MOLECULAR CHARACTERIZATION AND GENOTYPING OF HEPATITIS C VIRUS FROM SUDANESE END-STAGE RENAL DISEASE PATIENTS

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, 2018

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for Masters of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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(Signature of candidate)

____28th_____day of___August____2018_____in__University of the Witwatersrand Johannesburg

Presentations

1. Symposium: China-RSA International Symposium **(**Hepatitis in Africa: No room for complacency**).** Wits Club, University of Witwatersrand, South Africa. November 29th – 30th, 2016.

   Presentation: Trodia Zitha. Molecular characterization and genotyping of hepatitis C virus from Sudanese end-stage renal disease patients
2. Symposium: China-RSA International Symposium (HCC related HBV mutations in People Living with HIV/AIDS). Center for Disease Control and Prevention in Nanning, Guangxi Republic of China. May 8th -14th, 2017.

   Presentation: Trodia Zitha. Molecular characterization and genotyping of hepatitis C virus from Sudanese end-stage renal disease patients

Acknowledgements

Firstly, my appreciation goes to my family for their support. To my Mom thanks for the unconditional love, thank you for being the best mom. If it wasn't for you I wouldn't be writing this today, you have always made sacrifices for me to be able to get a better life and for that you will always be my superwoman. To Decious, I am really grateful for the support you have given to me. Thanks for teaching me what it means to care about someone. To my sister, Cornelia thank you for the endless support you gave to me.

To my supervisor Prof Kramvis, I don't know where to start but I have to start somewhere. Thank you for believing in me, for giving me this chance to be part of your HVDRU family, for making me believe in myself. Thank you for being more than just a supervisor to me, I will forever be grateful for the things you have done for me.

To Dr Chen, when I came to the HVDRU I was almost as good as clueless but you held my hand and walked me through all the necessary techniques I needed to and for that thank you. To the HVDRU family, thank you for all the knowledge you have imparted to me. I feel honoured to be part of this family. Dr Constance, thanks mommy dearest.

To the National Research Foundation, Poliomyelitis Research Foundation, Deutsche Forschungsgemeinschaft (DFG) grant and the University of the Witwatersrand thank you for the financial support to complete this study.

Above all, I would like to thank God, the Almighty for giving me the strength to carry on even though it was not easy. *Khanimamba.*

Abstract

Hepatitis C virus (HCV) is a global problem with approximately 11 million people in Africa infected (WHO, 2017). There is limited information on the prevalence of HCV in Sudan. In the general population, Sudan has been estimated to have a HCV prevalence ranging from 2 % to 4.8 % (Osman et al., 2013, Mudawi et al., 2007b). Fewer studies have been conducted in the Sudanese haemodialysis population. The haemodialysis population in Sudan have a higher prevalence as compared to the general population. HCV in the haemodialysis population showed the prevalence of 14.3% (El-Amin et al., 2007) and 23.7% (Alrahman and Gassoum, 2015). A recently published study demonstrated a decline of 6% in the prevalence of HCV as compared to the previously published results (Hammad et al., 2016b).

The high prevalence of HCV in the Sudanese haemodialysis population drew a need for further research to be conducted. The aim of the study was to genotype and molecularly characterize HCV isolated from end-stage renal disease (ESRD) patients from Sudan. The study was set out to determine the prevalence of HCV in Sudanese ESRD patients. We aimed at providing information on whether haemodialysis units are settings for HCV transmission and to determine HCV viral loads in haemodialysis patients. To achieve the aim set out in the study, 541 haemodialysis patients were recruited and tested for anti-HCV, of which 93 patients were found to be anti-HCV positive. The study participants were recruited from nine haemodialysis units at Khartoum, Sudan: Soba, Salma, Elakadeeemi, Elturki, Lbn sena, Tropical hospital, Alnaw, Police hospital and Elshurta.

