HCV RNA in the plasma samples

From 1520 HIV-infected adults undergoing HBsAg testing, 236 consecutive HBsAg positive subjects and 172 HBsAg negative subjects were tested for HCV RNA using plasma samples. Overall, 270/408 (66.2%) subjects were females and the median (interquartile range, IQR) age was 40 (35-47) years.

HCV RNA was detected in 4/82 plasma pools, with each pool comprising five samples. Testing of the individual samples of positive pools detected one positive sample in each pool, yielding a HCV RNA prevalence of 0.98% (95% confidence interval, CI, 0.02-1.94%).

4.3.2. Prevalence of HCV RNA in DPS samples

Of the 1047 HBsAg negative subjects, 875 were tested for HCV RNA using DPS. HCV RNA was detected in 5/175 DPS pools, with each pool comprising five samples. Testing of the individual samples from the positive pools detected one HCV RNA positive sample in each pool, yielding a prevalence of 0.57% (95% CI, 0.07-1.07%).

The overall prevalence of HCV RNA in the entire cohort studied was 0.70% (95% CI, 0.24-1.16%). Overall, HCV RNA prevalence was 3/236 (1.27%; 0.0-2.7%) and 6/1047 (0.57%; 0.11-1.03%) in HBsAg-positive vs. HBsAg-negative patients, respectively (p=0.22). The difference in prevalence between plasma samples and DPS was not significant (p=0.477)

4.3.3. Molecular characterisation of HCV

The nine HCV RNA positive samples – four from plasma and five from DPS – underwent population sequencing of the HCV core and NS5B genes to determine the HCV genotypes present. Sequences from both genes were obtained from all four plasma samples and four of the five DPS specimens. However, one DPS sample did not yield an NS5B PCR product despite repeated attempts.

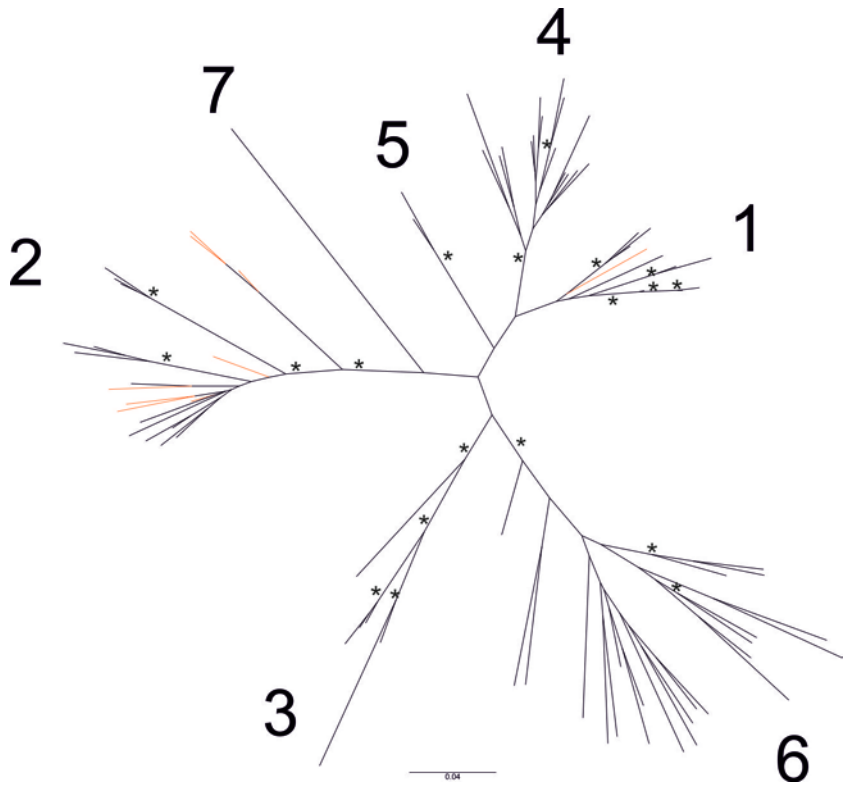

Figure 4.1. Phylogenetic relationship between HCV core sequences. Ghanaian sequences (in red) were aligned to reference sequences from the Los Alamos database. The seven major HCV genotypes (1-7) are indicated along the branches. Bootstrap values ≥80% are indicated by an asterisk.

Eight of the Ghanaian strains (89%) clustered with genotype (Gt) 2 reference sequences with a bootstrap value of 83.2% over 1,000 replicates for the core region (Figure 4.1). The remaining one strain (11%) clustered with Gt1 reference sequences with a bootstrap value of 81.1% over 1,000 replicates for the core gene. The results were concordant in NS5B sequences (Figure 4.2), with bootstrap values of 80.8% and 99.5% for Gt1 and Gt2 strains, respectively.

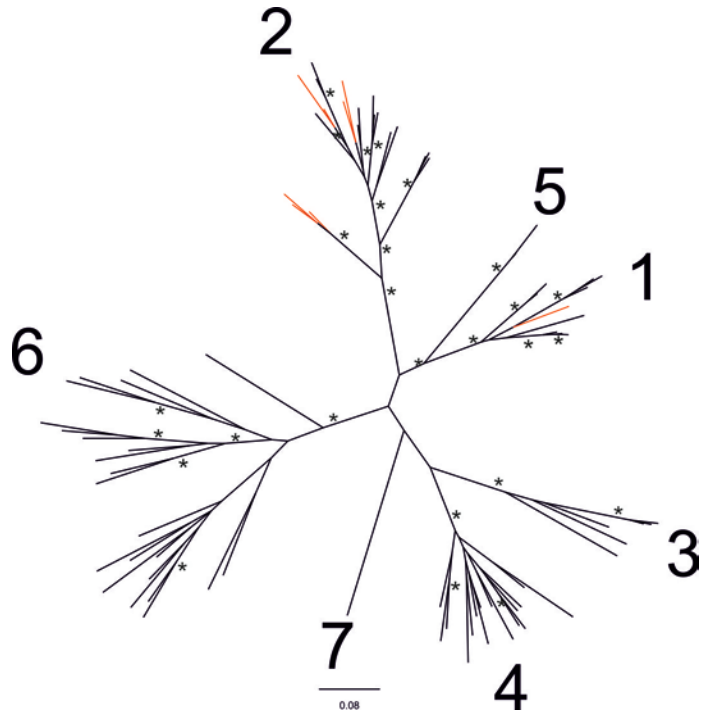

Figure 4.2. Phylogenetic relationship between HCV NS5B sequences. Ghanaian sequences (in red) were aligned to reference sequences from the Los Alamos database. The seven major HCV genotypes (1-7) are indicated along the branches. Bootstrap values ≥80% are indicated by an asterisk.

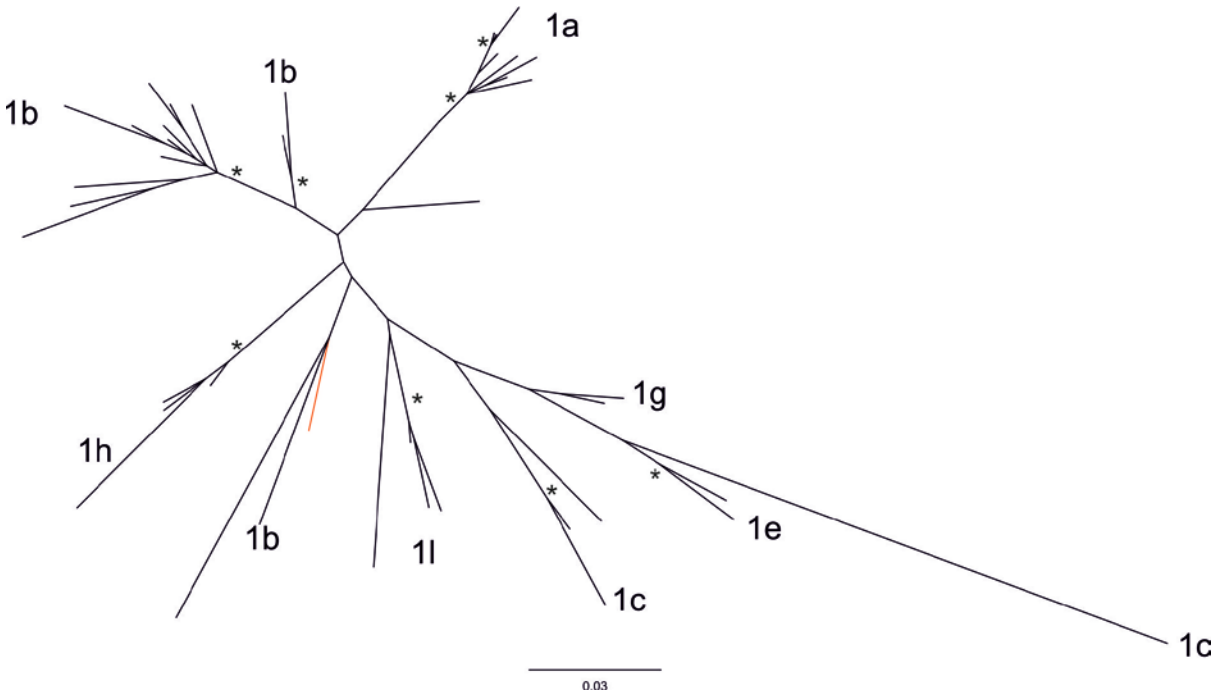

Figure 4.3. Phylogenetic relationship between HCV genotype 1 core sequences. The genotype 1 Kumasi sample (in red) was aligned against genotype 1 sequences from throughout the world obtained from the Los Alamos database. HCV genotype 1 subtypes are indicated along the branches. Nodes/clusters without a subtype label have an unassigned subtype. Bootstrap values ≥80% are indicated by an asterisk.

The Ghanaian Gt1 strain was distinct from Gt1 sequences from elsewhere in the world and did not cluster with any previously defined subtype with either the core (42.7% bootstrap value) (Figure 4.3) or NS5B sequence (38.3% bootstrap value) (Figure 4.4). There were two related African Gt1 sequences in the Los Alamos database, one from Nigeria (core; accession number U31232) and one from Equatorial Guinea (NS5B; accession number AJ851228), with sequence homologies of 92% and 86.1%, respectively (bootstrap values of 42.7% and 38.3%). A previously defined Gt1 strain from Kumasi, Ghana (Candotti et al., 2003) also

clustered with this strain in the core (bootstrap value 26.7%) analysis and shared sequence homology of 91.3%.

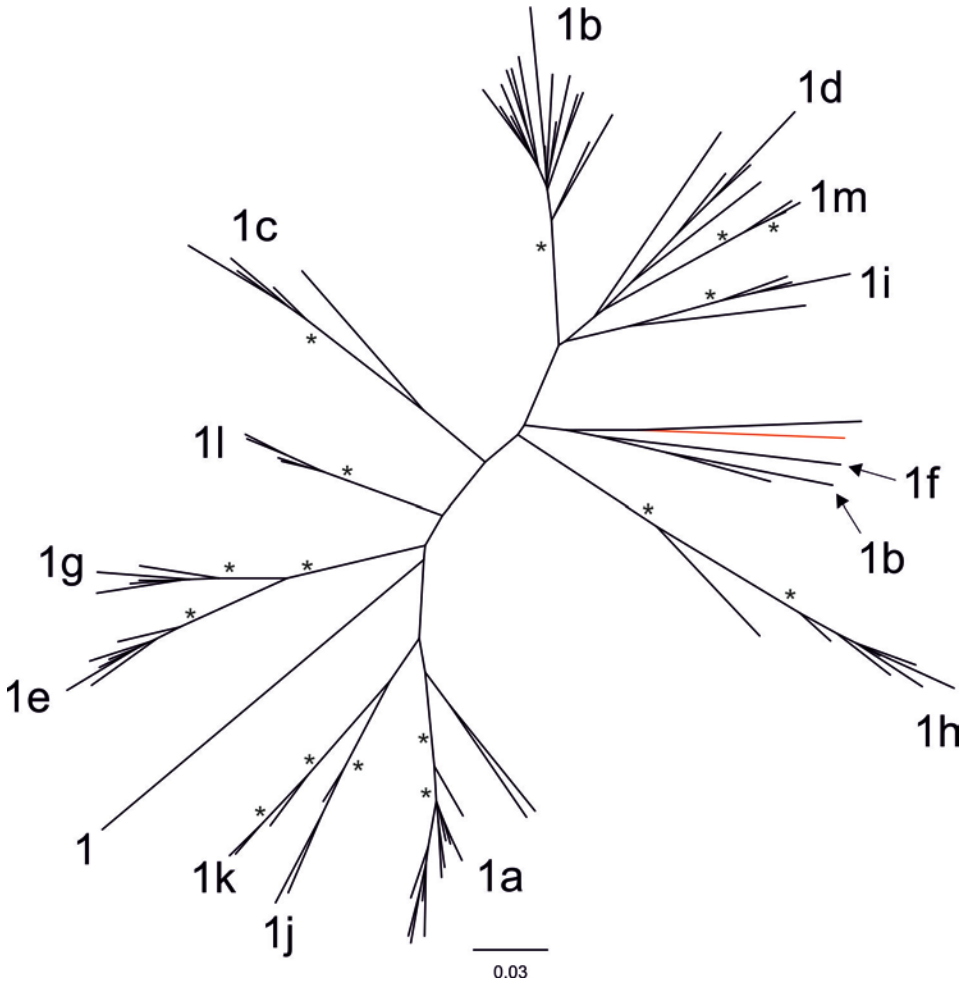

Figure 4.4. Phylogenetic relationship between HCV genotype 1 NS5B sequences. The genotype 1 Kumasi sample (in red) was aligned against genotype 1 sequences from throughout the world obtained from the Los Alamos database. HCV genotype 1 subtypes are indicated along the branches. Nodes/clusters without a subtype label have an unassigned subtype. Bootstrap values ≥80% are indicated by an asterisk.

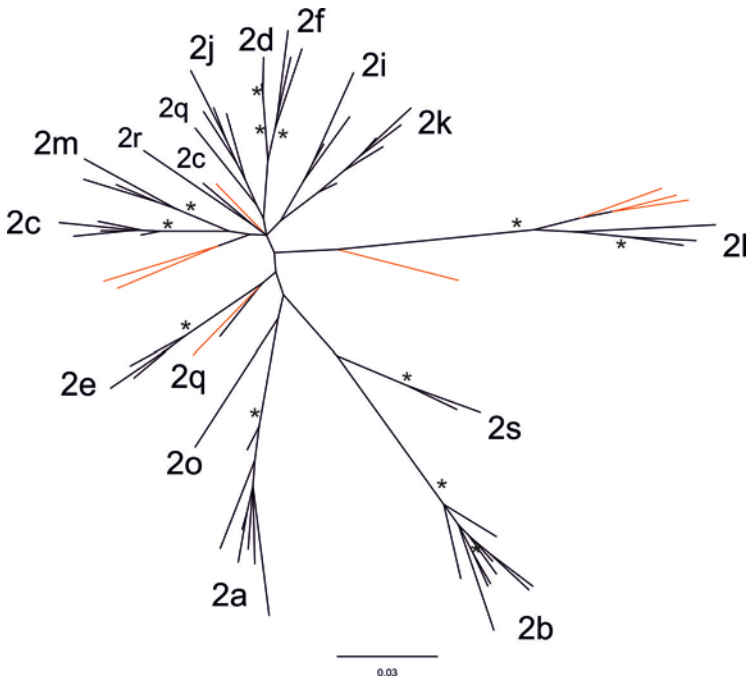

Figure 4.5. Phylogenetic relationship between HCV genotype 2 core sequences. The genotype 2 Kumasi samples (in red) were aligned against genotype 2 sequences from throughout the world obtained from the Los Alamos database. HCV genotype 2 subtypes are indicated along the branches. Nodes/clusters without a subtype label have an unassigned subtype. Bootstrap values ≥80% are indicated by an asterisk.

Upon further analysis against Gt2 sequences from throughout the world, the Ghanaian Gt2 strains displayed great diversity (Figures 4.5 and 4.6). Three of the strains (GH03, GH07 and GH08) clustered with subtype 2l (bootstrap values of 100% and 99.9% for core and NS5B respectively). The three strains core sequences shared an average sequence homology of 95.1% whilst the NS5B sequences averaged 89.2% sequence identity. A previously detected strain (core accession number AY236365; NS5B accession number AY236381) from a blood donor in Kumasi (Candotti et al., 2003) also clustered closely with these three strains in both core (100% bootstrap value) and NS5B (49.1% bootstrap value) analyses. GH03,

GH07 and GH08 showed sequence homologies of 91.6%/83.7%, 93%/85.1% and 91.8%/84% with the core/NS5B sequences of the aforementioned Ghanaian strain from a blood donor.

The other five Gt2 strains had less defined subtype assignment with poor bootstrap support. Two Ghanaian strains (GH04 and GH05) clustered together in both analyses sharing sequence homology of 92.7% and 88.5% in the core and NS5B genes, respectively. Unsubtyped strains previously identified in Kumasi blood donors (accession numbers AY236374, AY236375, AY236380, AY236386) clustered with these two Ghanaian sequences in the NS5B analysis (7.9% bootstrap value), whereas the two strains clustered on their own in the analysis of the core region (17.1% bootstrap value).

GH06 did not cluster with any known subtype (100% bootstrap value) in the core analysis. Two of the strains showed discordant results between core and NS5B analyses. The GH02 NS5B sequence clustered most closely with subtype 2j (bootstrap value of 20.6%), whereas the core sequence formed a cluster with two strains of subtypes 2c and 2r (5.9% bootstrap value). The second strain (GH09) clustered most closely with subtype 2q (39.7% bootstrap value) in the core analysis whilst the NS5B sequence clustered most closely with subtype 2l and an unassigned strain from a Kumasi blood donor (Candotti et al., 2003) (17.8% bootstrap value).

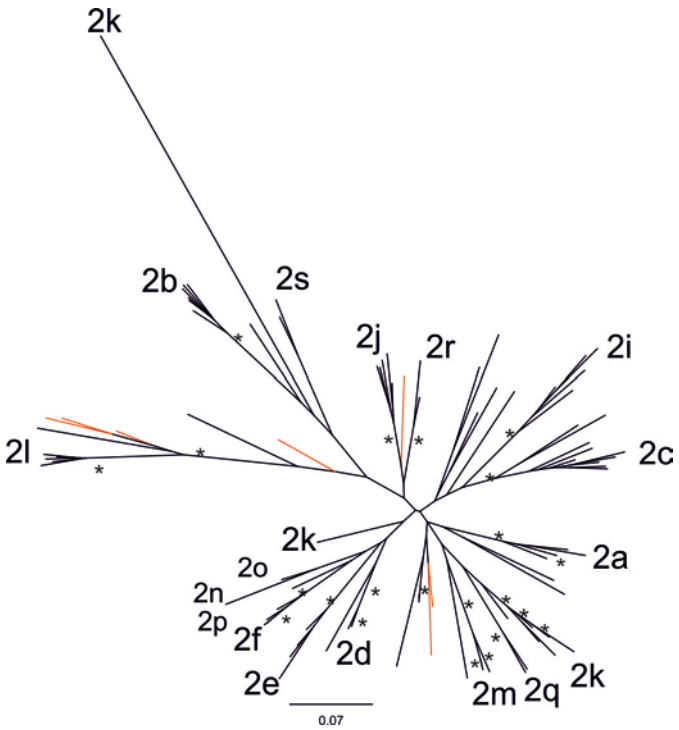

Figure 4.6. Phylogenetic relationship between HCV genotype 2 NS5B sequences. The genotype 2 Kumasi samples (in red) were aligned against genotype 2 sequences from throughout the world obtained from the Los Alamos database. HCV genotype 2 subtypes are indicated along the branches. Nodes/clusters without a subtype label have an unassigned subtype. Bootstrap values ≥80% are indicated by an asterisk.

4.3.4. HCV RNA positive subjects

Four of the HCV positive subjects with an active infection completed a questionnaire to determine risk factors associated with HCV infection in this population. The remaining five HCV positive subjects could not be contacted despite repeat attempts. The HCV positive individuals with demographic data available comprised two males and four females aged 25-46 years (Table 4.1). Using a structured questionnaire, the reported risk factors for HCV infection were surgical procedures and blood transfusion and these had occurred ≥12 months prior to HCV testing. No patient

reported any history of jaundice, intravenous or illicit drug use, needle stick injury, therapeutic or cosmetic cuts, or body tattooing.

Table 4.1. Demographic and clinical characteristics of the subjects from the Kumasi HIV cohort that tested HCV RNA positive

ID	Gender	Age	HBsAg status	CD4 count	ALT	HCV		
						Genotype	Core Subtype	NS5B Subtype
GH01	F	46	Negative	ND	ND	1	Unassigned	Unassigned
GH02	F	32	Positive	110	168	2	2c/2r	2j
GH03	M	43	Positive	441	50	2	2l	2l
GH04	M	41	Positive	385	44	2	Unassigned	Unassigned
GH05	NA	NA	Negative	ND	ND	2	Unassigned	Unassigned
GH06	NA	NA	Negative	ND	ND	2	Unassigned	NA
GH07	F	25	Negative	602	ND	2	2l	2l
GH08	F	26	Negative	207	ND	2	2l	2l
GH09	NA	NA	Negative	ND	ND	2	2q	2l

NA= Not available; ND = Not done

4.4. Discussion

This study is the first to employ HCV RNA testing to determine HCV prevalence in HIV-positive patients in Ghana. It is also the first to use DPS from a sub-Saharan African, resource-limited setting for HCV RNA screening and molecular characterisation. It was found that around 0.7% of subjects were positive for HCV RNA, thus indicating an active infection. These findings are consistent with the previously reported HCV RNA prevalence of 0.6-0.9% among blood donors in Ghana (Ampofo et al., 2002, Candotti et al., 2003).