HCV RNA was extracted from the serum samples received from Sudan followed by cDNA synthesis. After reverse transcription, the 5'UTR and NS5B region were amplified using nested polymerase chain reaction (PCR). Amplified PCR products were detected in 1% agarose gel

electrophoresis. PCR products which were found to be positive for PCR amplification were sequenced and bioinformatics analysis was performed. To determine the viral loads present in the study, quantification using real-time PCR was performed. Statistical analysis was performed using SPSS version 25.0 software. Fischer's exact and Chi-square tests were used to determine the significant difference between categorical variables. A p-value of <0.05 was considered as statistically significant. Out of the 93 HCV positive samples we managed to amplify 64 samples in the 5'UTR region and 44 in the NS5B region.

The high amplification in the 5'UTR as compared to NS5B region can be explained by the conserved nature of the 5'UTR region. Phylogenetic tree constructed using MEGA7 showed the presence of genotypes 1, 3, 4 and 5 with subtypes 1a, 1b, 1e, 3a, 4a, 4b, 4n, 4o and 4t among the Sudanese haemodialysis population. The genotypes and subtypes from the same haemodialysis units were seen clustering together in the phylogenetic tree displaying a possibility of a nosocomial infection. The presence of a group of sequences not clustering with any known genotypes were observed in the phylogenetic tree. At this stage we could not confirm if the group was representing a new genotype in the haemodialysis centers. Very low viral loads were detected in the study.

We recommend that stringent measures should be put in place to control the transmission of HCV in the haemodialysis population and also that constant testing of HCV RNA should be performed in patients who tested negative for antibody HCV at the outset. The HCV-positive patients should be isolated during their haemodialysis treatments to prevent patient to patient transmission. Genotyping of HCV is also recommended as this will be able to confirm transmissions and enable effective treatment using direct acting anti-viral (DAA).

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List of Abbreviations

aa	Amino acid
CDC	Centers for Disease Control
DAAs	Direct acting antivirals
dNTP	Deoxy-nucleotide-tri phosphate
E1	Envelop glycoprotein 1
ER	Endoplasmic reticulum
ESRD	End-stage renal disease
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
IDU	Intravenous drug user
IFN	Interferon
IRES	Internal ribosome entry site
IU	International unit
kb	kilo base
kDa	kilo Dalton
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
NANBH	Non-A, non-B hepatitis
NCR	Non-coding region
NS	Non structural
nt	Nucleotide
ORF	Open reading frame

PCR Polymerase chain reaction

RdRp RNA-dependent RNA polymerase

RNA Ribonucleic acid

µL micro litre

µM micro molar

UTR Untranslated region

Chapter 1 : Introduction

1.1 History of Hepatitis C virus

Hepatitis C, is an infectious blood borne disease caused by Hepatitis C virus (HCV) that infects the liver. HCV was discovered as the major cause of non-A, non-B hepatitis and isolated in 1989 (Choo et al., 1989). Infection with HCV may lead to chronic liver disease, cirrhosis or hepatocellular carcinoma (Chen and Morgan, 2006, Maasoumy and Wedemeyer, 2012). Researchers identified transfusion of blood products and intravenous drugs as the major transmission routes for HCV (Westbrook and Dusheiko, 2014). Although blood screening measures employed in developed countries has decreased the prevalence of hepatitis (Muktar et al., 2006), infection with HCV continues to be a global disease burden (WHO, 2017).

1.2 Epidemiology of HCV worldwide, in Africa and Sudan

In 2015, there were 1.75 million people newly infected with HCV, increasing the total number of people living with chronic Hepatitis C infection worldwide to 71 million (WHO, 2017). Between the years 1990-2005 there has been an increase in the HCV sero-prevalence from >122 million to >185 million cases (Mohd et al., 2013). Despite the high HCV sero-prevalence, which has been reported globally between the years 1990-2005 in 2016 (Petruzziello et al., 2016) has reported a decline in HCV sero-prevalence from 185-175 million with 118.9 million cases of HCV RNA positive individuals (Petruzziello et al., 2016). The Centres for Disease Control (CDC) reported the prevalence of HCV infection is <2% in most developed countries. On the contrary, prevalence is higher (≥2%) in developing countries (CDC, 2016).The prevalence of HCV in Africa remains high, the World Health Organization has reported that about 11 million people in Africa were infected with HCV in 2015 (WHO, 2017). The prevalence of HCV among end-stage renal disease (ESRD) patients

varies between developed (3.4 %) and developing countries (75–80%) (Burra et al., 2014), which is higher than in healthy general population (CDC, 2016) suggesting that dialysis patients are at greater risk of contracting HCV infection.