Previous work within our group has shown variable performance between three widely used commercial assays on a subset of the samples tested in this study (King et al., 2015). The seroprevalence rate of 6.7% found in HIV-infected patients in West Africa from a meta-analysis (Rao et al., 2015) is almost ten-fold higher than the prevalence found in this study. However, the previous work from our group and the prevalence determined by real-time PCR call into question the reliability of this estimate alongside others from Ghana (Brandful et al., 1999, Nkrumah et al., 2011, Kubio et al., 2012, Sagoe et al., 2012). Results of two previous studies from Uganda and Malawi are in agreement with these findings (Chasela et al., 2012, Mullis et al., 2013). Among 500 HIV-positive subjects in Uganda, anti-HCV prevalence by the Ortho assay was 7.5%. However, none of the anti-HCV reactive samples had detectable HCV RNA (Mullis et al., 2013). Among 2041 HIV-positive pregnant women in Malawi, HCV antibody prevalence by a chemiluminescence immunoassay was 5.3%, but none of the women tested HCV RNA positive (Chasela et al., 2012).

Nucleic acid amplification technology (NAT) has proved useful in determining an accurate assessment of the prevalence of HCV in various populations in a range of SSA countries (Ampofo et al., 2002, Candotti et al., 2003, Agwale et al., 2004, Laurent et al., 2010, Abreha et al., 2011, Zeba et al., 2011, Chasela et al., 2012) and represents a gold-standard test for the screening of active HCV infections. Unfortunately, it is often cost prohibitive for standard screening in resource-limited settings. In Ghana, HCV RNA testing is currently available in large research facilities and private laboratories, although the recommended introduction of routine HIV-1 RNA monitoring may in the future expand access to molecular assays in the region. Ghana is described as a lower middle income country with a gross national income per capita of US$1,600 (World Bank, 2014). Thus, measures to reduce the cost of diagnostic methods are necessary. This was achieved in two ways in this study, i) through the use of pooled samples; and ii) using DPS for screening.

Sample pooling strategies have been shown before to be effective for surveying low prevalence populations. Many developed countries utilise pool screening systems to screen blood donations for the presence of HIV, HBV and HCV (Gentili et al., 2001, Ohnuma et al., 2001, Stolz et al., 2003). Although effective at detecting the presence of blood-borne viruses, the sensitivity of the assay is reduced when samples are pooled. After extensive optimisation detailed in Chapter 3, our pooling approach proved to be effective for HCV RNA screening, reducing cost and labour, and confirming the previous observation that pooled testing is a realistic strategy for identifying HCV carriers in low-prevalence settings (Brant et al., 2008, Keys et al., 2014). In the case of plasma samples, pooling reduced the sensitivity of the assay by five-fold, which could result in the misdiagnosis of patients with a low HCV RNA

load. However, a previous study indicated that among 20 Kumasi blood donors infected with HCV Gt1 or Gt2, HCV RNA levels were a median 2.5×10^5 IU/ml and ranged from 3.3×10^3 to 9.0×10^6 IU/ml (Candotti et al., 2003), which is still above the lower limit of detection (550 IU/ml with pooled plasma samples) of our assay. Also, the cohort in this study had never been tested for HCV and was therefore naïve to all anti-HCV therapy, reducing the probability of a false negative misdiagnosis.

DBS have been used in limited-resource settings in South Africa to detect HIV RNA and DNA in infants, helping to expand access to these important diagnostic tests (Sherman et al., 2005). Furthermore, a study in Malawi used pooled DBS to monitor the HIV viral load of patients being treated with ART, a strategy that would reduce the cost of HIV virological monitoring by $345,000 per year if implemented (Pannus et al., 2013). In South Africa, use of pooled DPS has been shown to be successful in reducing the cost of monitoring HIV whilst still maintaining a high level of accuracy during testing (van Zyl et al., 2011).

DBS have been successfully used to screen a cohort of injecting drug users for both anti-HCV antibody and HCV RNA (Hope et al., 2011) although DPS have not been previously employed to study the prevalence of HCV. Although DPS still incur the cost of processing blood to plasma, like DBS, they do not require the cold storage for shipment, which, in resource-limited settings, would be beneficial if samples needed to be shipped to a central testing facility. This has been a proven approach in Haiti, where DBS from infants have been shipped overseas to the Netherlands for diagnostic testing of HIV (Ivers et al., 2008).

Employing DPS for screening does reduce the sensitivity of the assay. In the case of our assay, 2500 IU/ml was the lower limit of detection when using single DPS

specimens as the testing medium. As with pooling plasma samples, there is a risk of missing an active infection when utilising DPS. However, as mentioned above, the median viral load of samples from Ghana was much higher than the aforementioned limit of detection.

There was a difference in the prevalence of infection tested by plasma and DPS, although this was not significant. It is likely that the difference is due to the patient characteristics, as the plasma samples also contained HBsAg-positive patients, which had approximately double the prevalence of HCV infection compared to HBsAg-negative patients. Although there was a loss in sensitivity when using DPS for screening, the number of patients with a low enough viral load to be missed would be very few to none and not enough to substantially impact upon the prevalence.

As shown in this study, the combination of these two strategies could be easily adapted to allow routine testing of HCV whilst reducing costs.

It should be noted however that HCV prevalence was nearly double among HBsAg-positive subjects relative to those who tested HBsAg negative, although the difference did not reach statistical significance. This trend has also been examined in a cohort of blood donors from Kumasi (Layden et al., 2015), possibly reflecting shared transmission routes of the two viruses. There are an estimated 250,000 patients infected with HIV in Ghana (UNAIDS, 2014b), of whom 16.7% are co-infected with HBV (Geretti et al., 2010). Thus, the data here suggest an estimated 530 subjects with triple HIV/HBV/HCV co-infection and 1,750 subjects with HIV/HCV co-infection are living in Ghana. The triple infection prevalence of 1.27% found in this study is higher than many of the reported rates from other African countries, which are commonly under 1% (Cunha et al., 2007, Harania et al., 2008,

Patel et al., 2011). Larger studies are required to confirm an association between HBV and HCV co-infection in this population.

Taken together with previous results (Ampofo et al., 2002, Candotti et al., 2003), our findings do not suggest a marked excess risk of HCV infection in HIV-positive subjects in the region. The conclusion is in line with the observation that the reported risk factors for HCV infection were healthcare-related, with several of the identified strains showing clustering with strains previously detected in Kumasi blood donors (Candotti et al., 2003). Despite this agreement, certain limitations should be considered before classifying HCV as low prevalence in SSA. Previous studies have observed fluctuating HCV RNA levels during the early stages of infection (McGovern et al., 2009, Loomba et al., 2011). Consequently, it is possible that a patient with an acute infection may be misdiagnosed as non-infected if tested during a trough in HCV RNA levels. A pooled testing strategy with a higher limit of detection such as the one used in this study may increase the chance of this occurring. Secondly, HCV variants in Africa have showed a wide degree of variation in both the present study and previous studies, with many of the observed subtypes seemingly unique to this region (Ruggieri et al., 1996, Jeannel et al., 1998, Candotti et al., 2003, Markov et al., 2009). Although the primers used here in the real-time PCR target a conserved region of the genome, there is a concern that the African subtypes may not be detected by these. Designing primers based on African strains may increase the prevalence of HCV observed in this population.

Molecular characterization of the HCV positive specimens revealed that the majority of HCV-positive samples were Gt2 (89%), whilst a small proportion were Gt1

(11%). Overall, there was high molecular diversity among the strains and only three readily clustered with a well-defined subtype (2l). This was observed in the analysis of both of the sequenced genes. These three subtype 2l strains also clustered closely together and with another previously identified strain from a blood donor from the same region in Ghana. This could indicate a possible transmission network, especially as two of the risk factors identified were healthcare-related i.e. blood transfusion and surgery. The diversity is similar to findings from Ghanaian blood donors (Candotti et al., 2003) and other West African countries (Ruggieri et al., 1996, Jeannel et al., 1998, Markov et al., 2009). The proposed long-term endemicity and evolution of HCV genotypes 1 and 2 in West Africa provides an explanation for this diversity (Jeannel et al., 1998, Markov et al., 2009). A recent study placed the origin of Gt2 in Ghana, with subsequent spread to other West and North African countries (Purdy et al., 2015). Similar patterns to this can be seen in Gt6 found in Southeast Asia, which is thought to be its origin (Pybus et al., 2001).

Upon further analysis, the Gt1 sample did not cluster with any known subtype. However, the bootstrap value for this was under the 80% threshold in both core and NS5B analyses so it would be difficult to conclude that it is a new subtype without further sequence analysis. Possible new subtypes of HCV Gt1 have been previously characterised from SSA countries (Candotti et al., 2003, Bracho et al., 2006). According to nomenclature guidelines, further characterisation of this sample would need to be carried out, alongside further evidence from independently infected individuals, before the designation of a new subtype (Simmonds et al., 2005). However, the determination of another subtype may not be necessary if there is no clear benefit to clinical or epidemiological studies (Bracho et al., 2006). The low

prevalence of Gt1 found in this population may indicate that it is not endemic to the region and therefore of limited clinical value to classify new subtypes. Regions such as Ghana seem to contain such a high molecular diversity within genotypes that the number of subtypes would become unwieldy if new classifications of subtypes continued. The Gt1 strain may represent a variant that originated in Cameroon and spread to West Africa (Ndjomou et al., 2003, Bracho et al., 2006), although full genome sequencing is required to provide a more detailed molecular characterisation.

The choice and duration of HCV treatment is dependent on a number of viral factors, including the genotype, subtype, mutational profile and HCV viral load (European Association for the Study of the Liver, 2015). Some of the newly licensed DAAs are pan-genotypic (Lawitz et al., 2013, Rodriguez-Torres, 2013, Vince et al., 2014). However, other DAAs are only effective against certain genotypes or show greater efficacy against certain subtypes of the same genotype. For example, simeprevir is more effective against Gt1b than Gt1a (Jacobson et al., 2014). Consequently, the distribution of genotypes is not only important from an evolutionary perspective but also for the healthcare system. The lack of subtype assignment to the Gt1 strain may pose subsequent problems in the selection of the most appropriate treatment. Treatment of HCV infection in Africa is extremely limited and thus the response of widely diverse strains is unknown. Although Gt2 has historically been shown to respond well to treatment with peg-interferon and ribavirin (Dalgard et al., 2004, Mangia et al., 2005), African-American populations have a high proportion of the TT allele at the IL28B site, which negatively affects treatment response to interferon (Ge et al., 2009). New DAAs have shown high SVR rates against this genotype

(Lawitz et al., 2013), offering hope that chronic HCV infections in this region of the world may be easily treated as long as such drugs become available at an affordable level.

There are limitations to this study. The number of patients identified with current infections was small, leaving limited scope for a detailed analyses of factors associated with HCV infection alongside molecular characterisation. Large-scale studies are necessary in order to define the epidemiological characteristics of HIV/HCV co-infection in Ghana and SSA. The additional time and cost involved in processing whole blood to plasma restricts the use of DPS for screening purposes to populations that already have stored plasma samples, such as HIV-positive patients. Nevertheless, this still represents an important demographic as, unlike Western cohorts, HIV-positive patients in SSA countries are not routinely screened for HCV co-infections. For the purposes of screening a new cohort or the general population for HCV RNA, DBS would represent a more suitable and practical approach unless the extra time and expense to obtain plasma could be justified. In a previously reported approach, plasma which has been separated by gravity without the aid of machines has been used for the detection of HIV (Mwebaza et al., 2013). This strategy could form the preliminary step to preparing DPS with little associated cost and the use of already existent infrastructure. However, further tests would be required to ensure the techniques give comparable results when using DPS prepared from both centrifuged and non-centrifuged plasma. Taking these factors into account, the data presented here still provide novel information with implications for both public health and clinical care.

In summary, the use of a pooling strategy to screen both plasma and DPS for HCV RNA enabled a large cohort of HIV-infected individuals from Ghana to be tested in a shorter timescale and at a reduced cost than individual screening of each sample, thus providing laboratories with a useful diagnosis procedure in resource-limited settings. DPS testing also allows the recovery of RNA for sequencing, allowing the identification of possible transmission networks and further characterisation of the virus.

Chapter Five

Determining the prevalence of the HCV NS3 Q80K polymorphism in a UK population

The work presented in this chapter has been published in the following article:

Beloukas, A.*, King, S.*, Childs, K., Papadimitropoulos, A., Hopkins, M., Atkins, M., Agarwal, K., Nelson, M. & Geretti, A. M. 2015. Detection of the NS3 Q80K polymorphism by Sanger and deep sequencing in hepatitis C virus genotype 1a strains in the UK. *Clin Microbiol Infect*, 21(11), 1033-9.

*Joint authorship

5. Determining the prevalence of the HCV NS3 Q80K polymorphism in a UK population

Abstract

Background The Q80K polymorphism in the hepatitis C virus (HCV) NS3 gene reduces susceptibility to simeprevir and has potential effects on other novel protease inhibitors. The aims of this chapter were to i) determine the prevalence of Q80K in treatment-naïve patients infected with HCV genotype 1a in the North-West (NW) and South-East (SE) of England, ii) investigate the occurrence of Q80K as a minority variant (>1%), and iii) characterise the phylogeny of NS3 sequences from the United Kingdom (UK).

Methods Plasma samples from genotype 1a infected subjects, naïve to all anti-HCV therapy, underwent Sanger sequencing and deep sequencing (Illumina MiSeq, 1% interpretative cut-off) of the NS3 gene. Sequences underwent phylogenetic analysis to determine their distribution within the two genotype 1a clades.

Results Q80K occurred in 34/200 subjects (17.0%, 95% confidence interval 11.8%-22.2%), including 9/32 (28.1%) in the NW and 25/168 (14.9%) in the SE (p=0.076), with no difference by HCV RNA load and HIV status. The frequency of the polymorphism in the patients' samples was >40% in all cases. Phylogenetic analysis identified the two recognised HCV genotype 1a lineages with UK sequences being interspersed with sequences from both Europe and USA.

Conclusions Q80K occurs at high prevalence among treatment-naïve genotype 1a HCV patients in England, and conventional sequencing provides sufficient sensitivity to detect the polymorphism for pre-treatment screening. UK sequences are highly interspersed with sequences from elsewhere in Europe and USA, and their phylogeny is consistent with multiple introductions from different areas.

5.1. Introduction

The NS3/4A protease is an essential enzyme for the replication of HCV and represents a target for several direct acting antivirals (DAAs) (Schneider and Sarrazin, 2014). In genotype (Gt) 1 infection, traditional therapy of pegylated-interferon and ribavirin (P/R) had a limited success rate of around 50% after 48 weeks of therapy (Fried et al., 2002). Sustained virological response (SVR) rates increased to approximately 70% following introduction of first generation NS3 protease inhibitors (PIs) boceprevir and telaprevir for use as third agents with P/R (Jacobson et al., 2011, Poordad et al., 2011). Although first generation PIs proved effective, tolerability was problematic due to anaemia with boceprevir and skin rashes with telaprevir (7-11% treatment discontinuation). The introduction of the PI simeprevir further increased SVR rates to 80% in Gt1, treatment-naïve patients whilst decreasing treatment discontinuation rates due to adverse events to 3% (Jacobson et al., 2014).

Naturally occurring mutations in NS3 that reduce the susceptibility of HCV to PIs have previously been observed in treatment-naïve patients (Kuntzen et al., 2008, Cento et al., 2012). One notable polymorphism, glutamine to lysine at position 80 (Q80K) in NS3, increases the resistance to the newly licensed PI simeprevir by approximately 8-fold *in vitro* (Lenz et al., 2010). Whilst other resistance associated mutations (RAMs) seem to occur at relatively low frequencies in the population, Q80K is more common and has been observed at a natural prevalence of up to 48% in Gt1a patients from the USA (Lenz et al., 2015). The overall prevalence in Europe is 20% although this differs by country (Paolucci et al., 2012, Vicenti et al., 2012, Palanisamy et al., 2013, Sarrazin et al., 2015b). Phase III trials of treatment with

simeprevir plus P/R observed a lower SVR in patients harbouring Q80K than in those without the mutation (Forns et al., 2014, Jacobson et al., 2014, Manns et al., 2014). In a randomised trial of P/R plus simeprevir or placebo for treatment-naïve patients, the SVR measured 12 weeks after treatment completion (SVR_{12}) was similar between placebo and simeprevir recipients when the Q80K polymorphism was present at baseline (Jacobson et al., 2014). Consequently, current guidelines indicate that Gt1a patients should be tested for Q80K before starting therapy with simeprevir and if the polymorphism is present, for an alternative therapy to be considered (European Association for the Study of the Liver, 2015).

Previous studies have produced prevalence estimates for Q80K using population (Sanger) sequencing. This technique is limited to detecting variants that are present at ≥10-20% of the total viral strains in a sample. The existence of HCV as a quasispecies results in the circulation of many different variants during infection. Consequently, drug resistant variants will be present but often at too low a frequency to be detected by population sequencing. The development of next generation sequencing (NGS) technologies now offers improved sensitivity, enabling the detection of low frequency HCV variants, an approach defined as “deep sequencing”. Relatively few studies have used this technique to study low-frequency HCV RAMs in treatment-naïve individuals (Fonseca-Coronado et al., 2012, Bartolini et al., 2013, Dierynck et al., 2014, Jabara et al., 2014, Margeridon-Thermet et al., 2014) and none have specifically focussed on Q80K. The high prevalence and impact of the polymorphism warrants further investigation to determine its presence at low-level in patients with dominant wild-type virus.

The prevalence of the Q80K polymorphism has been well described in the United States and Europe (Cento et al., 2012, Berger et al., 2014, Lenz et al., 2015, Sarrazin et al., 2015b) but there is limited data available on the prevalence in the UK, especially using NGS. This study aimed to gain a better understanding of the natural prevalence of the Q80K mutation in a UK population from two regions of England. A second aim was to utilise deep sequencing to investigate the rate at which Q80K exists as a low frequency variant and therefore determine whether traditional population sequencing provides a sufficient level of sensitivity to screen patients prior to the commencement of simeprevir therapy. Finally, we aimed to determine the phylogeny of NS3 sequences from the UK in relation to sequences from the USA and Europe.

5.2. Methods

5.2.1. Patient population

The study was performed retrospectively using stored plasma samples from adults that attended for care at the Royal Liverpool University Hospital in Liverpool (North-West region) and at King's College Hospital, Chelsea & Westminster Hospital, and Charing Cross Hospital in London (South-East region) between 2006 and 2013. Eligible patients were infected with Gt1a HCV and naïve to all anti-HCV therapy, including pegylated-interferon and ribavirin, at the time of sampling. HIV status and HCV RNA load were retrieved from the clinics' databases. Ethics permission was granted by the South Berkshire Regional Ethics Committee to conduct the study after removing personal identifiable information from the samples.

5.2.2. Population sequencing

HCV RNA was extracted from 100µl plasma using the Nuclisens easyMAG system (bioMérieux, Boxtel, Netherlands) in a 60µl elution, further detailed in Chapter 2.7.1. A partial NS3 region (727bp) was amplified by nested PCR as detailed in Chapter 2.7.2. PCR products were purified and sequenced using the Applied Biosystems 3730 DNA Analyser as outlined in Chapter 2.8-2.12. The cycle sequencing reaction and Sanger sequencing of 63 samples was carried out by Thanos Papadimitropoulos.

5.2.3. Next generation sequencing

Samples were extracted, amplified and purified as detailed in Chapter 2.15-2.20 in preparation for next generation sequencing. Amplicons were prepared using the

Nextera XT Sample Preparation kit and the Illumina MiSeq platform was used to deep sequence the products (Chapter 2.21). A 727bp region of a plasmid control encompassing the full length NS3 gene was amplified and sequenced in four replicates to determine the error rate of the assay.

5.2.4. Sequence analysis

Consensus sequences from population sequencing were assembled using SeqScape (v2.7) and tested for Q80K using geno2pheno (http://hcv.geno2pheno.org/index.php). NGS reads underwent initial preliminary processing at The Centre for Genomics Research, University of Liverpool. BAMStats (http://sourceforge.net/projects/bamstats/files/) was used to calculate the read statistics. The VirVarSeq pipeline (Verbist et al., 2015) was used for the analysis of the deep sequencing reads, full details of which are in Chapter 2.22. Descriptive statistics were used to analyse the frequency of mutants in each patient's sample (as the proportion of deep sequencing reads showing the mutation). The plasmid control yielded an average of 174,799 reads, with a mean coverage of 46,268 reads per nucleotide. The average error rate, calculated by counting false positive reads at the codon level over NS3 amino acids 1-181, was 0.6% (SD ±0.2%). A threshold of 1% was used as a cut-off for minority variants.

5.2.5. Phylogenetic analysis

Gt1a HCV sequences from the USA and Europe were retrieved from the Los Alamos HCV Database. Multiple sequences from the same patient i.e. from clones or timepoints during therapy, were discarded along with sequences from non-human hosts. The resulting sequences were aligned against the sequences obtained from this

study using Clustal W. Where available, the consensus sequence from deep sequencing reads was used. The alignment was trimmed to nucleotide positions 3420-4014 (amino acids 1-198 of NS3). Sequences with strings of N >20bp in length were discarded. The resulting alignment was phylogenetically analysed using the PHYLIP v3.69 package (Felsenstein, 1989) as described in Chapter 2.13. Regional clusters were identified as clusters containing ≥3 sequences unique to either the NW or SE region and supported by a bootstrap value of ≥80%. Inter-regional clusters contained sequences from both the NW and SE regions.

5.2.6. Statistical analysis

Fisher's exact test was used to compare the proportion of subjects with Q80K by geographic location and HIV status. The Mann-Whitney U test was used to compare the HCV RNA load and CD4 counts (in HIV co-infected only) of patients with and without Q80K. The analysis was performed using SPSS version 21 (IBM Corporation, Armonk, NY, USA).

5.3. Results

5.3.1. Patient population

Overall, 200 adults (median age 43 years, interquartile range, IQR, 39-50) infected with Gt1a HCV were studied, including 32/200 (16%) in the North-West (NW) and 168/200 (84%) in the South-East (SE). At the time of testing, HCV RNA load was median (IQR) 6.3 (5.8-6.7) $\log_{10}$ IU/ml. Subjects were naïve to all anti-HCV therapy. A total of 61/200 (30.5%) subjects, all from the SE, were co-infected with HIV. A summary of the patient population is detailed in Table 5.1.

Of the 200 patient samples, 22 were population sequenced only, 63 were deep sequenced only and 115 samples underwent both population and deep sequencing.

Table 5.1. Clinical characteristics of study population

	Cohort		Total
	NW	**SE**	
Number	32	168	200
Median age (IQR)	41 (38-44)	44 (39-51)	43 (39-50)
Average HCV VL ($\log_{10}$ IU/ml), median (IQR)	5.9 (5.5-6.6)	6.3 (5.9-6.8)	6.3 (5.8-6.7)
HIV co-infection	0	61 (36.3%)	61 (30.5%)
Q80K detected[a]	9 (28.1%)	25 (14.9%)	34 (17.0%)

[a]Detection by Sanger and deep sequencing (frequency ≥1%)

5.3.2. Overall Q80K prevalence

Overall, the Q80K polymorphism was identified in 34/200 (17%, 95% confidence interval, CI 11.8%-22.2%) samples (Table 5.2). The prevalence of Q80K in the North-West was nearly double of that in the South East region, with 9/32 (28.1%) and 25/168 (14.9%) patients harbouring the polymorphism, respectively (p=0.076). When comparing results by HIV status in the SE, Q80K prevalence was 18/107 (16.8%) in HCV mono-infected subjects and 7/61 (11.5%) in HIV/HCV co-infected subjects (p=0.379). In HIV/HCV co-infected subjects, there was no significant difference (p=0.80) in the CD4 count of samples with and without the polymorphism, with medians (IQR) of 519 (199-701) vs. 474 (318-646) cells/mm^3, respectively. The median HCV RNA load was 6.4 (IQR 5.6-6.7) vs. 6.2 (IQR 5.8-6.7) log_{10} IU/ml in samples with vs. samples without Q80K, respectively (p=0.63).

Table 5.2. Prevalence of the Q80K polymorphism in each region by Sanger and deep sequencing

Region	Number with Q80K				Mutant frequency (%) within Illumina reads
	Sanger only (n=22)	Illumina only (n=63)	Sanger and Illumina (n=115)		
			Sanger	Illumina	
North West	2	0	7	7	All ≥99.8%
South East	4	8	13	13	41.1%, 45.8%, Remaining ≥98.9%
Total	6	8	20	20	

5.3.3. Q80K prevalence by population sequencing

Of the 137 samples that were population sequenced, the Q80K polymorphism was found in 26, yielding a prevalence of 19.0% (95% CI 12.4%-25.6%) samples. Two samples positive for Q80K displayed a mixed first base of codon 80; one had a dominant C and the other had an equal distribution of C and A at this position, as determined by manual inspection of the electropherogram peaks. One sample (0.7%) had the Q80L mutation.

5.3.4. Q80K prevalence by next generation sequencing

A subset of 178 samples underwent deep sequencing using the Illumina MiSeq. An average of 58,585 reads per sample was obtained (median; 53,413, IQR; 40,740-70,255) with an average coverage of 16,107 per nucleotide. The Q80K polymorphism was observed in 28/178 (15.7%, 95% CI 10.4%-21.0%) samples (Table 5.2). Of the samples deep sequenced, 115 had also undergone population sequencing; 20 of which showed the Q80K polymorphism. All samples showing the polymorphism by population sequencing showed Q80K by deep sequencing, at frequencies ≥98% in 18/20 cases. Of the two samples showing a Q/K mixed population by population sequencing, one displayed a Q:K ratio of 54.2:45.8 and the other showed Q:K:L at a ratio of 56.8:41.1:2.1, by deep sequencing.

NGS revealed a mixed population of Q/L at a ratio of 11.8:88.2 in the sample showing Q80L by population sequencing.

None of the samples that had Q at position 80 by population sequencing showed the polymorphism by deep sequencing, when an interpretative cut-off of 1% was applied. Introducing a lower interpretative cut-off increased the prevalence of Q80K; a further 2.0% (3/150) and 10.0% (15/150) of samples displayed the polymorphism

at cut-off values of ≥0.5% *x* <1%, and ≥0.2% *x* <0.5%, respectively. However, these cut-offs fell within the estimated error rate of the assay (0.6%, SD ±0.2%).

A further six samples showed low levels of other resistance mutations at position 80 comprising L (5.6%, 5.1% and 1.4%) and R (1.7% [n=2] and 1.1%).

5.3.5. Phylogeny of NS3 sequences

In order to determine the distribution of sequences containing the Q80K polymorphism within the two distinct clades of Gt1a, a phylogenetic analysis was performed. The analysis also allowed the assessment of the relatedness of the Q80K sequences present in the UK to other sequences from the USA and Europe. The NS3 sequences obtained from sequencing of UK samples (n=200) were phylogenetically analysed against Gt1a reference sequences (Figure 5.1). There was no difference in the proportion of the samples clustering in clade 1 between the two regions (NW; 17/32, 53.1% vs. SE; 74/168, 44.0%, p=0.44). Of the 34 sequences with the Q80K polymorphism present (either dominant or as a mixture), 32 (94.1%) clustered within clade 1 of Gt1a. The remaining two sequences clustered in the other Gt1a clade. These two sequences were the samples which showed a mixed population of both Q and K at position 80. An additional 54 samples without the Q80K polymorphism at any level >0.2% clustered within clade 1. Of 18 samples showing Q80K at levels ≤1%, 13 (72.2%) aligned with clade 2; the lineage of Gt1a not associated with the Q80K polymorphism. The UK sequences did not form a distinct cluster, but were interspersed with sequences from the USA and Europe, suggesting that they arose from multiple introductions. Six regional clusters were identified in the UK sequences - all comprising sequences from the SE - the largest of which contained 20 sequences from co-infected subjects from the SE, representing a possible

transmission network. One inter-regional cluster was identified, comprising two sequences from the SE and one from the NW. The sequences in this inter-regional cluster all contained the Q80K polymorphism. Of the remaining 29 samples with Q80K in clade 1, 11 (37.9%) were present in regional or inter-regional clusters, although these did not reach the 80% bootstrap threshold.

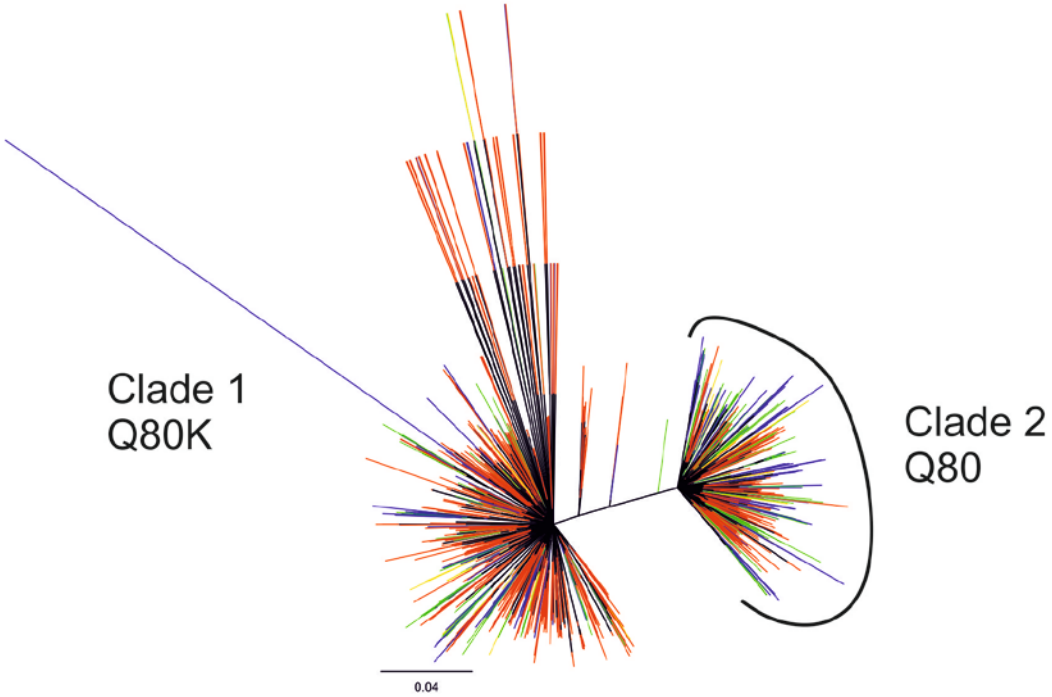

Figure 5.1. Phylogenetic relationship between genotype 1a NS3 sequences. Sequences were aligned against genotype 1a NS3 reference sequences from the USA and Europe. Sequences from the USA are highlighted red, Europe are highlighted blue, NW region are highlighted yellow and SE sequences are highlighted green.

5.4. Discussion

This study expands upon the knowledge of Q80K in a UK population of patients with chronic HCV infection and offers novel insights into the impact deep sequencing can have on the detection. In this study, we determined that Q80K was present in a large proportion of the HCV infected treatment-naïve UK population, particularly in the North West. In the UK, data from Phase II/III clinical trials of telaprevir and simeprevir showed 23% of Gt1a patients had Q80K (Sarrazin et al., 2015b) whilst a small study of HIV/HCV co-infected patients with acute HCV infection observed a prevalence of 16% with the polymorphism (Leggewie et al., 2013). The highest prevalence in Europe (45%) was seen in a Berlin cohort comprising Gt1a, HCV mono-infected and HIV/HCV co-infected patients (n=42) (Ehret et al., 2014), although many of the previous estimates from other European countries remain under 20% (Trevino et al., 2011, Vallet et al., 2011, Paolucci et al., 2012, Vicenti et al., 2012, Palanisamy et al., 2013) (Table 5.3). The lowest prevalence previously identified was 5.7% from a cohort of PI-naïve, Gt1a infections (n=53) in Sweden (Palanisamy et al., 2013). An Italian study determined a prevalence of 16.4% in Gt1a patients (Vicenti et al., 2012) and a similar prevalence of 16% was observed in a Spanish cohort of HIV/HCV co-infected patients with Gt1 infection (Trevino et al., 2011). The prevalence of this polymorphism in North America was observed at 48.1% in Gt1a patients (Lenz et al., 2015); approximately three times the value of many estimates from Europe included the one observed in this study. These data, along with the observations from this study, indicate that the UK has among the highest prevalence of Q80K in Europe.

Table 5.3. Prevalence of Q80K polymorphism in Europe, listed by country.

Country	Gt1a population	Q80K prevalence	Gt1b population	Q80K prevalence	Reference
Austria	109	18 (16.5%)	94	1 (1.1%)	(Sarrazin et al., 2015b)
Belgium	61	11 (18%)	147	1 (0.7%)	(Sarrazin et al., 2015b)
Bulgaria	14	0	35	0	(Sarrazin et al., 2015b)
France	275	38 (13.8%)	244	3 (1.2%)	(Sarrazin et al., 2015b)
France	46	3 (6.5%)	35	2 (5.7%)	(Vallet et al., 2011)
France	447	25 (5.6%)	203	6 (3.0%)	(Hajji et al., 2015)
Germany	42	19 (45.2%)	-	-	(Ehret et al., 2014)
Germany	241	70 (29%)	345	2 (0.6%)	(Sarrazin et al., 2015b)
Italy	31	3 (9.7%)	39	0	(Paolucci et al., 2012)
Italy	60	12 (20%)	150	1 (0.7%)	(Sarrazin et al., 2015b)
Italy	67	11 (16.4%)	42	0	(Vicenti et al., 2012)
Italy	103	23 (22.3%)	80	0	(Ruggiero et al., 2015)
Netherlands	26	3 (11.5%)	14	0	(Sarrazin et al., 2015b)
Norway	21	1 (4.8%)	5	0	(Sarrazin et al., 2015b)
Poland	20	15 (75%)	444	2 (0.5%)	(Sarrazin et al., 2015b)
Portugal	37	3 (8.1%)	9	0	(Sarrazin et al., 2015b)
Romania	-	-	92	1 (1.1%)	(Sarrazin et al., 2015b)
Russia	6	4 (66.7%)	177	0	(Sarrazin et al., 2015b)
Spain	82	7 (8.5%)	181	0	(Sarrazin et al., 2015b)
Spain	43	8 (18.6%)	20	1 (5%)	(Trevino et al., 2011)
Sweden	53	3 (5.7%)	-	-	(Palanisamy et al., 2013)
Sweden	46	7 (15.2%)	13	0	(Sarrazin et al., 2015b)
UK	38	6 (15.8%)	-	-	(Leggewie et al., 2013)
UK	159	36 (22.6%)	39	0	(Sarrazin et al., 2015b)

In this study, the difference in prevalence of the polymorphism between the two regions of the UK approached significance and, as described above, prevalence rates also differ markedly between European countries. Multiple studies have identified two distinct clades within Gt1a (Pickett et al., 2011, Vicenti et al., 2012, De Luca et al., 2015, McCloskey et al., 2015), and the Q80K polymorphism is associated with one of these clades (McCloskey et al., 2015). The circulation of these two clades may provide some explanation as to the differences in Q80K prevalence observed between European countries and regions within countries (Sarrazin et al., 2015b). However, in this study, we did not observe a difference in the proportion of samples clustering in the Q80K-associated clade between the NW and SE regions. This is despite the fact that the prevalence of the polymorphism in the NW was nearly double that of the SE. Our observations suggest that the distribution of clades may not be the determining factor in the prevalence of Q80K although larger studies should be performed to assess the distribution of the two clades within the UK and Europe in order to confirm this.

The results from our study are in concordance with these previous results, with all of the sequences containing a dominant (>98%) Q80K polymorphism aligning in clade 1. Interestingly, the two samples showing mixed amino acids (Q/K) were the only two sequences that clustered in the other Gt1a clade. One possible explanation for this may be that the Q80K mutation in these two strains is a *de novo* mutation, in contrast to the majority of circulating Q80K strains which are likely to have descended from a common ancestor.

The Q80K polymorphism is believed to have originated in the United States in a period from the 1940s to 1950s (McCloskey et al., 2015). Given the estimated date

of origin, this substitution would not have arisen through the pressure of drug selection but appears to be a naturally occurring polymorphism with an increased resistance to drugs (McCloskey et al., 2015). One of the possible hypotheses to explain the high prevalence of Q80K is that the variant is transmissible (McCloskey et al., 2015). If this hypothesis is correct, then it is likely that the spread of the polymorphism did not occur until much later than it arose, when international travel and transmission events became more common. If the polymorphism is transmissible, then it may be considered that a proportion of the circulating strains in the UK have either descended from transmission events from the USA or involved migrants from the USA in the UK. Our phylogenetic analysis showed that the UK sequences carrying Q80K were interspersed with sequences from both the USA and Europe and did not cluster with strains from one particular country. This suggests that there have been multiple introductions of Q80K into the UK. One inter-regional cluster, containing 3 sequences with the polymorphism, was identified. Other clusters containing Q80K sequences were identified but were less well supported by bootstrap values. These data indicate that Q80K has spread through transmission events within the UK although a large scale phylogenetic study with more UK sequences is required to confirm this hypothesis.

Another hypothesis is that the mutation frequently arises *de novo* during infection (McCloskey et al., 2015) although this seems less likely due to the large difference in the prevalence of the polymorphism between countries that has been observed. The emergence of *de novo* mutations may be influenced by pressure from the immune system. The epitope which includes the Q80 site is restricted by HLA A24 (Ward et al., 2002). However, a study of acute HCV infections found no association between this HLA type and substitutions at position 80 although only a small number of

patients were studied (Applegate et al., 2015). If the polymorphism did emerge *de novo* during infections, the prevalence would be expected to be less diverse between different parts of the world.

Currently, it is recommended that patients with a Gt1a infection undergo resistance testing for Q80K using traditional Sanger sequencing prior to the commencement of therapy with simeprevir (European Association for the Study of the Liver, 2015). The data from NGS showed that all Q80K mutants existed at high frequencies (>40%) allowing their detection by population sequencing. This finding is consistent with the observation that, *in vitro*, variants harbouring the polymorphism have a replication capacity similar to that of wild-type virus (Lenz et al., 2010), which would allow transmissibility and persistence at high frequency within the viral quasispecies despite the absence of drug selective pressure (McCloskey et al., 2015). There was no increase in prevalence of Q80K in the cohort below the detection threshold of population sequencing and above 1%, which was the threshold set for analysis. This value was above the assay average error rate of 0.6% and is a recommended cut-off to distinguish between true mutations and those caused by PCR and sequencing errors (Gianella et al., 2011). The application of lower thresholds resulted in an increased proportion of samples with Q80K. These data are in concordance with a previous study which found that of 21 patients harbouring the Q80K polymorphism, it occurred at levels >99% in 17, with only four carrying the mutation at levels lower than 1% (Jabara et al., 2014). A similar finding was observed in an Italian cohort, where 5 (18%) patients carried Q80K at levels ≥20% but the polymorphism existed as a minority variant (0.49-0.91% frequency) in only 3 (11%) patients (Bartolini et al., 2013). A large study of 185 patients from throughout

the world identified only two additional patients carrying the Q80K mutation (1% and 14% frequency) through the use of NGS when compared to population sequencing (Dierynck et al., 2014). The majority of the ultra-low frequency (≤1%) Q80K substitutions observed in the present study aligned with clade 2 of Gt1a, which is not associated with the polymorphism. This suggests that they were not biologically meaningful substitutions and were errors introduced by the assay or were *de novo* mutations present at the time of sampling with no significance.

Q80K reduces susceptibility to simeprevir by approximately 8 fold *in vitro* (Lenz et al., 2010) and the presence of the polymorphism reduces SVR rates of treatment with simeprevir plus P/R in both treatment-naïve (from 85% to 52%) and treatment-experienced patients (from 79% to 47%) (Forns et al., 2014, Jacobson et al., 2014). A recent phase III trial employing simeprevir plus sofosbuvir for the treatment of Gt1-infected patients with cirrhosis reported higher SVR rates in patients without Q80K versus those with the mutation (92% vs. 74%, respectively) (Lawitz et al., 2015b). This is an alarming observation, which may further reduce treatment options for patients harbouring Q80K. The polymorphism also confers a low-level (3-fold) decrease in susceptibility to paritaprevir *in vitro* (Pilot-Matias et al., 2015). The mutations Q80L and Q80R were found at low levels (≥1%) in patient samples. These mutations confer a 2- and 7-fold change in susceptibility to simeprevir *in vitro*, respectively (Lenz et al., 2010). Q80R has also been observed in a small number of patients experiencing failure of simeprevir therapy, when it occurred alongside major resistance mutations at position 155 and/or position 168 (Lenz et al., 2015).

It is unclear whether the presence of the Q80K polymorphism at an ultra-low frequency would impact upon treatment response. A previous study using deep sequencing to track the changes in RAMs in Gt1b infected patients (n=18) undergoing simeprevir therapy found that ultra-low frequency variants (0.2%-0.3%) of Q80L and Q80R persisted at low background levels with no increase in frequency in two patients whilst another patient displayed an increase of Q80L from 1.0% at baseline to 76.0% during treatment (Akuta et al., 2014b). Failure of treatment due to simeprevir-resistant mutants could not be predicted at baseline and was mostly due to the emergence of *de novo* variants (Akuta et al., 2014b). More studies into the dynamics of low-level variants during treatment are required to confirm this observation although, if found to be true, the use of deep sequencing for routine diagnostic testing of minority variant Q80K strains is not necessary as they are unlikely to impact on treatment.

In conclusion, we have demonstrated that populations from two regions in the UK, including those with HIV co-infection, have a high prevalence of Q80K, adding valuable data to previous estimates from Europe. The use of deep sequencing only increased the prevalence of the polymorphism over that detected by Sanger sequencing when frequencies under 1% were included and these are likely to be technical artefacts. The UK sequences did not form a distinct cluster, instead aligning with sequences from both the USA and Europe, indicating multiple transmission events.

Chapter Six

The impact of HIV co-infection on the prevalence of baseline HCV drug resistance associated mutations

6. The impact of HIV co-infection on the prevalence of baseline HCV drug resistance associated mutations

Abstract

Background Co-infection with HIV is known to accelerate disease progression and increase the viral load of HCV. Despite this, the effect of co-infection of resistance associated mutations (RAMs) has been poorly studied. This study aimed to determine the impact of HIV co-infection on the prevalence of RAMs at both high and low frequencies.

Methods Samples from HCV mono-infected (n=177) and HIV/HCV co-infected patients (n=188) were collected from sites throughout the UK and Europe. Next generation sequencing was used to determine the prevalence of RAMs in two genes (NS3 and NS5A), using an interpretative cut-off value of ≥1%.

Results Overall, RAMs were relatively common in both the NS3 and NS5A gene, with prevalences of 58.4% and 26.8%, respectively. There was no difference in the prevalence of high frequency RAMs between mono- and co-infected patients in either NS3 (46.9% vs. 55.3%, p=0.12) or NS5A (17.5% vs. 10.6%, p=0.07). The same was true of low frequency RAMs in NS3 (14.7% vs. 9.6%, p=0.15) and NS5A (16.4% vs. 14.4%, p=0.66).

Conclusion Generally, there was no difference in the prevalence of RAMs between mono-infected and co-infected patients. Consequently, the presence of HIV co-infection should not compromise treatment with direct-acting antivirals.

6.1. Introduction

The HCV NS3 gene plays a vital role in the replication of the virus by encoding the viral protease, which cleaves the HCV polyprotein into the individual components, thus allowing the production of infectious virions (Lindenbach and Rice, 2005). The role of the HCV NS5A protein has not yet been fully elucidated. Although the protein does not have enzymatic activity, it is thought to play a key role in both the synthesis of viral RNA and the assembly or secretion of infectious virions (Scheel and Rice, 2013). It is known that NS5A can down-regulate the translation of viral RNA, through interactions between amino acids and RNA (Hoffman et al., 2015a, Hoffman et al., 2015b), and is also responsible for binding to host Cyclophilin A (CypA), which is involved in the subcellular distribution of NS5A (Dujardin et al., 2015).