There is limited information on the prevalence of HCV in Sudan. In the general population. Sudan has been estimated to have a HCV prevalence ranging from 2 % to 4.8 % (Osman et al., 2013, Mudawi et al., 2007b). Fewer studies have been conducted in the Sudanese haemodialysis population. The haemodialysis population in Sudan has a higher prevalence as compared to the general population. HCV in the haemodialysis population showed the prevalence of 14.3% (El-Amin et al., 2007) and 23.7% (Alrahman and Gassoum, 2015). A recently published study demonstrated a decline of 6% in the prevalence of HCV as compared to the previously published results (Hammad et al., 2016a).

1.3 Transmission of HCV

HCV is primarily transmitted through exposure to infected blood and blood products. The mode of transmission includes sharing of injection equipment - mostly drug exchange among intravenous drug users, transfusion of unscreened blood and blood products, organ transplantation, haemodialysis units, unintentional exposure, sexual contact and to a lesser extent vertical transmission from infected mother to baby at birth (Moreira et al., 2005)

Health care workers and laboratory personnel are also at a greater risk of HCV transmission. HCV transmission due to blood transfusions has decreased since the initiation of blood screening in 1990. Before donor

screening, organ transplantation was one of the major sources of HCV transmission (Pereira et al., 1992)

1.4 Clinical Features of HCV Infection

The infection has an incubation period that can last up to 6 - 8 weeks and is often referred to as the “silent disease” because it can go undetected for a long period (Sharma, 2010). HCV infection can either be acute or chronic. The acute infection is asymptomatic or clinically-mild and can occur a few days after exposure to the virus (Sharma, 2010). Symptoms of acute hepatitis include jaundice, malaise, and nausea. Ranging from 54% -86% of infected individuals fail to clear and can progress from acute to chronic hepatitis (Sabahi, 2009).

Chronic HCV infection is a prominent cause of end-stage liver diseases, liver failure or hepatocellular carcinoma (HCC), which may eventually lead to liver-related death and thus may require liver transplantation (Sabahi, 2009). Chronic HCV patients are often characterized by elevated alanine aminotransferase (ALT) levels (Leone and Rizzetto, 2005).

1.5 Diagnosis of HCV

HCV is diagnosed using serologic and molecular assays. Serologic assays are used to detect the presence of antibodies specific to HCV, while molecular assays are used to detect the presence of HCV RNA. Enzyme immunoassays (EIA) are examples of serological assays, they are highly sensitive assays used to test the presence of specific antibodies to HCV. EIA are highly sensitive with a detection rate greater than 99%. As sensitive as it may be, testing for anti-HCV in immunosuppressed populations (like haemodialysis patients) can produce false negative results (Pawlotsky, 2002, Gharny et al., 2009, Agnello et al., 1992).

Diagnosis of HCV using molecular techniques include: reverse transcription of RNA followed by amplification of DNA using the polymerase chain reaction (PCR), quantification of the HCV RNA or genotyping. Quantification of the HCV RNA is useful in patients on therapy and have low titres. The detection limit of qualitative assays is very low with exceptional specificity (Weiner et al., 1990). Genotyping can be useful in determining specific medicines that can be used to treat HCV infection as different genotypes of HCV react differently to treatment (Pawlotsky, 2002, Gharny et al., 2009).

1.6 Hepatitis C Virus

1.6.1 HCV viral particle

HCV belongs to the family *Flaviviridae* and genus *Hepacivirus*. The HCV genome is comparable to that of the pestiviruses and flaviviruses (Choo et al., 1991). HCV is spherical in structure consisting of spike-like projections. When visualised under the electron microscope, HCV has been found to be between 55-60 nm in diameter (Kaito et al., 1994, Blanchard et al., 2002). The HCV virus contains an envelope made up of lipid bilayer and inside the envelope, a capsid is enclosed (Kaito et al., 1994). The capsid functions in protecting the genetic material of the virus, which is a positive single stranded RNA (ssRNA) genome, of approximately 9.6 kb in length (Choo et al., 1991, Sharma, 2010) (Figure 1.1).