In recent years, direct acting antivirals (DAAs) have been developed which specifically target HCV proteins including NS3 and NS5A. Protease inhibitors (PIs) such as telaprevir (now discontinued), boceprevir (soon to be discontinued), simeprevir and paritaprevir compete with the polyprotein substrate of the NS3 protease's active site, blocking the binding and subsequent cleavage of the polyprotein (Raney et al., 2010). NS5A inhibitors such as daclatasvir, ledipasvir and ombitasvir display extremely high potency (Guedj et al., 2013). The exact mechanism of action of NS5A inhibitors is still unclear although daclatasvir has been shown to block hyperphosphorylation of the NS5A protein, which is essential to the proper functioning of the protein (Fridell et al., 2011a, Lemm et al., 2010).

Clinical trials exploring the efficacy of PIs and NS5A inhibitors with either pegylated-interferon and ribavirin (P/R) or in interferon-free combination regimens have observed far greater SVR rates than the previous standard of care combination of peg-IFN and RBV (Afdhal et al., 2014b, Forns et al., 2015, Lawitz et al., 2015a). The combination of ledipasvir (NS5A inhibitor) and sofosbuvir (NS5B polymerase inhibitor), for instance, has lead to SVR rates >90% in both treatment-naïve and treatment experienced patients (Afdhal et al., 2014a, Afdhal et al., 2014b).

Combination therapy with multiple DAAs offers an effective treatment regimen, although instances of treatment failure have been identified and, in such cases, variants harbouring resistance mutations to one or all of the antivirals used during therapy have been observed at the time of treatment failure (Black et al., 2015, Krishnan et al., 2015c). Through the assessment of the prevalence of variants carrying resistance to more than one DAA before the initiation of therapy, the influence of the selective pressure exerted by antiviral therapy can be determined.

HCV not only displays great diversity between genotypes and subtypes at the population level, but also within an individual infection. The HCV RNA-dependent RNA polymerase is extremely error prone and lacks proofreading capacity, generating an error rate of approximately $1.2x10^{-4}$ to $2.5x10^{-5}$ mutations per nucleotide per genome replication *in vivo* (Cuevas et al., 2009, Ribeiro et al., 2012). When combined with its high replication rate of 10^{12} virions per day, every possible resistance-associated mutation (RAM) could, in theory, arise multiple times per day in an infected host (Rong et al., 2010). As a result, HCV exists in a host as a quasispecies – a mixture of related but genetically diverse variants (Martell et al.,

1992). The replicative fitness of a proportion of the spontaneously generated variants will be impaired by the mutation to such a degree that they either die out or persist at a low frequency in the quasispecies (Tong et al., 2006, Lenz et al., 2010). In the event of the introduction of a selective pressure such as antiviral therapy, such low-frequency variants acquire a replication advantage, providing the potential to emerge as the dominant strains in the quasispecies (Halfon and Sarrazin, 2012). The same process has been described for HIV and hepatitis B virus. However, in the case of HCV, variants often still retain a sufficient replicative capacity to allow them to exist as the dominant strain as single or even double mutants, prior to the introduction of selective pressure.

Many of the studies examining the presence of drug resistance associated mutations (RAMs) in treatment-naïve HCV-positive individuals have utilised population (Sanger) sequencing technology, allowing the detection of variants present at a frequency of ≥10-20%. In some studies, naturally occurring drug-resistant variants carrying mutations in the NS3 gene have been found by Sanger sequencing to occur at prevalences ranging up to 19% in treatment-naïve patients with HCV genotype (Gt) 1 (Vicenti et al., 2012). A growing number of studies have also examined the NS5A gene and its associated RAMs, and commonly detected mutations in baseline samples from antiviral-naïve patients (Plaza et al., 2012, Suzuki et al., 2012, Paolucci et al., 2013, McCormick et al., 2015). Naturally occurring RAMs in this gene have been observed at prevalences up to 53% in Gt1b patients (Paolucci et al., 2013), while Gt1a patients generally harbour a lower prevalence of mutations (Plaza et al., 2012, Paolucci et al., 2013).

The relatively high detection threshold of Sanger sequencing means that there is the potential for drug resistant variants present at low frequencies in a patient's sample to go undetected. Other methods such as clonal sequencing can detect lower frequencies but are costly and labour intensive (Halfon and Locarnini, 2011). Recently, next generation sequencing (NGS) has enabled the identification of variants at levels undetectable by traditional population sequencing techniques, with costs per Mb of data as low as US$0.5 (Loman et al., 2012). This represents a relatively affordable technique for the sensitive detection of resistant variants, without the increase in labour associated with other methods, although still requiring complex bioinformatics for analysis and interpretation.

Co-infection with HIV is relatively common, and from a global perspective an estimated 4% of HCV infected patients carrying both viruses (Fernandez-Montero and Soriano, 2012). HIV is known to promote HCV replication and accelerate the progression of liver fibrosis (Lin et al., 2013). There have been few studies comparing the quasispecies complexity and presence of RAMs between HCV mono-infections and HIV/HCV co-infections and those studies that have performed comparisons have found conflicting results (Shuhart et al., 2006, Xu et al., 2012, Bartolini et al., 2013, Jabara et al., 2014). Depressed immune function may reduce immunological selective pressure and the diversity of the HCV quasispecies in HIV co-infected patients. Conversely, the increased replication of HCV observed during co-infection with HIV (Eyster et al., 1994, Beld et al., 1998) may be postulated to increase the likelihood of mutants emerging, increasing the diversity of circulating variants and therefore the risk of a co-infected patient harbouring baseline RAMs to DAAs. If this concern was realised, it would add further complications to the

treatment of HCV in co-infections and may increase the need for baseline screening of patients for resistance substitutions. Reassuringly, to date treatment responses to DAA-based therapy have shown to be as high as those seen in HIV-negative individuals, with SVR rates exceeding 90% in many studies (Antonini et al., 2015, Wyles et al., 2015).

This study aimed to assess the influence of HIV co-infection on the detection of RAMs in the NS3 and NS5A genes of HCV in a large cohort of chronically infected individuals, naïve to all forms of anti-HCV therapy, and to relate the findings to the subjects' CD4 cell counts. An NGS assay was developed and tested to allow the determination of low-frequency variants, thus increasing the sensitivity of assessment relative to Sanger sequencing.

6.2. Methods

6.2.1. Preliminary testing of the NGS assay

To determine the sensitivity of detection of low-frequency resistant variants, mock specimens were constructed using two NS3 DNA plasmids. The plasmid encoded a 2897bp region, encompassing the full length NS3 gene, in a pCR 2.1 vector (Life Technologies, Carlsbad, CA, USA). One plasmid was classified as a 'wild-type' sequence. The other encoded an identical sequence with the addition of 5 RAMs: V36A, T54S, V55A, R155K, and A156S. The plasmids were obtained from Andrea Rosi at the University of Siena, Italy. The constructed specimens comprised mixtures of the wild-type plasmid with the RAM-containing plasmid at frequencies of 1%, 2%, 5% and 10%. The plasmids were mixed at the appropriate proportions prior to amplification using the nested PCR protocol and the NGS protocol detailed in Chapter 2. The error rate of the assay was determined by testing four replicates of the wild-type plasmid and counting false positive reads (mutations relative to the input wild-type sequence) at the codon level over NS3 amino acids 1-181.

6.2.2. Study population

The study was performed retrospectively using stored plasma samples from the Royal Liverpool Hospital in Liverpool, and King's College Hospital, Chelsea & Westminster Hospital, and Charing Cross Hospital in London (UK); Hospital Carlos III in Madrid (Spain) and the University Hospital in Siena (Italy). Specimens were collected from treatment-naïve adults with HCV Gt1 infection with or without HIV co-infection who were naïve for all forms of anti-HCV therapy and attended for care between 2006 and 2013. Overall, 367 samples were tested; 178 HCV mono-infected

and 189 HIV/HCV co-infected. Of the HIV-positive patients, 24/189 (12.7%) were ART-naïve. A sub-set of 107 mono-infected samples were matched 1:1 with 107 co-infected samples by HCV genotype (1a or 1b) and HCV RNA load (±0.1 $\log_{10}$ IU/ml).

6.2.3. Next generation sequencing

Full details of the NGS protocol are given in Chapter 2. Briefly, samples underwent nucleic acid extraction using the QIASymphony automated extraction platform (Qiagen, Hilden, Germany), and subjected to reverse transcription into cDNA before amplification using nested PCR. The amplicons comprised a 727bp region of NS3 and a 1474bp (Gt1a) or 1378bp (Gt1b) region of NS5A. The NS3 amplicon encoded the region from aa180 of NS2 to aa204 of NS3 and did not include the helicase domain. The Gt1a NS5A amplicons encoded the entire NS5A gene (aa218 of NS4B to aa447 of NS5A); the Gt1b amplicons omitted the final 30 amino acids of the NS5A gene (aa218 of NS4B to aa416 of NS5A). The two amplicons for each sample were pooled together in equimolar amounts before undergoing library preparation using the Nextera XT sample preparation kit (Illumina, San Diego, CA, USA). Normalised libraries were sequenced at the Centre for Genomic Research, University of Liverpool, UK on an Illumina MiSeq instrument. The NS3 wild-type plasmid (see above) was included in each run, which allowed for the checking of cross-contamination in the downstream analysis.

6.2.4. NGS data analysis

Reads trimmed of the adaptor sequences underwent preliminary filtering of poor quality reads at the Centre for Genomics Research. The variant-calling pipeline

VirVarSeq (Verbist et al., 2015) was used to analyse the samples at the codon level. VirVarSeq utilises the quality of the sequencing run to filter poor quality individual bases from the reads thereby reducing the false positive rates. Further steps of the pipeline employed by VirVarSeq are detailed in chapter 2. BAMStats (http://sourceforge.net/projects/bamstats/files/) was used to calculate the read statistics.

Nineteen amino acid positions of the NS3 protease gene and nine amino acid positions in domain I of the NS5A gene were analysed for the presence of substitutions known to cause resistance to DAAs. The positions and RAMs analysed are listed in Tables 6.1 and 6.2. Mutations listed on geno2pheno (http://hcv.geno2pheno.org/index.php) as 'resistant' or 'possibly resistant' (according to version updated March 2015) were included alongside other mutations which have been observed to cause resistance *in vitro* and/or *in vivo* in Gt1 infections based on published studies. For the purposes of this study, a major RAM was defined as any mutation listed as 'resistant' on geno2pheno whilst 'possibly resistant' mutations were considered minor RAMs. RAMs were defined as high-frequency or low-frequency if occurring in ≥10% or <10% of deep-sequencing reads, respectively.

Table 6.1. Table of resistance associated mutations in NS3. Mutations were obtained from geno2pheno and other sources, indicated accordingly.

Position	Wild-type amino acid	Resistance mutations	Possible resistance mutations
36	V	A/G/L/M	
41	Q		R
43	F	I/L/S/V	
54	T	A/C/G/S	
55	V	A	I[1]
80	Q	K	G[2]/H/L[2]/R
107	V		I[1]
109	R		K[2]
117	R		H
122	S	R	
132	I[a]/V[b]		V[3a]
138	S		T
155	R	G/I/K/M/Q/T	
156	A	F/G/N/S/T/V	
158	V		I[1]
168	D	A/E/H/I/N/Q/T/V/Y	G
170	V/I	A/T	
174	N[a]/S[b]	F	S[a]
175	L[a]/M[b]		L[b]

[1] (Barnard et al., 2013)

[2] (Lenz et al., 2010)

[3] (Kieffer et al., 2012)

[a] Genotype 1a only

[b] Genotype 1b only

Table 6.2. Table of resistance associated mutations in NS5A. Mutations were obtained from geno2pheno and other sources, indicated accordingly.

Position	Wild-type amino acid	Resistance mutations	Possible resistance mutations
24	K		$G^{a1}/N^{a1}/R^{a1}$
28	M^{a}/L^{b}	A^{a}/T	$A^{b}/G^{a1}/M^{b2}/V^{a}$
30	Q^{a}/R^{b}	$D^{a}/E/H/K^{a}/R^{a}$	$G^{a1}/L^{a1}/T^{a1}$
31	L	$I/M/V^{a}$	$F^{b}/G^{b3}/V^{b}/W^{b3}$
32	P	L	
38	S		F^{a1}
58	H^{a}/P^{b}	D	P^{a4}/S^{b}
92	A	K^{b}	T^{a1}
93	Y	$C^{a}/H/N/S/T^{a}$	F^{a1}

[1] (dvory-Sobol et al., 2014)

[2] (Fridell et al., 2010)

[3] (Walker et al., 2014)

[4] (Fridell et al., 2011b)

[a] Genotype 1a only

[b] Genotype 1b only

The Shannon entropy of each specimen was calculated to assess the complexity of the quasispecies. This value represents the number of variants and the evenness of their distribution; an increase in the number of variants or unevenness results in a higher entropy value.

The formula used to calculate the Shannon entropy is:

$$H = -\sum_{i}^{n} p_i \, ln \, p_i$$

where p_i is the probability of the amino acid at that position in the sequence. The Shannon entropy of each specimen was calculated using the output of VirVarSeq. The entropy of the NS3 protease region (amino acid 1-181) and NS5A Domains I and II (amino acids 1-342) were calculated for each patient using a custom script written by Sam Haldenby of the CGR, University of Liverpool.

6.2.5. Statistical analysis

Descriptive statistics were used to analyse the prevalence of mutants in the study population (as the proportion of subjects showing the mutation) and the frequency of mutants in each patient's sample (as the proportion of deep sequencing reads showing the mutation). Fisher's exact test was used to examine differences between groups. The Mann-Whitney U test was used to assess differences in the CD4 cells counts and HCV viral load of subjects with vs. those without detectable RAMs. Spearman's rank correlation coefficient was used to assess the association between the Shannon entropy and the CD4 cell count or HCV viral load. A p-value of ≤ 0.05 was considered significant. Statistical analysis was performed using SPSS version 21 (IBM Corporation, Armonk, NY, USA).

6.3. Results

6.3.1. Preliminary testing of the NGS assay

To test the sensitivity of the NGS assay for low-frequency variants, mock specimens consisting of mixtures of two wild-type and RAM-containing NS3 plasmids were tested, where the input of the RAMs-containing plasmid ranged from 1% to 10%. The plasmids were sequenced with an average of 233,331 reads (median 224,041; interquartile range, IQR 212,148-243,831). In each of the assays, the lowest input of the RAMs-containing plasmid was detected consistently. The estimated frequency of each RAM in the tested samples was approximately double the input (Table 6.3).

The NS3 wild-type plasmid was used to determine the assay error rate. The average error rate was 0.6% (SD ±0.2%) at the amino acid level. As a result, a cut-off value of 1% was chosen for analysis, which is also consistent with previous studies (Gianella et al., 2011, Dierynck et al., 2014).

The sequences obtained through Sanger sequencing of 115 samples (Chapter 5) were compared against the NGS reads to determine the frequency at which traditional sequencing fails to detect the variant. All RAMs present at a frequency ≥20% in the deep sequencing reads were also detected in the Sanger sequences. Of the 115 samples, two had a RAM at a frequency >10% and <20%. Both RAMs could also be identified in the Sanger sequences. Of the 10 RAMs present at a frequency <10% (range 1%-8.7%), none were detected in the Sanger sequences. Consequently, the threshold for low-frequency variants was set at <10% of NGS reads.

Table 6.3. The identification of low frequency variants in mock specimens comprising mixtures of wild type and resistant HCV NS3 plasmids

	Proportion of RAM-containing plasmid											
RAM	**1%**			**2%**			**5%**			**10%**		
	Replicate 1 (%)	**Replicate 2 (%)**	**Mean (%)**	**Replicate 1 (%)**	**Replicate 2 (%)**	**Mean (%)**	**Replicate 1 (%)**	**Replicate 2 (%)**	**Mean (%)**	**Replicate 1 (%)**	**Replicate 2 (%)**	**Mean (%)**
V36A	3.4	1.7	2.6	4.1	4.5	4.3	11.0	10.8	10.9	20.8	16.5	18.7
T54S	2.9	1.5	2.2	3.7	4.2	4.0	10.4	9.9	10.2	19.5	15.6	17.6
V55A	3.7	1.8	2.8	4.4	5.2	4.8	11.9	11.8	11.9	22.3	18.4	20.4
R155K	2.9	1.5	2.2	3.7	3.9	3.8	8.8	9.7	9.3	18.7	15.1	16.9
A156S	3.2	1.5	2.4	3.9	4.3	4.1	9.1	10.3	9.7	19.7	15.9	17.8

Each mock specimen was constructed according to the column heading i.e. 1% drug resistance contained the plasmid encoding drug resistance mutations at a frequency of 1% of the total population. The proportion of reads showing the mutation is shown in the replicate and average columns.

6.3.2. Study population

Of the 178 HCV mono-infected samples, 118 (66%) were Gt1a and 60 (34%) Gt1b. Of the 189 HIV/HCV co-infected samples, 156 (83%) were Gt1a and 33 (17%) were Gt1b. The average (median; IQR) HCV RNA load in mono-infected and co-infected samples was 6.1 (6.2; 5.7-6.5) and 6.4 (6.5; 6.1-6.8) $\log_{10}$ IU/ml, respectively. The average (median; IQR) CD4 count of HIV-positive individuals was 524 (468; 313-656) cells/mm^3. The subset of samples that were matched for genotype and HCV RNA load comprised 80 Gt1a and 27 Gt1b patients from the mono-infected and co-infected cohorts. Further details are summarised in Table 6.4.

Table 6.4. Characteristics of the matched subset of the study population

	HCV Mono-infected (n=107)	**HIV/HCV Co-infected (n=107)**
Genotype		
1a	80	80
1b	27	27
HCV Viral Load ($\log_{10}$ IU/ml), median [IQR]	6.30 [5.94-6.60]	6.33 [5.94-6.60]
CD4 count (cells/mm^3), median [IQR]	NA	455 [276-668]

NA – Not available

6.3.3. Next generation sequencing

Overall, 365 clinical samples yielded NGS results, with an average of 233,912 reads (median 228,482; IQR 179,019-277,616). One Gt1b sample from a HIV/HCV co-infected subject failed to amplify during the library preparation step. One HCV mono-infected sample did not align to either Gt1a or Gt1b reference sequences during the analysis and was excluded from further analysis.

6.3.4. Prevalence of RAMs in NS3

Nineteen positions in the NS3 gene known to cause a decrease in susceptibility to either linear and/or macrocyclic PIs were analysed (Table 6.1). RAMs occurring at any frequency ≥1% within the Illumina reads were identified at 14/19 recognised resistance sites, and involved NS3 codons 36, 54, 55, 80, 107, 109, 117, 132, 155, 158, 168, 170, 174, and 175. No RAMs were found at positions 41, 43, 122, 138, and 156. Gt1a strains displayed a significantly higher prevalence of RAMs (193/274, 70.4%) compared to Gt1b infections (20/91, 22.0%) (p=0.0001). The difference was driven mainly by the high prevalence of Q80K and N174S in Gt1a strains. Whereas Gt1a displayed a significantly higher prevalence of Q80K than Gt1b strains (p=0.0001), the RAM R117H was observed in a higher proportion of Gt1b patients than Gt1a patients (p=0.026) (Table 6.5).

In Gt1a, overall 37/274 (13.5%) subjects showed dual mutants, 10/274 (3.6%) subjects displayed triple mutants, and 2/274 (0.7%) displayed quadruple mutants (Table 6.6). In Gt1b, 3/91 (3.3%) patients harboured double mutants. No triple or quadruple mutants were observed in Gt1b patients.

When analysing prevalence of RAMs by HIV co-infection status, in Gt1a infections 71.2% (84/118) of mono-infected patients vs. 69.9% (109/156) of co-infected patients displayed ≥1 RAM (p=0.89); in Gt1b infections, prevalence of ≥1 RAM was 18.6% (11/59) vs. 28.1% (9/32), respectively (p=0.30). Major RAMs were identified in 28.0% (33/118) mono- and 28.2% (44/156) co-infected subjects with Gt1a infection. In Gt1b infections, 6.8% (4/59) and 9.4% (3/32) of mono- and co-infected patients, respectively, harboured a major RAM.

Multiple major RAMs were identified in 2.7% (10/365); 4/118 Gt1a mono-infected, 1/59 Gt1b mono-infected and 5/156 Gt1a co-infected patients. All of these patients harboured two major NS3 RAMs except one Gt1a mono-infected patient who harboured four major RAMs; two at high frequency and two at low frequency.

Table 6.5. Difference in NS3 RAM prevalence between Gt1a and Gt1b infected patients. Subtype-specific RAMs are not included

RAM	Prevalence in Gt1a (n=274) n (%)	Prevalence in Gt1b (n=91) n (%)	Significance (P-value)
V36A	-	1 (1.1)	0.25
V36L	4 (1.5)	-	0.58
V36M	6 (2.2)	-	0.34
T54S	7 (2.6)	2 (2.2)	1.00
V55A	9 (3.3)	1 (1.1)	0.46
V55I	6 (2.2)	-	0.34
Q80H	-	1 (1.1)	0.25
Q80K	44 (16.1)	-	0.0001*
Q80L	9 (3.3)	1 (1.1)	0.46
Q80R	3 (1.1)	1 (1.1)	1.00
V107I	2 (0.7)	3 (3.3)	0.10
R109K	1 (0.4)	-	1.00
R117H	3 (1.1)	5 (5.5)	0.026*
R155K	1 (0.4)	-	1.00
V158I	-	1 (1.1)	0.25
D168E	3 (1.1)	2 (2.2)	0.60
I/V170A	1 (0.4)	1 (1.1)	0.44
I/V170T	3 (1.1)	-	0.58
N174F	-	2 (2.2)	0.06

* indicates a significant difference (P-value <0.05) between the two subtypes

Table 6.6. Prevalence of multiple mutations in the NS3 and NS5A genes

Mutations	HCV Mono-infected		HIV/HCV Co-infected	
	1a (n=118)	1b (n=59)	1a (n=156)	1b (n=32)
	n (%)	n (%)	n (%)	n (%)
NS3				
Double	18 (15.3)	2 (3.4)	19 (12.2)	1 (3.1)
Major	3 (2.5)	1 (1.7)	5 (3.2)	-
Triple	6 (5.1)	-	4 (2.6)	-
Major	-	-	-	-
Quadruple	1 (0.8)	-	1 (0.6)	-
Major	1 (0.8)	-	-	-
NS5A				
Double	8 (6.8)	3 (5.1)	5 (3.2)	-
Major	1 (0.8)	1 (1.7)	2 (1.3)	-
Triple	2 (1.7)	-	2 (1.3)	-
Major	-	-	-	-
Quadruple	-	-	-	-
Major	-	-	-	-

6.3.5. Prevalence of high-frequency RAMs in NS3

Overall, high-frequency RAMs were found at 11/19 sites analysed, comprising 36, 54, 55, 80, 107, 117, 132, 155, 168, 170, and 174. Of 177 HCV mono-infected patients, 83 (46.9%) harboured ≥1 RAM at a high frequency compared to 104/188 (55.3%) co-infected patients (p=0.12). There was no difference in the prevalence of RAMs when comparing mono-infected and co-infected patients by HCV Gt1 subtypes. HCV Gt1a strains showed RAMs in 76/118 (64.4%) mono-infected subjects vs. 100/156 (64.1%) co-infected patients (p=1.00) (Figure 6.1); HCV Gt1b strains showed prevalences of 7/59 (11.9%) vs. 4/32 (12.5%) respectively (p=1.00) (Figure 6.2). Tables detailing the frequencies of RAMs observed can be found in the Appendix.

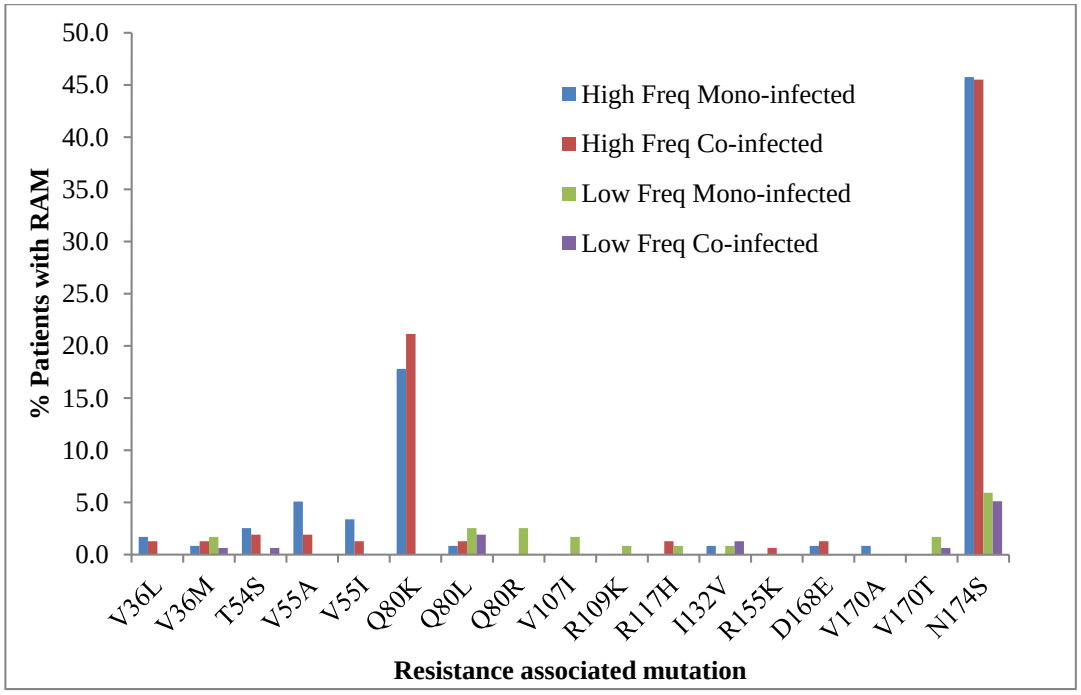

Figure 6.1. The prevalence of Gt1a infected patients harbouring resistance mutations in the NS3 gene at high frequency (≥10%) and low frequency (≥1% *x* <10%).

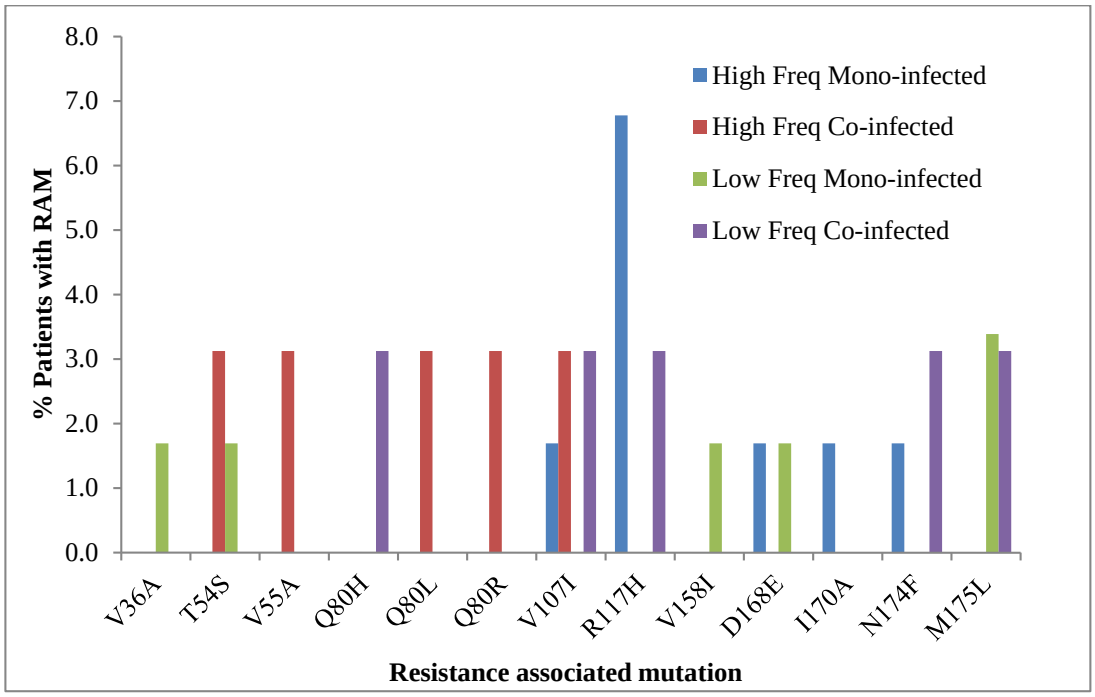

Figure 6.2. The prevalence of Gt1b infected patients harbouring resistance mutations in the NS3 gene at high frequency (≥10%) and low frequency (≥1% *x* <10%).

The subtype-specific substitution N174S was the most common mutation observed in Gt1a samples from both mono- and co-infected subjects, with a prevalence of 45.8% (54/118) and 45.5% (71/156), respectively. The polymorphism Q80K was the second most prevalent substitution observed in the cohort, with a prevalence of 19.7% (54/274) in Gt1a and 0% (0/91) in Gt1b samples (p=0.0001). Within HCV Gt1a strains, 21/118 (17.8%) HCV mono-infected patients harboured the Q80K polymorphism whilst 33/156 (21.2%) HIV/HCV co-infected patients harboured this substitution (p=0.54). Excluding the substitutions N174S and Q80K, high frequency RAMs were identified in 22/177 (12.4%) mono-infected subjects; 15/118 (12.7%) for HCV Gt1a and 7/59 (11.9%) for HCV Gt1b. Of 188 co-infected samples, 20 (10.6%) harboured a RAM; 16/156 (10.3%) Gt1a and 4/32 (12.5%) Gt1b. Again, there was no difference in the prevalence of high-frequency RAMs between mono- and co-infected subjects with either Gt1a infection (15/118, 12.7% vs. 16/156, 10.3%; p=0.57) or Gt1b infection (7/59, 11.9% vs. 4/32, 12.5%; p=1.00).

In Gt1a infections, major RAMs were identified in 27.1% (32/118) and 27.6% (43/156) mono- and co-infected patients, respectively (p=1.00). A lower proportion of Gt1b patients harboured major RAMs; 3.4% (2/59) mono-infected and 6.3% (2/32) co-infected subjects (p=0.61). Patients harbouring dual major RAMs at high frequencies were rare, with only 2.5% (3/118) and 1.9% (3/156) of Gt1a mono- and co-infected patients showing such resistance profile. No Gt1b patients were identified as having multiple major RAMs.

In Gt1a patients, 15/118 (12.7%) and 19/156 (12.2%) of mono-infected and co-infected patients harboured dual mutants whilst 2/118 (1.7%) and 2/156 (1.3%)

patients were identified with triple mutants, respectively. One co-infected Gt1b patient was found to harbour two high frequency RAMs in the NS3 gene.

Patients with Gt1a infections displayed a significantly higher prevalence of high-frequency RAMs (176/274, 64.2%) compared to Gt1b (11/91, 12.1%) (p=0.0001). However, excluding the N174S and the Q80K polymorphism, which did not occur in Gt1b infections, there was no significant difference between Gt1a and Gt1b in the prevalence of other RAMs (31/274, 11.3% vs. 11/91, 12.1%; p=0.85).

Upon comparing the HCV RNA load of patients with and those without a high frequency RAM, no difference was found (median [IQR], 6.33 [5.94-6.70] vs. 6.27 [5.84-6.65] $\log_{10}$ IU/ml, respectively) (p=0.39).

Among HIV/HCV co-infected subjects, prevalence of RAMs was 11/24 (45.8%) in ART-naïve subjects ([T54S (n=1), Q80K (n=3) and N174S (n=10)]. There was no difference between the prevalence of RAMs in ART-naïve vs. ART-experienced patients, when Q80K and N174S were either included (11/24, 45.8% vs. 93/164, 56.7%; p=0.38) or excluded (1/24, 4.2% vs. 19/164, 11.6%; p=0.48). When RAM prevalence was analysed by CD4 cell counts, there was no difference between those patients harbouring a RAM vs. those without (p=0.92). The median (IQR) CD4 count was 464 (301-661) vs. 469 (313-658) cells/mm^3 in patients with vs. patients without RAMs, respectively.

6.3.6. Prevalence of low-frequency RAMs in NS3

Of the 19 NS3 positions analysed, 12 showed ≥1 low-frequency RAM, occurring at positions 36, 54, 80, 107, 109, 117, 132, 158, 168, 170, 174, and 175. No low-frequency RAMs were observed at positions 41, 43, 55, 122, 138, 155, and 156. Among the HCV mono-infected patients, 26/177 (14.7%) harboured ≥1 low-frequency RAM compared to 18/188 (9.6%) HIV/HCV co-infected patients (p=0.15). The detected RAMs are detailed in Figures 6.1 and 6.2. Tables detailing the frequencies of RAMs observed can be found in the Appendix. When analysing the prevalence of low-frequency RAMs by HIV co-infection status and HCV Gt1a subtype, HCV mono-infected patients had a significantly higher prevalence of low-frequency RAMs than HIV/HCV co-infected patients: 17.8% (21/118) vs. 8.3% (13/156), respectively (p=0.026). This difference was still significant upon the exclusion of N174S (14/118, 11.9% vs. 7/156, 4.5%) (p=0.037). Within HCV Gt1b, the prevalence of low-frequency RAMs was approximately double in HIV/HCV co-infected patients (5/32, 15.6%) compared to HCV mono-infected patients (5/59, 8.5%) although the difference was not significant (p=0.31). As was the case for high frequency RAMs, the substitution N174S was also the most commonly observed low-frequency RAM present in 5.9% (7/118) mono- and 5.1% (8/156) co-infected Gt1a individuals.

Major RAMs were not as prevalent at low frequency as they were at high frequency in Gt1a infections; with only 2.5% (3/118) and 1.9% (3/156) of mono- and co-infected patients harbouring a major RAM, respectively (p=1.00). No difference was observed between the prevalence of low-frequency major RAMs in mono and co-infected subjects (2/59, 3.4% vs. 1/32, 3.1%, p=1.00). Two patients were identified

as harbouring dual major RAMs at low frequency; one (0.8%) Gt1a mono-infected patient and one (1.7%) Gt1b mono-infected patient.

Dual low frequency mutants were identified in 1/118 (0.8%) mono-infected and 2/156 (1.3%) co-infected Gt1a patients and 1/59 (1.7%) Gt1b mono-infected patient. Of the 44 patients harbouring ≥1 low-frequency RAM, 18 (36.7%) showed also ≥1 high-frequency RAM.

Upon comparing HCV Gt1 subtypes, 34/274 (12.4%) of Gt1a samples and 10/91 (11.0%) Gt1b samples harboured ≥1 low-frequency RAM (p=0.85).

There was no difference in the HCV viral load between patients showing a low-frequency RAM and those not displaying a RAM (median [IQR], 6.29 [6.03-6.71] vs. 6.31 [5.90-6.68] $\log_{10}$ IU/ml, respectively) (p=0.60).

Four (16.7%) ART-naïve subjects harboured at least one RAM at a low abundance; Q80L (n=1), I132V (n=1), N174S (n=2) and M175L (n=1). This was no different to the prevalence of 14/164 (8.5%) observed in ART-experienced patients (p=0.26). The prevalence of low-frequency RAMs did not differ by CD4 cell count, with medians (IQR) of 397 (273-556) vs. 486 (314-664) cells/mm^3 in individuals with vs. without RAMs, respectively (p=0.25).

6.3.7. Detection of NS3 RAMs below the interpretative cut-off of 1%

The utilisation of a lower interpretative cut-off resulted in 22/177 mono-infected and 15/188 co-infected samples harbouring a RAM at a level between 0.5% and 1%. In

Gt1a individuals, there was no significant difference between mono- (18/118, 15.3%) and co-infected (14/156, 9.0%) individuals (p=0.13). This was also true of Gt1b individuals; 8.5% (5/59) mono-infected vs. 3.1% (1/32) co-infected (p=0.42). Further analysis with a lower interpretative cut-off of 0.1% revealed 62.1% of mono-infected subjects displayed a RAM compared to 69.1% of co-infected individuals. As before, there were no significant differences between mono- and co-infected patients with Gt1a infection (67.8% vs. 71.8%, p=0.51) or with Gt1b infection (50.8% vs. 56.3%, p=0.67).

6.3.8. Prevalence of RAMs in NS5A

Nine sites known to harbour RAMs to NS5A inhibitors were analysed (Table 6.2). Overall, RAMs occurring at any frequency were observed at 7/9 sites analysed, comprising 24, 28, 30, 31, 32, 58, and 93. No RAMs were found at positions 38 and 92 at any level. The prevalence of RAMs did not differ between HCV mono-infected and HIV/HCV co-infected patients with either HCV Gt1a infections (33/118, 28.0% vs. 35/156, 22.4%, p=0.32) or Gt1b infections (21/59, 35.6% vs. 9/32, 28.1%, p=0.50). Although HCV Gt1b strains displayed a higher prevalence of RAMs than Gt1a infections; 30/91 (33.0%) vs. 68/274 (24.8%), respectively, the difference was not significant (p=0.14). Overall, in Gt1a infections, double mutants were identified in 14/274 (5.1%) patients and triple mutants in 4/274 (1.5%) patients (Table 6.6). Quadruple mutants were not identified in any Gt1a infection. In Gt1b infections, 3/91 (3.3%) patients showed double mutants. Triple or quadruple mutants were not identified in any Gt1b patient.

Table 6.7. Difference in NS5A RAM prevalence between Gt1a and Gt1b infected patients. Subtype-specific RAMs are not included.

RAM	Prevalence in Gt1a (n=274) n (%)	Prevalence in Gt1b (n=91) n (%)	Significance (P-value)
M/L28T	4 (1.5)	-	0.58
Q/R30H	6 (2.2)	-	0.34
L31I	-	1 (1.1)	0.25
L31M	6 (2.2)	4 (4.4)	0.27
L31V	1 (0.4)	-	1.00
P32L	-	1 (1.1)	0.25
Y93H	2 (0.7)	14 (15.4)	0.0001*

* indicates a significant difference (P-value <0.05) between subtypes

Only one RAM showed a difference in prevalence between the two subtypes (Table 6.7). There was a significantly higher prevalence of Y93H in Gt1b strains compared to Gt1a strains (p=0.0001).

In Gt1a infections, major RAMs were identified in 11.9% (14/118) mono-infected and 8.3% (13/156) co-infected patients. Gt1b infections showed prevalences of 23.7% (14/59) and 15.6% (5/32) in mono- and co-infection, respectively. Multiple major RAMs were rare, with only 1.1% (4/365) of patients harbouring them; 1/118 Gt1a mono-infected, 1/59 Gt1b mono-infected and 2/156 Gt1a co-infected.

6.3.9. Prevalence of high-frequency RAMs in NS5A

Overall, high-frequency RAMs were found at 6/9 sites, comprising codons 24, 28, 30, 31, 58 and 93. Of 177 mono-infected patients, 31 (17.5%) harboured at least 1 high frequency RAM whilst 20/188 (10.6%) co-infected patients were identified as having ≥1 RAM (p=0.07). Of the Gt1a patients, 18/118 (15.3%) mono-infected

patients harboured ≥1 RAM at a high frequency whilst prevalence was 13/156 (8.3%) in co-infected patients (p=0.085) (Figure 6.3). In Gt1b infected patients, the prevalence of high frequency RAMs was 22.0% (13/59) vs. 21.9% (7/32) in mono- and co-infected patients, respectively (p=1.00) (Figure 6.4). Tables detailing the frequencies of RAMs identified can be found in the Appendix.

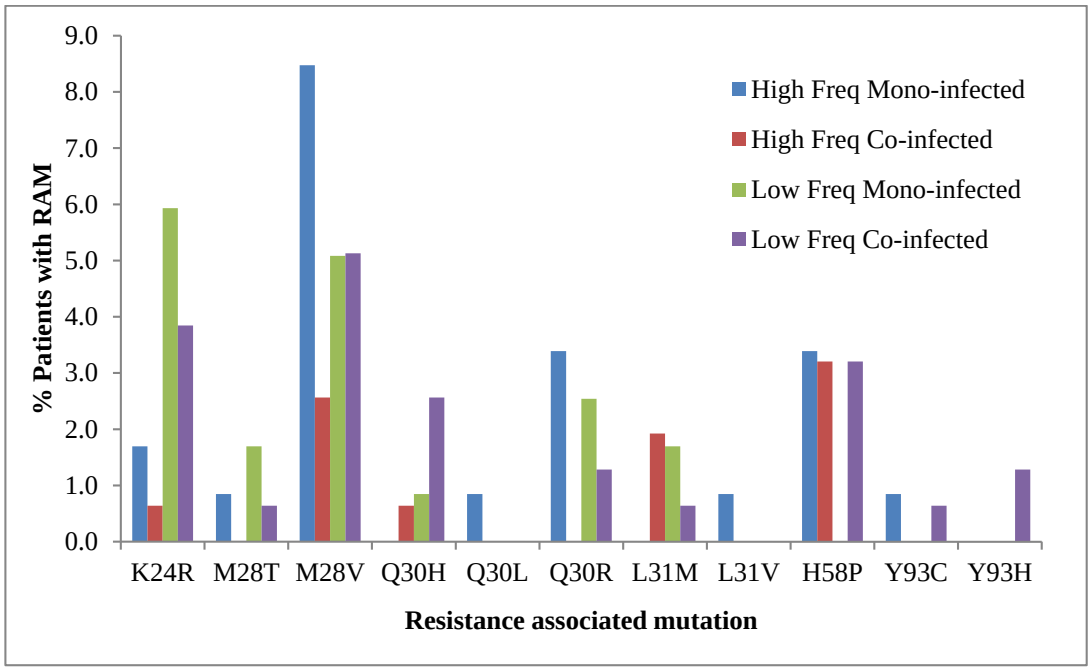

Figure 6.3. The prevalence of Gt1a infected patients harbouring resistance mutations in the NS5A gene at high frequency (≥10%) and low frequency (≥1% *x* <10%).

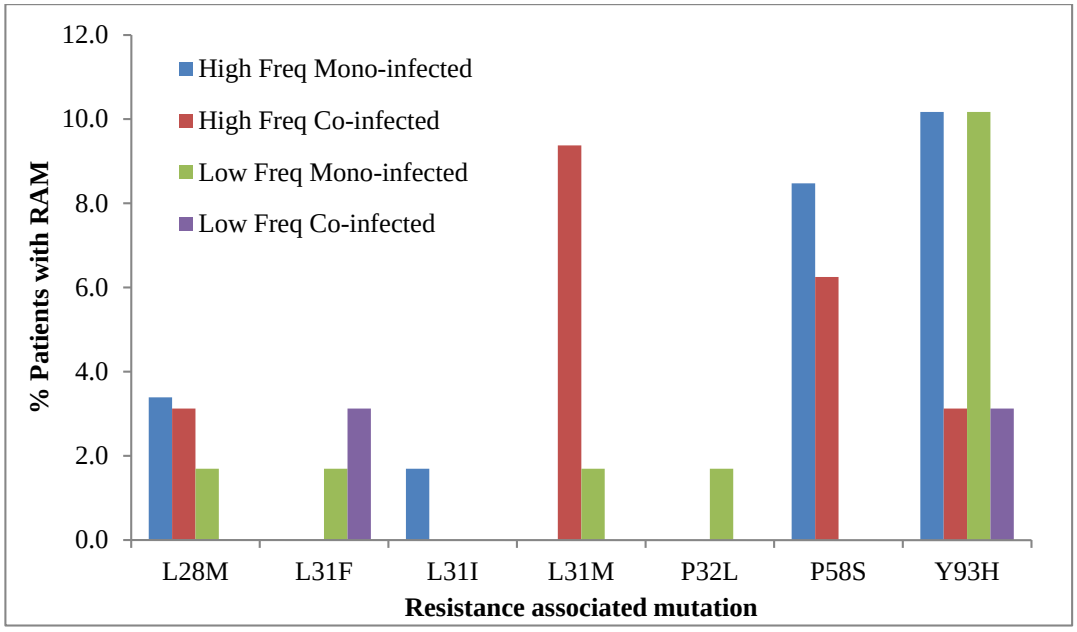

Figure 6.4. The prevalence of Gt1b infected patients harbouring resistance mutations in the NS5A gene at high frequency (≥10%) and low frequency (≥1% *x* <10%).

The most common substitution was M28V in Gt1a patients, observed in 10/118 (8.5%) mono-infected and 4/156 (2.6%) co-infected patients. In Gt1b patients, Y93H was the most common substitution seen in 6/59 (10.2%) and 1/32 (3.1%) mono- and co-infected patients, respectively.

Major RAMs were observed in 5.9% (7/118) and 2.6% (4/156) mono- and co-infected patients infected with Gt1a (p=0.22). In Gt1b infections, 11.9% (7/59) mono-infected and 12.5% (4/32) co-infected patients showed a major RAM at high frequency (p=1.00). No patients were identified as having multiple major RAMs at high frequency.

Gt1b patients showed significantly higher prevalence of high frequency RAMs (20/91, 22.0%) compared to Gt1a patients (31/274, 11.3%) (p=0.015). The Y93H

mutation was not observed at high frequencies in any Gt1a patients in comparison to 7/91 (7.7%) Gt1b patients.

The HCV viral load did not differ between patients harbouring a high frequency RAM (median [IQR], 6.37 [5.90-6.75] log_{10} IU/ml) and those not showing a high frequency RAM (median [IQR], 6.30 [5.92-6.68] log_{10} IU/ml) (p=0.65).

Among HIV/HCV co-infected subjects, no ART-naïve patient harboured a high frequency RAM in the NS5A gene. There was no difference in RAM prevalence between ART-naïve vs. ART-experienced patients (0/24, 0% vs. 20/164, 12.2%; p=0.081). No difference was found between the CD4 count of HIV-positive patients with and without a high frequency RAM (median [IQR], 561 [271-718] vs. 455 [312-652] cells/mm^3) (p=0.60).

In Gt1a infected patients, dual RAMs were identified in 4/118 (3.4%) mono-infected and 1/156 (0.6%) co-infected patients. Of Gt1b infected patients, only one mono-infected patient harboured two mutants. One patient was identified with three high frequency RAMs; one Gt1a mono-infected patient.

6.3.10. Prevalence of low frequency RAMs in NS5A

Low frequency RAMs were observed at 7/9 positions analysed; 24, 28, 30, 31, 32, 58, and 93. Of 177 mono-infected patients, 29 (16.4%) harboured ≥1 low frequency RAM compared to 27/188 (14.4%) co-infected patients (p=0.66). When analysing prevalence of low-frequency RAMs by HIV status and HCV subtype, there was no difference between HCV mono-infected and HIV/HCV co-infected patients. In Gt1a

infections, 20/118 (16.9%) mono-infected patients showed ≥1 RAM compared to 25/156 (16.0%) HIV/HCV co-infected subjects (p=0.87) (Figure 6.3). In Gt1b infections, 9/59 (15.3%) mono-infected patients vs. 2/32 (6.3%) co-infected patients harboured ≥1 low frequency RAM (p=0.32) (Figure 6.4). Tables detailing the frequencies of RAMs identified can be found in the Appendix.

Major RAMs present at a low frequency were identified in 6.8% (8/118) and 5.1% (8/156) of mono- and co-infected patients, respectively, with Gt1a infection. In Gt1b, despite mono-infected subjects displaying a higher prevalence of major RAMs than co-infected subjects (8/59, 13.6% vs. 1/32, 3.1%), this difference was not significant (p=0.15).

Two patients were identified with dual major RAMs at low frequencies; both were Gt1a co-infected subjects. There was no difference in prevalence of low frequency RAMs between Gt1a and Gt1b infected subjects (45/274, 16.4% vs. 11/91, 12.1%, respectively) (p=0.40).

No difference in HCV RNA load was observed between patients with vs. those without a low frequency RAM (median [IQR], 6.36 [6.02-6.71] vs. 6.27 [5.90-6.68] $\log_{10}$ IU/ml, respectively) (p=0.23).

The proportion of ART-naïve patients displaying a low frequency RAM (2/24, 8.3%) was not different to that of ART-experienced patients (25/164, 15.2%) (p=0.54). Both RAMs in ART-naïve patients were found in Gt1a patients; K24R (n=1) and M28V (n=1).

Among HIV-positive patients, there was no difference in the CD4 count of patients harbouring a low frequency RAM to those who did not harbour one (median [IQR], 419 [281-550] vs. 474 [312-670] cells/mm^3) (p=0.24).

In Gt1a patients, 1/118 (0.8%) and 1/156 (0.6%) were identified as having two low frequency RAMs. One (1.7%) Gt1b mono-infected patient harboured two RAMs. Two patients, both Gt1a co-infected, were identified with three RAMs present at low frequency. Of the 54 patients harbouring a low frequency substitution in NS5A, 9 (16.7%) also carried ≥1 high frequency mutation.

6.3.11. Detection of NS5A RAMs below the interpretative cut-off of 1%

RAMs occurring at a frequency of ≥0.5% and <1% were found in 13.0% and 13.8% of mono- and co-infected subjects, respectively. In Gt1a individuals, there was no significant difference between mono- (18/118, 15.3%) and co-infected (24/156, 15.4%) individuals (p=1.00). This was also true of Gt1b individuals, with a prevalence of 11.9% (5/59) in mono-infected and 6.3% (2/32) in co-infected patients (p=1.00). Mutations at even lower levels of ≥0.1% and <0.5% were present in 34.5% in mono-infected subjects and 64.9% in co-infected individuals. In Gt1a, a significantly higher proportion of co-infected patients harboured a mutation compared to mono-infected subjects; (116/156, 74.4% vs. 50/118, 42.4%, respectively) (p=0.0001). No significant difference was found in the prevalence of such low-level resistance mutations between mono-infected (11/59, 18.6%) and co-infected patients (6/32, 18.8%) in Gt1b infections (p=1.00).

6.3.12. Prevalence of dual-class NS3/NS5A resistance

The occurrence of high-frequency RAMs in both NS3 and NS5A was uncommon: 7.9% (14/177) mono-infected and 5.3% (10/188) co-infected patients harboured such dual-class substitutions. Low-frequency RAMs or a combination of high- and low-frequency RAMs occurring in both genes were present in 10.7% (19/177) mono-infected and 11.7% (22/188) co-infected patients. Of the 57 patients harbouring multiple RAMs, N174S was the only NS3 RAM present in 28 (49.1%) of them.

6.3.13. Quasispecies complexity of NS3

Overall, co-infected patients showed a trend towards a higher complexity than mono-infected patients in the NS3 protease region (median [IQR], 3.10 [2.54-4.34] vs. 2.82 [2.27-4.17], respectively, p=0.05).

In Gt1a infections, mono-infected patients showed a median (IQR) complexity of 2.83 (2.26-4.13) compared to 3.01 (2.46-4.34) of co-infected patients (p=0.11). In Gt1b infections, the median (IQR) complexity was 2.80 (2.38-4.34) and 3.44 (2.76-4.55) in mono- and co-infected patients, respectively (p=0.16). Among HIV-positive subjects, the entropy value showed no association with the CD4 count (Spearman's rank=-0.02, p=0.79). There was a weak, positive correlation between the entropy values and the HCV viral load (Spearman's rank=0.26, p=0.0001).

6.3.14. Quasispecies complexity of NS5A

Overall, there was no difference in quasispecies complexity between mono-infected and co-infected subjects (median [IQR] 7.67 [5.77-11.02] vs. 8.43 [6.20-11.46], respectively, p=0.27). As was the case with NS3 complexity, co-infected patients showed a non-significant increase in NS5A complexity compared to mono-infected

patients when split by subtype. In Gt1a infections, mono-infected subjects showed a median (IQR) complexity of 7.41 (5.62-10.12) against 8.30 (6.13-11.15) of co-infected subjects (p=0.15). In Gt1b infections, the median (IQR) complexity was 8.57 (5.98-12.53) vs. 9.78 (6.81-12.88) for mono-infected vs. co-infected patients, respectively (p=0.60). Shannon entropy values showed a very weak, positive correlation with CD4 count (Spearman's rank=0.08, p=0.27). There was a weak, positive correlation between the entropy and the HCV viral load (Spearman's rank=0.25, p=0.0001)

6.3.15. Prevalence of RAMs and quasispecies complexity in the matched subgroup of patients

A subgroup of samples was matched according to the HCV Gt1 subtype and the HCV RNA load to enable a direct comparison to be made between HCV mono-infected and HIV/HCV co-infected patients.

In this subset, there was no difference in the prevalence of high-frequency NS3 RAMs between mono-infected and co-infected subjects in Gt1a (51/80, 63.8% vs. 53/80, 66.3% p=0.87) or Gt1b (5/27, 18.5% vs. 3/27, 11.1%, p=0.70). However, the prevalence of low- frequency RAMs was significantly higher in mono-infected patients than co-infected patients in Gt1a (16/80, 20% vs. 3/80, 3.8%, respectively, p=0.0025). Again, this significance was still present upon the exclusion of N174S (11/80, 13.8% vs. 3/80, 3.8%, p=0.047). In Gt1b strains, there was no difference in prevalence between mono- and co-infected patients (3/27, 11.1% vs. 3/27, 11.1%, p=1.00).

Upon comparing high frequency RAMs in the NS5A gene of Gt1a infected patients, mono-infected subjects showed approximately double the prevalence compared to co-infected subjects (14/80, 17.5% vs. 6/80, 7.5%) although this difference did not reach statistical significance (p=0.092). Among Gt1b infections, there was no difference between mono-infected (6/27, 22.2%) and co-infected (7/27, 25.9%) patients (p=1.00). When analysing prevalence of low-frequency RAMs, there was no difference between HCV mono-infected and HIV/HCV co-infected patients in either subtype. In Gt1a infections, 14/80 (17.5%) mono-infected patients showed ≥1 RAM compared to 16/80 (20.0%) HIV/HCV co-infected subjects (p=0.84). In Gt1b infections, mono-infected patients displayed double the prevalence of low-frequency RAMs compared to co-infected patients (5/27, 18.5% vs. 2/27, 7.4%) although the difference was not significant (p=0.42).

In the matched subset of patients, there was no difference in NS3 quasispecies complexity between mono- and co-infected subjects (median [IQR] 3.05 [2.43-4.31] vs. 2.93 [2.50-4.39], respectively, p=0.84). Difference in NS3 complexity in mono-infected patients vs. co-infected patients did not reach statistical significance for either Gt1a (median [IQR] 3.08 [2.34-4.31] vs. 2.80 [2.25-4.39]) (p=0.68) or Gt1b (median [IQR] 2.84 [2.48-4.41]) vs. 3.42 [2.74-4.67] (p=0.32). Similarly, NS5A complexity in co-infected patients (median [IQR] 8.08 [6.06-11.41]) was not different from that of mono-infected patients (8.58 [5.95-12.38]), (p=0.65). In Gt1a strains, the median (IQR) complexity was 8.57 (5.93-10.94) vs. 7.66 (5.96-11.00) in mono-infected vs. co-infected patients (p=0.52). The median (IQR) in Gt1b strains was 9.83 (6.08-14.05) vs. 9.58 (6.77-13.10) in mono-infected vs. co-infected patients, respectively (p=0.99).

6.4. Discussion

In this large cross-sectional cohort study of HCV mono-infected and HIV/HCV co-infected patients, we determined the prevalence of recognised HCV RAMs in HCV treatment-naïve patients using an NGS approach. This is the largest study to date to use NGS technology to determine the impact of HIV on the natural prevalence of HCV RAMs in two key genes. Overall, RAMs were relatively common in both NS3 and NS5A, occurring in 58.4% and 26.8% of patients, respectively. Multiple NS3 RAMs were seen in 14.2% while multiple NS5A RAMs were observed in 5.8%. Major RAMs were seen in approximately half of patients harbouring ≥1 RAM. It was observed that, generally, there was no significant difference in the prevalence of high or low frequency RAMs between HIV/HCV co-infected patients and HCV mono-infected patients. Viral load and CD4 count did not differ between those patients with and those patients without a RAM.

During the preliminary testing of the NGS protocol, the frequency of the RAMs in the RAM-containing plasmid was estimated at double the input. This may reflect preferential amplification, which is known to account for a skew in the frequency measurement of HIV variants, typically by 2- to 15-fold (Jabara et al., 2011). However, the wild-type and RAMs-containing plasmids did not differ in nucleotides within the primer binding region, which is believed to account for the skew during PCR (Kanagawa, 2003). One alternative explanation lies with the underquantification of the plasmids prior to NGS, which may have lead to an increase in the input of the RAMs-containing plasmid. It is nevertheless important to emphasise that the assay was still able to detect variants present at low frequencies. The average error rate of the assay was determined as 0.6% at the codon level. This

is consistent with the recommended threshold of 1% used in previous studies examining HIV variants (Gianella et al., 2011). A threshold of 1% provides a useful cut-off for two main reasons: i) levels below this fall into the error rate of the assay and, as such, variants may be artefacts and ii) true variants at ultra-low levels <1% may have no biological significance in the treatment of HCV (Akuta et al., 2014a, Akuta et al., 2014b, Murakami et al., 2014).

The VirVarSeq pipeline used for the analysis of the deep sequencing reads in this study has previously been validated for the detection of minority variants present at frequencies as low as 0.5% in a population (Verbist et al., 2015). The pipeline also includes the production of a consensus sequence of the sample in question, to which sequencing reads can be subsequently mapped (Verbist et al., 2015). This procedure increases the coverage in samples which differ from the reference sequence, thus greatly reducing the likelihood of false negative results. These features make this pipeline a suitable tool for the analysis of the deep sequencing reads in the present study. Furthermore, the reporting of variants at a codon level makes VirVarSeq suitable for a high-throughput clinical setting, where the immediate detection of drug resistance would be important.

NS3 RAMs were relatively common in both mono- and co-infected patients, with 58.4% of patients showing ≥1 RAM at a frequency ≥1%. One of the most common RAMs observed in NS3 was Q80K, which, as discussed in the previous chapter, confers an ~8 fold change (FC) in resistance to simeprevir *in vitro* and also reduces responses to simeprevir treatment (Lenz et al., 2010, Forns et al., 2014, Jacobson et al., 2014, Manns et al., 2014). The N174S substitution was highly prevalent in HCV

Gt1a strains, occurring in 51.1% of patients. This finding is consistent with previous studies (Vallet et al., 2011, Paolucci et al., 2012). N174S is a minor RAM and believed to play an accessory role in drug resistance. It has been observed as an emerging mutation at the time of treatment failure in a small minority of patients failing treatment with simeprevir plus P/R, but only in combination with a mutation at positions 80, 155 and/or 168 (Lenz et al., 2015).

Major RAMs were observed at a prevalence of 23.0% in our cohort. The mutation R155K, conferring a 30- and 37-FC in resistance to simeprevir (Lenz et al., 2010) and paritaprevir (Pilot-Matias et al., 2015), respectively, was only observed in one patient at a high frequency. The RAM D168E was identified in five patients; three of which harboured it at high frequency. This mutation decreases susceptibility to simeprevir and paritaprevir by 40- and 14-FC *in vitro* (Lenz et al., 2010, Pilot-Matias et al., 2015). Both of these RAMs have been observed at treatment failure with paritaprevir, ombitasvir and dasabuvir combination therapy (Krishnan et al., 2015c). RAMs at positions 36(A/L/M), 54(S) and 55(A/I) were more commonly seen mutations in patients, with 7.7% of patients harbouring at least one of these RAMs. Decreases in susceptibility to simeprevir and paritaprevir are no higher than 3-fold *in vitro* in any of these mutations (Lenz et al., 2010, Pilot-Matias et al., 2015). Several mutations at position 156 are known to confer high levels of resistance to current PIs *in vitro*, although none were observed in this study.

Upon excluding the Q80K and N174S polymorphisms, the overall prevalence of high-frequency NS3 RAMs (11.5%) lies somewhere between the range of prevalences seen in other studies using traditional population sequencing (Bartels et

al., 2008, Kuntzen et al., 2008, Paolucci et al., 2012, Vicenti et al., 2012, Palanisamy et al., 2013). One reason for the higher prevalence seen here than in some of the other studies (Bartels et al., 2008, Kuntzen et al., 2008, Paolucci et al., 2012) is probably due to a larger number of sites associated with resistance being studied relative to these earlier studies. The study population is also likely to account for differences. In a recent study, the prevalences of NS5A RAMs were seen to differ between populations from different countries (Svarovskaia et al., 2015).

RAMs were found at seven of the nine key sites associated with resistance in domain I of NS5A, with positions 38 and 92 showing no mutations at frequencies ≥1%. Overall, 26.8% of patients harboured a RAM in the NS5A gene. The M28V mutation was the most common substitution observed at both high and low frequencies. This RAM only occurs in Gt1a strains, and confers low-level (1.3FC) resistance to daclatasvir and a 58-fold reduced susceptibility to ombitasvir *in vitro* (Fridell et al., 2011b, Krishnan et al., 2015a). M28V has been observed as a variant present at the point of virological failure in a study of paritaprevir, ombitasvir and dasabuvir, either as the sole RAM or in combination with RAMs in other genes (Krishnan et al., 2015c). Y93H confers resistance to daclatasvir, ledipasvir and ombitasvir – all three NS5A inhibitors currently licensed for clinical use. The mutation causes a more dramatic reduction in susceptibility to the inhibitors in Gt1a strains, ranging from 1,677-FC to 41,383-FC compared to Gt1b, where the highest FC is 77 (Fridell et al., 2011b, Wong et al., 2013, Krishnan et al., 2015a). Y93H has also been observed in cases of virological failure in both Gt1a and Gt1b patients upon treatment with daclatasvir with P/R or in combination with asunaprevir (Suzuki et al., 2013, Hezode et al., 2015, Kosaka et al., 2015). Another Gt1a-specific RAM,

Q30R, was seen at a prevalence of 3.3%. This confers a high-level of resistance to all three NS5A inhibitors (range 632-FC to 1227-FC in susceptibility) (Fridell et al., 2011b, Wong et al., 2013, Krishnan et al., 2015a) and has also been observed in cases of virological failure during treatment with paritaprevir, ombitasvir and dasabuvir (Krishnan et al., 2015c). The Gt1a-specific K24R mutation, which confers low-level resistance to ledipasvir *in vitro* and has also been identified in treatment failures, was also relatively common being observed in 5.8% of Gt1a patients (dvory-Sobol et al., 2014, dvory-Sobol et al., 2015).

Previous studies using deep sequencing to study low-frequency RAMs in HCV infection have shown a large degree of variation in prevalences observed. However, the comparison of results is confounded by two factors; i) the difference in RAMs examined and ii) the differences in cut-off values used for the identification of minority variants. Through the examination of more sites associated with resistance, the likelihood of a patient harbouring a RAM increases. Our study examines nearly double the number of sites than that of a previous study (Dierynck et al., 2014) and thus contributes to the difference in prevalence observed. However, the additional RAMs observed confer only minor resistance to PIs. If only the same substitutions were examined, the overall prevalence of low frequency NS3 RAMs in our study would decrease from 13.4% to 4.9%; a value much closer to the prevalence of 3.8% observed in the aforementioned study (Dierynck et al., 2014). The field of resistance to DAAs is evolving rapidly as more drugs are licensed and more studies, both *in vitro* and *in vivo*, are performed to identify sites associated with resistance. This adds further complications to the comparison of RAM prevalences between studies.

Jabara et al., (2014) observed that 98% of 40 patients carried at least 1 low-frequency RAM using an interpretative cut-off value of 0.08% whilst another study observed a low-frequency RAM (<20%) prevalence of 33% (n=28) when using a cut-off value of 0.35% (Bartolini et al., 2013). However, in a larger study of baseline samples (n=185) using a cut-off value of 1%, it was found that only 3.8% of patients harboured a low-frequency RAM, not detected by Sanger sequencing (Dierynck et al., 2014). Our observation of 13.4% of patients harbouring a low-frequency NS3 RAM, using a 1% cut-off value, lies somewhere between two of the studies. All of the samples in our study were from patients naïve to all forms of anti-HCV therapy, meaning that none of the variants observed are expected to be a result of selection by previous treatment. Previous studies indicated that the majority of low-frequency RAMs observed existed at frequencies of below 1%. This is in concordance with our study, where applying lower interpretative cut-off values of 0.5% and 0.1% increased the proportion of samples showing ≥1 RAM. It should be noted that the ultra-low frequency (<1%) variants observed fell within the error rate of the assay, which makes the significance of such mutations doubtful. These variants are likely to be technical artefacts as a result of misincorporation during reverse transcription, PCR stages or a false call by the sequencing platform. NS5A variants present at a frequency between 0.1% and 0.5% were present in a significantly higher proportion of co-infected subjects than mono-infected patients. If the variants were truly artefacts, then one would expect a similar proportion in each of the two groups. Immunosuppression and a reduced ability to clear the virus in these co-infected patients may contribute to this difference. Nevertheless, substitutions at such low levels are not expected to impact on the treatment outcome (Akuta et al., 2014a,

Akuta et al., 2014b, Murakami et al., 2014) meaning that this association with co-infected patients should not affect the risk of virological failure.

Our study observed a significant difference between the presence of high frequency NS3 RAMs in Gt1a and Gt1b infections although this was due to the Gt1a-specific N174S mutation and the Q80K substitution. Whilst there was still a modest increase in the presence of high-frequency RAMs in Gt1a when these polymorphisms were excluded, the significance was lost. There was also no significant difference between subtypes upon the comparison of low frequency RAMs. This observation is in concordance with a previous study, which also used NGS to determine the differences in RAM frequencies between the two subtypes of Gt1 (Margeridon-Thermet et al., 2014). Apart from Q80K, the only other NS3 mutation with a significant difference in prevalence between the two subtypes was R117H. This was seen in more Gt1b patients than Gt1a (5.5% vs. 1.1%, respectively). This is a minor RAM, seen at long-term follow-up in a very limited number of patients (n=2) treated with telaprevir (Susser et al., 2012).

In comparison to the NS3 protease, the prevalence of patients with a high frequency RAM in NS5A was significantly higher in Gt1b patients than in Gt1a patients. This is in concordance with previous studies which observed the same association in smaller cohorts using population sequencing (Plaza et al., 2012, Paolucci et al., 2013). One of these studies observed a prevalence of 12.5% in Gt1a patients (n=32) compared to 53.3% of Gt1b patients (n=30) harbouring a RAM (Paolucci et al., 2013). In this study, only one NS5A resistance mutation had a significantly different prevalence between the two subtypes. Y93H was detected in a significantly higher

proportion of Gt1b samples compared to Gt1a samples (15.4% vs. 0.7%), an association which has previously been observed (Plaza et al., 2012, Paolucci et al., 2013).

Despite this apparent increase in the prevalence of NS5A RAMs, clinical trials have observed a higher SVR rate in Gt1b patients over Gt1a patients, when treated with NS5A inhibitor daclatasvir and P/R (Hezode et al., 2015). One possible explanation is that the mutations only confer a low-level resistance to inhibitors whilst having a reduced replicative capacity and as such can be overcome by the dose of DAA administered. Plaza et al., (2012) previously described no mutations being found in Gt1a samples from both their cohort and the Los Alamos database (n=189). This is a stark contrast to our study which found 11.3% of Gt1a patients harboured a RAM at levels ≥10%. This difference between subtypes was lost when observing the presence of low-frequency RAMs, with 16.4% and 12.1% of Gt1a and Gt1b patients, respectively, harbouring a low-frequency mutation(s). The differences between subtypes are likely due to a combination of factors, one such being the genetic barrier to resistance, which is used to describe the challenge of a virus acquiring specific mutations (Gotte, 2012). The lower the genetic barrier, the easier it is for a mutation to arise and different subtypes can display different genetic barriers at the same position. This is displayed at position 155 of the NS3 gene. In Gt1a, a single transition event (AGG to AAG) is required to substitute arginine for lysine. In comparison, a higher genetic barrier is present in Gt1b at the same position, where both a transition and a transversion (CGG to AAG) are required for the amino acid substitution. In this study, the V36M mutation in NS3 was observed at a higher prevalence in Gt1a than Gt1b (2.2% vs. 0%), although not significant. The genetic

barrier is lower in Gt1a requiring one transition compared to one transition and one transversion for Gt1b strains (Bartels et al., 2013). The NS5A mutation Q30H was also observed at varying proportions in each subtype (Gt1a, 2.2% vs. Gt1b, 0%). This pattern also follows the genetic barrier with one transversion required in Gt1a but one transition and one transversion necessary for the same mutation in Gt1b (Bartels et al., 2013). Such genetic barriers also play an important role in combination therapy. Through increasing the potency and number of DAAs used in treatment, the number of mutation events to confer resistance to the regimen is also raised (Gotte, 2012).

Treating co-infected individuals is more problematic due to possible drug-drug interactions between DAAs and ART. Therefore, the fewer resistance mutations are present, the wider the choice of possible drugs to use. There have been surprisingly few studies using NGS to examine the effect HIV co-infection has upon the presence of RAMs compared to mono-infected patients (Bartolini et al., 2013, Jabara et al., 2014), given the high rate of co-infection in some cohorts (Garten et al., 2004). Both of the aforementioned studies found no difference in the presence of NS3 RAMs between mono-infected and co-infected patients, although both studied only a limited number of patients (28 and 40, respectively). The co-infected patients were on ART in both studies with similar median CD4 counts of approximately 500cells/mm^3, a value similar to our study. The cut-off values used were lower than that used in this study, at 0.35% and 0.08%.

When analysed by HCV Gt1 subtype, mono- and co-infected subjects showed a similar prevalence of high-frequency RAMs in the NS3 gene. Upon analysing the

presence of low-frequency NS3 RAMs in Gt1a patients, mono-infected patients displayed a significantly higher prevalence compared to co-infected subjects. The only instance of co-infected subjects displaying a higher prevalence of RAMs than mono-infected patients was the determination of low frequency NS3 RAMs in Gt1b infected patients; where the prevalence was approximately double that of mono-infected patients although this did not reach statistical significance. The latter two observations are in contrast to the findings of the two previous studies, which found no difference in NS3 RAM frequency between mono- and co-infected subjects using ultra deep pyrosequencing (Bartolini et al., 2013, Jabara et al., 2014). Regardless of the subtype, there was either no difference or mono-infected patients displayed a higher prevalence of RAMs in NS5A than co-infected patients, although in these cases, the difference did not reach statistical significance.

In the subset of patients matched by HCV RNA load, the differences between mono-infected and co-infected patients reflected those already seen in the whole cohort. The only difference was in low frequency NS3 RAMs in Gt1b infected subjects. Whereas co-infected patients displayed double the prevalence compared to mono-infected patients when studying the whole cohort, there was no difference between the two groups in the matched subset. These observations that HIV/HCV co-infected patients are generally not at an increased risk of harbouring a drug resistant variant at either high or low frequency suggests that drug resistance should not present a greater challenge to treat co-infected individuals.

In this chapter, multiple hypotheses were tested on the same set of data, with a p-value ≤ 0.05 considered as significant and the null hypothesis being rejected. However, as more hypotheses are tested on the same set of data, the chance of

observing a significant result, and therefore a Type I error, increases (Bland and Altman, 1995). The Bonferroni correction method can be employed to counteract this problem, which, using the number of hypotheses being tested, lowers the p-value at which a result would be considered significant [Bland & Altman, 1995].

In this study, we determined the Shannon entropy of patients; a measure of complexity indicating the number of variants and evenness of their distribution in a population. In the whole cohort, it was observed that co-infected patients had a higher complexity than mono-infected patients, although this difference rarely reached statistical significance. This may partly be due to the HCV RNA load of the samples. The viral load had a weak, positive correlation with the entropy values and HIV-positive individuals had slightly elevated viral loads over mono-infected patients. The combination of these two factors is likely to play a role in the difference between mono- and co-infected patients. The correlation between viral load and quasispecies complexity may, at least in part, be technical. As the viral load increases, so does the likelihood of sampling a variant, contributing to a higher complexity. We attempted to reduce the influence of the HCV RNA load on the technical aspects of testing by matching a subset of sample by the HCV RNA load. In this group, the opposite was found, with co-infected patients generally displaying lower complexity than mono-infected patients. This is in concordance with a previous study employing deep sequencing to examine quasispecies complexity in the NS3 gene (Jabara et al., 2014).

Other studies examining different regions of the genome have also found a decreased complexity among co-infected patients, although most of these studies have used

small cohorts (Shuhart et al., 2006, Lopez-Labrador et al., 2007). Shuhart et al., (2006) studied the E2-HVR region of the HCV genome, finding that HIV-positive, ART-naïve subjects displayed a significantly lower quasispecies complexity than both HIV-positive patients receiving ART and HIV-negative individuals. The CD4 count also impacted upon the quasispecies, with an increase in the complexity upon the doubling of the CD4 count (Shuhart et al., 2006), suggesting that the immune system does play a role in driving evolution and diversity in the quasispecies. Upon comparison of HIV-negative and HIV-positive subjects receiving ART, no difference in the complexity of quasispecies was observed (Shuhart et al., 2006). Another study of the HVR-1 region also observed a non-significant decrease in quasispecies complexity in co-infected patients compared to mono-infections (Lopez-Labrador et al., 2007). In contrast, another study found an increased complexity in the core gene in HIV/HCV co-infected patients with low (<350 cells/µl) CD4 count compared to HCV mono-infected patients and co-infected patients with normal (>400 cells/µl) CD4 count (Xu et al., 2012).

The conflicting results between studies may be a consequence of the different genes examined and the pressure that each is subjected to from the immune system. The HVR region is subject to strong selection pressures and thus has a high mutation rate which aids its escape from the immune system (Kato et al., 1994, van Doorn et al., 1995). Therefore, in an environment with a diminished pressure such as an immunocompromised patient, it may be expected that co-infected individuals exhibit decreased diversity and complexity in this region.

A decrease in RAM prevalence and quasispecies complexity in co-infections may be because the patient's immune system is a selective force behind the emergence of mutations. Hence, during HIV infection, a compromised immune system would not present as much of a challenge to the virus, decreasing the number of different circulating variants. On the other hand, it is known that co-infection with HIV increases HCV replication (and HCV RNA load) compared to mono-infections (Neukam et al., 2012). This increase in viral load may increase the likelihood of a drug resistant variant or other mutation arising and existing as a minority variant or, if replicative fitness is high enough, the dominant variant. This can be seen in the difference in the prevalence of NS3 low frequency RAMs between the whole cohort and matched subset of Gt1b patients. In the matched subset, the elimination of the elevated viral load in the HIV-positive cohort lead to a reduction in RAMs to a prevalence equal to that of mono-infected patients. The diminished capability of the immune system in HIV infection may also contribute to an increase in RAMs and complexity through its reduced capacity to clear minority variants.

In both NS3 and NS5A, although ART-experienced patients tended to display higher RAM prevalences than ART-naïve patients, the differences never reached significance, although this is probably due to the small number (n=24) of ART-naïve patients tested. Further studies are required to confirm this association. If the difference between the two groups is true, then it may be a consequence of the impact of ART on the function of the immune system through restoration of CD4 cells, which may produce an evolutionary pressure on HCV. Some sites associated with resistance such as Q80 and R155 in NS3 are known to exist in epitopes exposed to the immune system and restricted by certain HLA types (Ward et al., 2002, Lauer

et al., 2004). This may provide sufficient pressure for resistant variants to dominate in the quasispecies, as the wild-type virus diminishes. One piece of contrasting evidence to this hypothesis is that we did not observe any difference between the CD4 cell counts of patients with and without RAMs at high or low frequency in either gene. In addition, there was only a very weak association between the CD4 count and Shannon entropy value. If CD4 cells were involved in selective pressure at these sites of resistance or in the overall complexity, then one would expect a higher CD4 count in the patients harbouring a RAM and a positive correlation between the CD4 count and the complexity. A previous case report of a co-infected patient found a dominant R155K in the NS3 gene of a co-infected patient (Kim et al., 2009). Following the re-initiation of ART the virus eventually reverted to the wild-type variant. This does not support the hypothesis that the immune system drives selection for drug resistance, as the reconstitution of the immune system eventually lead to the wild-type virus becoming dominant. This may be because an improved immune function actually aids in the clearance of resistant variants with an impaired replicative capacity. Of course, this reversion may also be a natural fluctuation in the levels of variants.

The impact of low frequency HCV variants on the outcome of treatment is largely unknown and has only been studied in small numbers of patients with pre-existing RAMs at baseline (Fevery et al., 2015). While it has been observed that the presence of PI-resistant variants at baseline may have negatively affected the response to therapy (Lenz et al., 2012), there is also evidence to suggest that low level pre-existing drug resistant variants are not selected for upon the initiation of treatment (Fevery et al., 2015). In one study, all four patients identified with minority (<20%)

RAMs at baseline achieved SVR when treated with simeprevir plus sofosbuvir (Fevery et al., 2015). Another study identified minority variants present at baseline which became dominant variants at the time of treatment failure in two patients who failed PI triple therapy (Larrat et al., 2015). In a trial of ledipasvir and sofosbuvir therapy, only one of the eight patients who did not achieve SVR harboured only one NS5A minority variant at baseline (Sarrazin et al., 2015a). Minority NS5A variants present at baseline during treatment with grazoprevir and elbasvir were consistent with the variants detected at the time of treatment failure in patients who did not achieve SVR (Black et al., 2015).

It is important to note that there were only a small number of patients with pre-existing RAMs at baseline in these studies, making it difficult to draw firm conclusions without the addition of more data.

NGS may still be useful in clinic. The use of NGS at early stages of treatment may aid clinicians in the detection of resistant variants starting to outgrow the wild-type and help to dictate the treatment options before the establishment of such variants. NGS also enables the quantification of variants which may aid in the determination of the impact of variants, guiding therapeutic decisions in the future. The threshold of variants which have an influence on therapy needs to be determined. Although the cost of NGS has reduced dramatically in recent years, the use of deep sequencing for the detection of low frequency variants in clinic remains limited. The initial outlay for a sequencing platform alongside the large amount of generated data which needs to be analysed present challenges which need to be overcome. If pre-existing mutations do not affect the outcome of treatment, as suggested above, then the

introduction of NGS into the clinical setting to screen baseline samples would be costly in return for very few benefits.

Regimens of multiple DAAs, each targeting a different region of the HCV genome have been explored to treat chronic HCV infections in both treatment-naïve patients and null responders to previous therapy and are now in clinical use (Afdhal et al., 2014a, Afdhal et al., 2014b, Feld et al., 2014). As more DAAs are licensed for clinical use, such combination therapies are likely to become more commonplace. Consequently, variants conferring resistance to multiple inhibitors may be of clinical significance. Although no currently licensed combination therapies comprise only a PI and NS5A inhibitor, one does combine a PI (paritaprevir), NS5A inhibitor (ombitasvir) and polymerase inhibitor (dasabuvir). In this study, we identified 6.6% of patients harbouring a high-frequency RAM in both the NS3 and NS5A gene. In 29.2% (7/24) of these patients, the combination of mutations detected conferred resistance to both the PI (paritaprevir) and NS5A inhibitor (ombitasvir). Variants conferring resistance in both genes at low frequencies or a mixture of high and low frequencies occurred in 11.2% of patients. However, due to the sequencing protocol, it is not possible to determine whether the RAMs are circulating in the same or different variants. If multiple variants are responsible for the RAMs, then a combination of inhibitors may still be effective as a variant carrying a resistance mutation in NS3 will be susceptible to an NS5A inhibitor and vice versa. Resistance has been observed in clinical trials of combination therapies utilising DAAs targeting multiple HCV regions, leading to virological failure in some patients (Krishnan et al., 2015c, Lenz et al., 2015). However, using chimeric mice, another study observed no resistance or viral breakthrough when higher concentrations of PI and NS5A

inhibitor were combined (Shi et al., 2013). The use of optimum concentrations of DAAs may aid in the prevention of viral breakthrough, even when resistance mutations are present in more than one gene.

In conclusion, this study provides a large amount of data on the prevalence of high- and low-frequency resistant variants in HCV infected patients naïve to all anti-HCV therapy. Our results, from the largest comparison of HCV mono-infected and HIV/HCV co-infected patients using NGS to date, suggest that the prevalence of both low- and high-frequency RAMs is similar in mono- and co-infected patients before the initiation of therapy. Consequently, the presence of an HIV co-infection should not affect treatment using DAAs. We have reduced the number of variables as much as possible by eliminating confounding factors such as co-infection with HBV, restricting the genotypes tested and, for a subset of samples, matching samples by viral load. Through doing so, we are able to draw a firmer conclusion of the impact of HIV co-infection on HCV variants.

Chapter Seven

General Discussion

7. General Discussion

The overall aim of this thesis was to investigate HCV genetic variation, both in terms of molecular epidemiology at the population level and the occurrence of resistance mutations that may impact of the effectiveness of direct-acting antiviral agents (DAAs) in individuals, and the influence of HIV co-infection on the findings. The study populations included patients from Ghana and the UK.

There is a scarcity of data concerning the epidemiology of HCV infection in sub-Saharan Africa and available studies have often reported unconfirmed data. The investigation therefore aimed first of all to determine the prevalence of HCV infection in patients receiving routine HIV care in Ghana, and through this assess the appropriate modalities for HCV screening in such settings, which could contain cost whilst remaining sensitive and specific.

The results are noteworthy on several accounts. Previous work from our research group demonstrated that gold-standard HCV antibody screening tests overestimate the true prevalence of HCV infection in this population likely due to false positivity, and that a number of strategies could be employed to improve specificity (King et al., 2015). Despite screening a large number of subjects for HCV RNA, the prevalence of HCV infection in the Ghana cohort was lower than 1% overall, and well below previous estimates based on standard HCV antibody screening assays. Indeed, HCV prevalence among the HIV co-infected patients was no higher than that previously reported for blood donors in the same region (Candotti et al., 2003). The prevention of transmission should complement the effort to diagnose and treat active HCV infections. In order to do this, and also in order to optimise the design of

targeted screening protocols, large surveys are required to identify the major risk factors of acquiring HCV in resource-limited settings. Blood transfusion and surgical procedures were identified as the risk factors from the limited number of HCV-positive patients determined in our study. Other studies suggest that risk factors for HCV infection in the general population of Ghana include a history of medical interventions (e.g., transfusions) and traditional practices (e.g., scarification), particularly in the northern regions of Ghana (Layden et al., 2015). Measures such as the proper sterilisation of surgical instruments and the appropriate screening of blood donations are increasingly adopted in Ghana and will greatly reduce the spread of HCV and prevent new infections. Screening of blood donations for infectious diseases has previously been multiplexed, allowing the simultaneous detection of HIV, HBV and HCV (Ohnuma et al., 2001). This process would also be advantageous in regions such as Ghana where HIV and HBV are relatively common.

Larger studies should seek to investigate risk factors for HCV infection in the HIV/HCV co-infected population, and to confirm whether co-infection with HBV is indeed associated with higher prevalence of HCV, as our prevalence data seemed to suggest. This is important, as co-existence of HCV and HBV infection would put patients with HIV at a very high risk of liver disease in a setting where both ascertainment and management options for viral hepatitis are very limited at present (Lemoine et al., 2015).

There were other important observations made in the Ghana cohort. In order to gain an accurate representation of the burden of HCV infection, there is a necessity for the utilisation of techniques that allow detection of HCV RNA. Unfortunately, the

restricted access to the necessary infrastructure and high cost limit availability in low income countries. Studies are required to investigate the applicability of immune assays for HCV antigen detection. Alternatively, the use of centralised testing facilities along with specimen pooling techniques demonstrated in this study could be used to address these problems. From a practical perspective, the study demonstrated the feasibility of performing large molecular epidemiology studies using dried plasma spots (DPS). This is helpful where molecular testing may only be available in centralised facilities: the use of DPS circumvents the requirement for stringent storage and transport conditions, without affecting the ability to detect HCV RNA in untreated patients, and the ability to recover virus suitable for sequencing.

Among the HCV positive subjects from the Ghana cohort, viral genetic diversity was assessed by sequencing two genetic regions. This enabled the identification of a possible new HCV strain that clustered within the HCV genotype (Gt) 1 group but appeared to be distinct from known Gt1 subtypes, thus furthering knowledge of the molecular epidemiology of HCV infection in West Africa. Full genome sequencing is required to confirm the structure of the unusual HCV Gt1 strain identified in this study, and in another study previously performed among blood donors in the same region of Ghana (Candotti et al., 2003). These data are important because they provide an insight into the patterns of HCV spread at the population level. They also provide a basis for raising the question of whether treatment strategies optimised for HCV strains circulating in Europe or North America will be equally effective against such poorly studied ‘African strains’.

The efficacy of treatment on the HCV strains found in Africa is currently unknown. Observations from our study and previous studies (Ruggieri et al., 1996, Candotti et al., 2003, Ndjomou et al., 2003) have shown a high diversity in the circulating strains, with a proportion being seemingly unique to those regions. This may impact upon treatment response rates. Several new DAAs are proposed to be pan-genotypic (Rodriguez-Torres, 2013, Sulkowski et al., 2014, Vince et al., 2014), whilst several retain at least partially genotype-specific activity.

Access to DAAs and other forms of HCV treatment is very limited in Ghana, as is the rest of sub-Saharan Africa. In order for DAA treatment to become accessible in such regions, it is necessary for pharmaceutical companies to either; i) allow the production of generic versions of their therapies for use in developing countries or ii) reduce the cost of their antiviral drugs to an affordable level for low-middle income countries (LMICs). The cost of sofosbuvir is adjusted based on the GDP of the country meaning that a 12-week course of the drug would cost approximately US$1000 in a LMIC compared to US$84,000 in the USA (Hill and Cooke, 2014). Despite this reduction, the price point of sofosbuvir is still unattainable for much of the population in LMICs.

Whilst interferon and ribavirin offer a cheaper alternative to DAAs, this approach may not be more cost effective than the use of new treatment options. African-American populations have a higher proportion of the unfavourable TT allele at the IL28B site, which negatively affects the response to interferon treatment (Ge et al., 2009). While good responses may be anticipated for the prevalence HCV Gt2 strains, interferon and ribavirin treatment are less efficacious against Gt1 infections compared to other genotypes (McHutchison et al., 1998), which are found in many parts of Africa (Jeannel et al., 1998, Bracho et al., 2006, Rouabhia et al., 2013).

As the yield of HCV infections was low in Ghana, further studies were completed with the UK cohort alone. In the UK HCV-positive cohort, the investigation aimed to characterise the diagnostic yield of deep sequencing compared with Sanger sequencing for the identification of naturally occurring HCV drug resistance. This provided information immediately relevant for clinical care, while also giving insights into the relative fitness of circulating HCV strains.

We analysed the occurrence of a common polymorphism – Q80K – in the NS3 gene of HCV Gt1a strains circulating in two regions of England. Determining the presence of Q80K is recommended prior to the use of the NS3 protease inhibitor simeprevir and testing is widely available through the use of conventional (Sanger) sequencing. We compared results obtained with Sanger sequencing with those obtained by the use of deep sequencing, with the aim of determining whether the mutant would occur as a low frequency variant in subjects showing wild-type virus by Sanger sequencing. Deep sequencing can achieve a sensitivity of 1% or lower for low-frequency variants, which compares with a sensitivity of 10-20% for Sanger sequencing. Thus, the first use of next generation sequencing in this thesis was for the purpose of determining whether the use of this technique could benefit the clinic in the detection of one specific polymorphism, Q80K in NS3. Our findings indicated that deep sequencing offers no specific benefit over traditional Sanger sequencing for the detection of Q80K, as the polymorphism existed in all samples tested at frequencies ≥40%. Therefore, current estimates of prevalence of this polymorphism and routine diagnostic applications are accurate.

The Q80K polymorphism is thought to have first arisen in the USA in the 1940s (McCloskey et al., 2015) with subsequent migration events allowing it to establish in other regions of the world. The phylogenetic analysis performed in this thesis determined that the UK HCV Gt1a sequences containing the polymorphism were interspersed with sequences from both USA and Europe. There appeared to have been multiple introductions of the polymorphism in this country, but the spread of this polymorphism was also due to transmission events within this country. Unfortunately, the lack of publically available UK sequences from other published studies (Leggewie et al., 2013, Sarrazin et al., 2015b, Shepherd et al., 2015) does not allow any comparison between our cohort and others. Larger phylogenetic analyses of UK sequences would enable transmission networks to be identified and provide further insight into the patterns of HCV spread. Meantime, the results were interesting for several reasons. We were able to demonstrate that a 1% cut-off should be applied to the interpretation of HCV deep sequencing data produced with the Illumina platform. The assay error rate as determined with a molecular clone was below this cut-off and any detection of Q80K variants below 1% yielded sequences that did not cluster within the Q80K lineage, further indicating that detection was most likely spurious. Given that one previous UK-based study has reported on Q80K detection below the 1%, our proposed cut-off should be used to re-analyse these previously published data (Leggewie et al., 2013). Thus, the limited data on this polymorphism in the UK allowed this study to greatly expand on the knowledge, whilst also adding the impact of utilising deep sequencing for detection of this substitution. From a more general perspective, the observation that Q80K variants occurred always as dominant strains and did not influence the HCV RNA load

measured in the sample provides support to the notion that these strains are highly fit and transmissible.

The third part of the investigation employed a deep sequencing approach to resolve the impact of HIV co-infection on the spontaneous occurrence of HCV drug resistance mutations, providing an opportunity to obtain a more definitive conclusion than the ones garnered from the few previous small-scale studies. This was the largest cohort to date to explore this effect using deep sequencing technology (Bartolini et al., 2013, Jabara et al., 2014). Generally, there were no differences in the prevalence of recognised drug resistance-associated mutations (RAMs) in NS3 and NS5A when comparing HCV mono-infected and HIV/HCV co-infected patients. These data suggest that it is likely that the two antagonistic mechanisms – an increase in HCV replication that may favour virus genetic evolution and escape, and the impaired selective pressure from the immune system – are in equilibrium. From a clinical perspective, this apparent lack of difference in RAM prevalence should mean co-infected individuals are not at risk of further complications for the treatment of HCV, and is in line with the excellent responses to DAA-based therapy observed in HIV/HCV co-infected patients (Antonini et al., 2015, Wyles et al., 2015).

Upon comparing the quasispecies complexity, co-infected patients displayed a higher complexity than mono-infected patients, although this rarely reached significance. This trend was reversed when the subset of samples matched by viral load were analysed, indicating the role that the viral load played in quasispecies complexity. It is difficult to ascertain whether the observed association between viral load and complexity is genuine or technical.

Although selective pressure from the immune system has been hypothesised to impact upon the evolution of HCV, we did not observe any association between the CD4 count and quasispecies complexity. There was also no difference in CD4 count between those with vs. those without a RAM. These observations cast doubt over the importance of the role that CD4 cells play in providing a selective pressure. However, other components of the immune system may still play a role in this mechanism, providing sufficient pressure to elicit a response.

Unlike Q80K, both NS3 and NS5A RAMs were relatively commonly detected at low frequencies (≥1% *x* <10%) in both HCV mono-infected and HIV/HCV co-infected patients naïve to all forms of anti-HCV therapy, and were detected at overall prevalences of 12.1% and 15.3%, respectively. In NS3 and NS5A, 7.1% and 12.9% of patients, respectively, harboured mutation(s) only at low frequency, providing an insight into the extent to which deep sequencing can increase prevalence rates.

Whether minority NS3 and NS5A RAMs affect responses to combination DAA therapy remains controversial as an effect has not been easily observed in clinical studies to date (Black et al., 2015, Fevery et al., 2015, Sarrazin et al., 2015a). The impact of low-frequency mutations present at baseline on the outcome of therapy needs to be further studied in larger cohorts, including patients with various clinical and virological characteristics i.e. previous non-responders, HIV/HCV co-infected patients, a range of genotypes. Meanwhile, testing of baseline samples may be indicated in patients who fail to respond to DAA therapy, in order to determine the effect of drug selective pressure in relation to pre-existing RAMs. This will also help the interpretation of the persistence of resistant variants after the cessation of

therapy. Recent studies have observed the persistence of resistance in NS3, NS5A and NS5B to varying degrees. One study found that 94.4% of patients still harboured a RAM in NS5A, detectable by deep sequencing, 96 weeks post-treatment, although the frequencies had declined over this period (dvory-Sobol et al., 2015). Persistence to this degree may restrict subsequent treatment options, especially if resistance is present in more than one of the genes targeted by the drugs. The codon position of the RAM was also found to impact upon its persistence, most likely due to the replicative fitness of each variant. In patients harbouring RAMs at the end of treatment, the R155K variant was found in 25% of patients at week 48 post-treatment in contrast to only 2% of patients harbouring D168V at the same time point (Krishnan et al., 2015b).

The data clearly indicate that deep sequencing can increase the detection of HCV RAMs in a sample. The technique is increasingly available, but not as yet part of routine diagnostics in most settings. There was a modest increase in the workload associated with the next generation sequencing protocol used in this thesis, compared to Sanger sequencing. These were mostly associated with quality control measures to ensure the input for the sequencing platform was of a high enough standard alongside the pooling of amplicons. The preliminary processing of reads, including the trimming of adapter sequences and quality filtering is an addition not necessary for Sanger sequencing. Although these additional procedures increase time and labour costs, the integration of deep sequencing into clinic should not present any major obstacles to overcome. As the technology is updated, it is likely that costs will be reduced further adding more incentive for implementation in clinics. The amplification of whole genome or a large fragment comprising the regions NS3

through NS5B would reduce the overall number of PCRs as well as the subsequent quality control measures, thereby reducing not only the workload but also the cost.

Next generation sequencing technologies can offer the clinic an enormous amount of data, although this data presents a need for an easily implemented, straightforward bioinformatics pipeline to ensure accuracy and quality control. VirVarSeq, the pipeline used in this study, presents a viable option. For routine clinical use, a consensus approach between national centres for both laboratory and analytical procedures would provide easily comparable results for patients.

The use of deep sequencing in clinic extends beyond the detection of minority variants. The ability to quantify the variants allows clinicians to assess changes in the dynamics of the quasispecies and identify the emergence of drug resistant variants as they occur during therapy. This may provide a capacity for the early cessation of treatment, in cases where the emergence of drug resistant variants alongside the viral load kinetics indicates that a sustained virological response may not be achieved. In doing so, the financial costs of the antiviral drugs alongside the associated laboratory tests necessary during treatment would be eliminated. Taking the aforementioned points into consideration, it would be appropriate to evaluate the cost-effectiveness of deep sequencing before implementation in routine clinical use.

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Appendix

9. **Appendix**

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
[illegible]DICAL SCIENCES
[illegible] RESEARCH PUBLICATION AND ETHICS

Our Ref: CHRPE/143/10 — September 20, 2010

Dr. Richard Odame Phillips
Department of Medicine
KATH, Kumasi

Dear Sir,

LETTER OF APPROVAL
Protocol Title: ***"A Study of Hepatitis B Co-Infection Among HIV-Positive Patients in KATH"***

Proposed Site: Department of Medicine, Komfo Anokye Teaching Hospital
Sponsor: The Leverhulme-Royal Society Africa Award

Your submission to the Committee on Human Research Publication and Ethics on the above named protocol refers.

The Committee has considered the ethical merit of your submission and approved the protocol. The approval is for a fixed period of one year, renewable annually thereafter. The committee may however, suspend or withdraw ethical approval at anytime if your study is found to contravene the approved protocol.

Data gathered for the study should be used for the approved purposes only. Permission should be sought from the committee if any amendment to the protocol or use, other than submitted, is made of your research data.

The Committee should be notified of the actual start date of the project and would expect a report on your study, annually or at close of the project, whichever one comes first. It should also be informed of any publication arising from the study.

Thank you Sir, for your application.

Yours faithfully,

Osonfuor Prof. Sir. J. W. Acheampong MD FWACP
CHAIRMAN

Room 8 Block J, School of Medical Sciences, KNUST, University Post Office, Kumasi, Ghana
Phone [illegible] Email: [illegible]

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08 June 2012

Professor Anna-Maria Geretti
Professor of Virology & Infectious Diseases
University of Liverpool & Royal Liverpool Hospital
Department of Clinical Infection, Microbiology & Immunology, Institute of Infection & Global Health
The University of Liverpool, The Apex Building
8 West Derby Street, Liverpool
L69 7BE

Dear Professor Geretti,

Study title:	**STUDY OF THE EVOLUTION OF HEPATITIS C VIRUS (HCV) QUASISPECIES UNDER DRUG PRESSURE (EVOHC) AND THE MECHANISMS OF INTERFERON ALPHA-INDUCED BLOCK TO HIV REPLICATION (INTAPHIR).**
REC reference:	**12/SC/0346**
Protocol number:	**UoL000864**

The Proportionate Review Sub-committee of the NRES Committee South Central - Berkshire reviewed the above application on 07 June 2012.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study:

1. Please amend the REC name to NRES Committee, South Central - Berkshire Research Ethics Committee.
2. Please include a statement in each PIS informing participants that 'this study is

partly doctoral research'.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Confirmation should also be provided to host organisations together with relevant documentation.

Approved documents

The documents reviewed and approved were:

Document	Version	Date
Evidence of insurance or indemnity	Novae: Period 01/08/2011 to 31/07/2012	25 August 2011
Investigator CV	Tomas Doyle	
Investigator CV	Simon King	
Investigator CV	Anna Maria	
Letter from Sponsor		16 April 2012
Other: Intention to sponsor NHS		19 March 2012
Other: Comments by Independent Referees		
Other: Response to review		
Participant Consent Form: HCV INTAPHIR		18 January 2012
Participant Consent Form: HCV		18 January 2012
Participant Information Sheet: HCV	1	18 January 2012
Participant Information Sheet: HCV INTAPHIR	1	18 January 2012
Protocol	1	24 January 2012
REC application		25 May 2012

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached

sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/SC/0346	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely,

Mr David Carpenter
Chair

Email: scsha.berksrec@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

After ethical review – guidance for researchers

Copy to: *Mrs Lindsay Carter*
Heather Rogers, Research Development & Innovation Department

NRES Committee South Central - Berkshire

Attendance at PRS Sub-Committee of the REC meeting on 07 June 2012

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mr David Carpenter	Social Scientist	Yes	
Mrs Liz Hunter	Midwifery Guidelines Co-ordinator	Yes	
Dr M.J. Proven	Coordinator for Quality Assurance in Research	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Ms Rae Granville	Committee Co-ordinator

NHS
Health Research Authority

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05 July 2012

Professor Anna-Maria Geretti
Professor of Virology & Infectious Diseases
University of Liverpool & Royal Liverpool Hospital
Department of Clinical Infection, Microbiology & Immunology, Institute of Infection & Global Health
The University of Liverpool, The Apex Building
8 West Derby Street, Liverpool
L69 7BE

Dear Professor Geretti,

Full title of study:	**STUDY OF THE EVOLUTION OF HEPATITIS C VIRUS (HCV) QUASISPECIES UNDER DRUG PRESSURE (EVOHC) AND THE MECHANISMS OF INTERFERON ALPHA-INDUCED BLOCK TO HIV REPLICATION (INTAPHIR).**
REC reference number:	**12/SC/0346**
Protocol number:	**UoL000864**
EudraCT number:	

Thank you for your letter of 05 July 2012. I can confirm the REC has received the documents listed below as evidence of compliance with the approval conditions detailed in our letter dated 07 June 2012. Please note these documents are for information only and have not been reviewed by the committee.

Documents received

The documents received were as follows:

Document	Version	Date
Participant Consent Form: Mechanisms of interferon alpha-induced block to HIV replication	2	11 June 2012
Participant Consent Form: Study Of Hepatitis C Virus (HCV) Variants Under Drug Pressure	2	11 June 2012
Participant Information Sheet: Study Of Hepatitis C Virus (HCV) Variants Under Drug Pressure	2	11 June 2012
Participant Information Sheet: Mechanisms of interferon alpha-induced block to HIV replication	2	11 June 2012

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

12/SC/0346	Please quote this number on all correspondence

Yours sincerely,

Ms Rae Granville
Committee Co-ordinator

E-mail: scsha.berksrec@nhs.net

Copy to: *Mrs Lindsay Carter*
Heather Rogers, Research Development & Innovation Department

Table 9.1. Presence of high frequency (≥10%) drug resistance NS3 mutations identified using next generation sequencing in genotype 1a samples

NS3 amino acid position	HCV mono-infected		HIV/HCV co-infected	
	Resistance associated mutation	% Frequencies (n)	Resistance associated mutation	% Frequencies (n)
36	L	99.9 (1), 100 (1)	L	99.9 (1), 100 (1)
	M	100 (1)	M	99.3 (1), 99.8 (1)
54	S	99.8 (1), 99.9 (2)	S	99.8 (1), 99.9 (2)
55	A	96.7 (1), 99.8 (2), 99.9	A	51.5 (1), 99.9 (1), 100 (1)
	I	(2), 100 (1)	I	99.7 (1), 99.9 (1)
		91.7 (1), 99.6 (1), 99.8		
		(1), 100 (1)		
80	K	41.1 (1), 45.8 (1), 99 (1),	K	40.2 (1), 72.3 (1), 98.3 (1), 99.6
		99.7 (1), 99.8 (2), 99.9		(1), 99.7 (2), 99.8 (6), 99.9
	L	(7), 100 (8)	L	(15), 100 (6)
		88.2 (1)		99.6 (1), 100 (1)
117			H	99.6 (1), 99.9 (1)
132	V	11.7 (1)		
155			K	97.5 (1)
168	E	99.9 (1)	E	99.8 (1), 99.9 (1)
170	A	36.1 (1)		
174	S	12.0 (1), 21.4 (1), 27.8	S	10.5 (1), 12.0 (1), 20 (1), 22.8
		(1), 28.1 (1), 40.9 (1),		(1), 25.9 (1), 27.2 (1), 56 (1),
		50.3 (1), 73.1 (1), 88 (1),		74.6 (1), 98.3 (1), 98.9 (1), 99
		90.4 (1), 93.4 (1), 94.6		(1), 99.2 (3), 99.4 (4), 99.5 (6),
		(1), 97.1 (1), 97.6 (1),		99.6 (4), 99.7 (9), 99.8 (17),
		98.5 (1), 98.9 (1), 99 (2),		99.9 (10), 100 (7)
		99.3 (2), 99.4 (1), 99.5		
		(5), 99.7 (2), 99.8 (3),		
		99.9 (14), 100 (10)		

Table 9.2. Presence of high frequency (≥10%) drug resistance NS3 mutations identified using next generation sequencing in genotype 1b samples

NS3 amino acid position	HCV mono-infected		HIV/HCV co-infected	
	Resistance associated mutation	% Frequencies (n)	Resistance associated mutation	% Frequencies (n)
54			S	13.7 (1)
55			A	99.9 (1)
80			L	100 (1)
			R	99.9 (1)
107	I	100 (1)	I	14.2 (1)
117	H	98.8 (1), 99.7 (1), 99.8 (1), 99.9 (1)		
168	E	76.1 (1)		
170	A	36.1 (1)		
174	F	99.9 (1)		

Table 9.3. Presence of low frequency (>1%) drug resistance NS3 mutations in genotype 1a samples determined by next generation sequencing

NS3 amino acid position	HCV mono-infected		HIV/HCV co-infected	
	Resistance associated mutation	% Frequencies (n)	Resistance associated mutation	% Frequencies (n)
36	M	1.1 (1), 2.8(1)	M	1 (1)
54			S	1.4 (1)
80	L	1.4 (1), 2.1 (1),	L	1 (1), 3.1 (1), 5.6(1)
	R	5.1 (1)		
		1.1 (1), 1.7 (2)		
107	I	1.7 (1), 2 (1)		
109	K	1.2 (1)		
117	H	3.4 (1)		
132	V	1.6 (1)	V	2.2 (1), 2.8 (1)
170	T	1.1 (1), 2.1 (1)	T	6.5(1)
174	S	1 (1), 1.3 (1), 1.6 (2), 1.8 (1), 2.1 (1), 5 (1)	S	1.1 (1), 1.5 (1), 2.2 (1), 2.7 (1), 2.9 (1), 3.5 (1), 5.6 (1), 8.7 (1)

Table 9.4. Presence of low frequency (>1%) drug resistance NS3 mutations in genotype 1b samples determined by next generation sequencing

NS3 amino acid position	HCV mono-infected		HIV/HCV co-infected	
	Resistance associated mutation	% Frequencies (n)	Resistance associated mutation	% Frequencies (n)
36	A	8.5 (1)		
54	S	7.6 (1)		
80			H	1.2 (1)
107			I	1.9 (1)
117			H	1.6 (1)
158	I	9 (1)		
168	E	1.1 (1)		
174			F	5.3 (1)
175	L	2.1 (1), 4.1 (1)	L	1.2 (1)

Table 9.5. High frequency (≥10%) drug resistance mutations present in the NS5A gene in genotype 1a samples, determined by deep sequencing

NS5A amino acid position	HCV mono-infected		HIV/HCV co-infected	
	Resistance associated mutation	% Frequencies (n)	Resistance associated mutation	% Frequencies (n)
24	R	47.5 (1), 98.9 (1)	R	16.3 (1)
28	T	70.8 (1)		
	V	14.1 (1), 20.8 (1), 62.5 (1), 71.7 (1), 93.3 (1), 97.6 (1), 98.6 (1), 99.9 (1), 100 (2)	V	26.1 (1), 34.7 (1), 39.9 (1), 99.9 (1)
30			H	99.8 (1)
	L	99.7 (1)		
	R	14.4 (1), 47.0 (1), 52.0 (1), 99.4 (1)		
31			M	99.3 (1), 99.6 (1), 99.8 (1)
	V	58.8 (1)		
58	P	10.0 (1), 75.2 (1), 99.7 (1), 99.8 (1)	P	30.4 (1), 36.7 (1), 54.6 (1), 99.8 (1), 99.9 (1)
93	C	94.9 (1)		

Table 9.6. High frequency (≥10%) drug resistance mutations present in the NS5A gene in genotype 1b samples, determined by deep sequencing

NS5A amino acid position	HCV mono-infected		HIV/HCV co-infected	
	Resistance associated mutation	% Frequencies (n)	Resistance associated mutation	% Frequencies (n)
28	M	15.2 (1), 99.4 (1)	M	99.8 (1)
31	I	100 (1)		
			M	70.0 (1), 99.7 (1), 99.9 (1)
58	S	96.6 (1), 96.8 (1), 99.6 (1), 99.8 (1), 99.9 (1)	S	10.6 (1), 24.4 (1)
93	H	10.5 (1), 12.3 (1), 27.3 (1), 27.6 (1), 73.9 (1), 88.0 (1)	H	17.9 (1)

Table 9.7. Low frequency (>1%) drug resistance mutations present in the NS5A gene in genotype 1a samples, determined by deep sequencing

NS5A amino acid position	HCV mono-infected		HIV/HCV co-infected	
	Resistance associated mutation	% Frequencies (n)	Resistance associated mutation	% Frequencies (n)
24	R	1.0 (3), 1.1 (1), 1.7 (1), 4.0 (1), 8.1 (1)	R	1.0 (1), 1.8 (2), 3.1 (1), 4.7 (1), 4.9 (1)
28	T	1.0 (1), 1.5 (1)	T	1.1 (1)
	V	1.0 (1), 1.3 (1), 1.7 (1), 3.1 (1), 4.6 (1), 5.4 (1)	V	1.0 (1), 1.3 (1), 1.6 (1), 2.0 (1), 2.9 (1), 4.1 (1), 5.0 (1), 7.9 (1)
30	H	2.1 (1)	H	1.1 (1), 1.5 (1), 3.3 (1), 6.6 (1)
	R	1.0 (1), 2.5 (1), 3.1 (1)	R	1.2 (1), 1.7 (1)
31	M	1.5 (1), 9.2 (1)	M	9.3 (1)
58			P	1.1 (1), 1.2 (1), 3.3 (1), 7.8 (1), 8.2 (1)
93			C	1.2 (1)
			H	7.4 (1), 9.9 (1)

Table 9.8. Low frequency (>1%) drug resistance mutations present in the NS5A gene in genotype 1b samples, determined by deep sequencing

NS5A amino acid position	HCV mono-infected		HIV/HCV co-infected	
	Resistance associated mutation	% Frequencies (n)	Resistance associated mutation	% Frequencies (n)
28	M	1.2 (1)		
31	F	7.0 (1)	F	1.9 (1)
	M	1.9 (1)		
32	L	6.4 (1)		
93	H	1.3 (1), 1.5 (1), 3.4 (1), 5.4 (1), 6.0 (1), 7.1 (1)	H	1.7 (1)