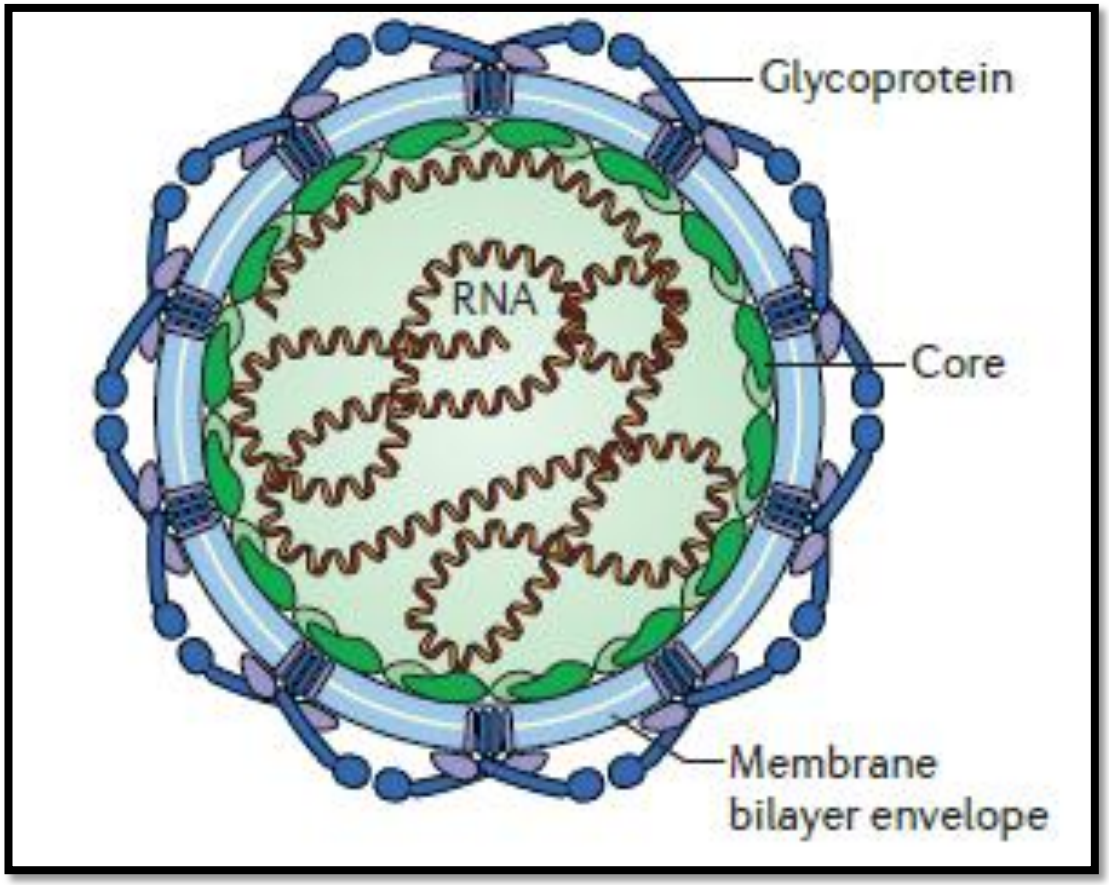


Figure 1.1: Hepatitis C virus particle. The virus particle contains a membrane bilayer envelope, the core and glycoprotein. (Lindenbach and Rice, 2013). Copyright permission requested from Springer Nature.

1.6.2 HCV genome

The HCV genome contains a long open reading frame (ORF), which has a 5' and 3' untranslated regions (UTR) at each end and a middle region approximately 9000 nucleotides that is translated into both structural and non-structural proteins (Figure 1.2) (Takamizawa et al., 1991, Atoom et al., 2014). This genome has high a mutation rate and can generate approximately 10^{-5} mutations per nucleotide per replication cycle (Espirito-Santo et al., 2007). This high mutation rate of the HCV genome can lead to as high as 10 to 100 replacements per site per year (Davis, 1999).

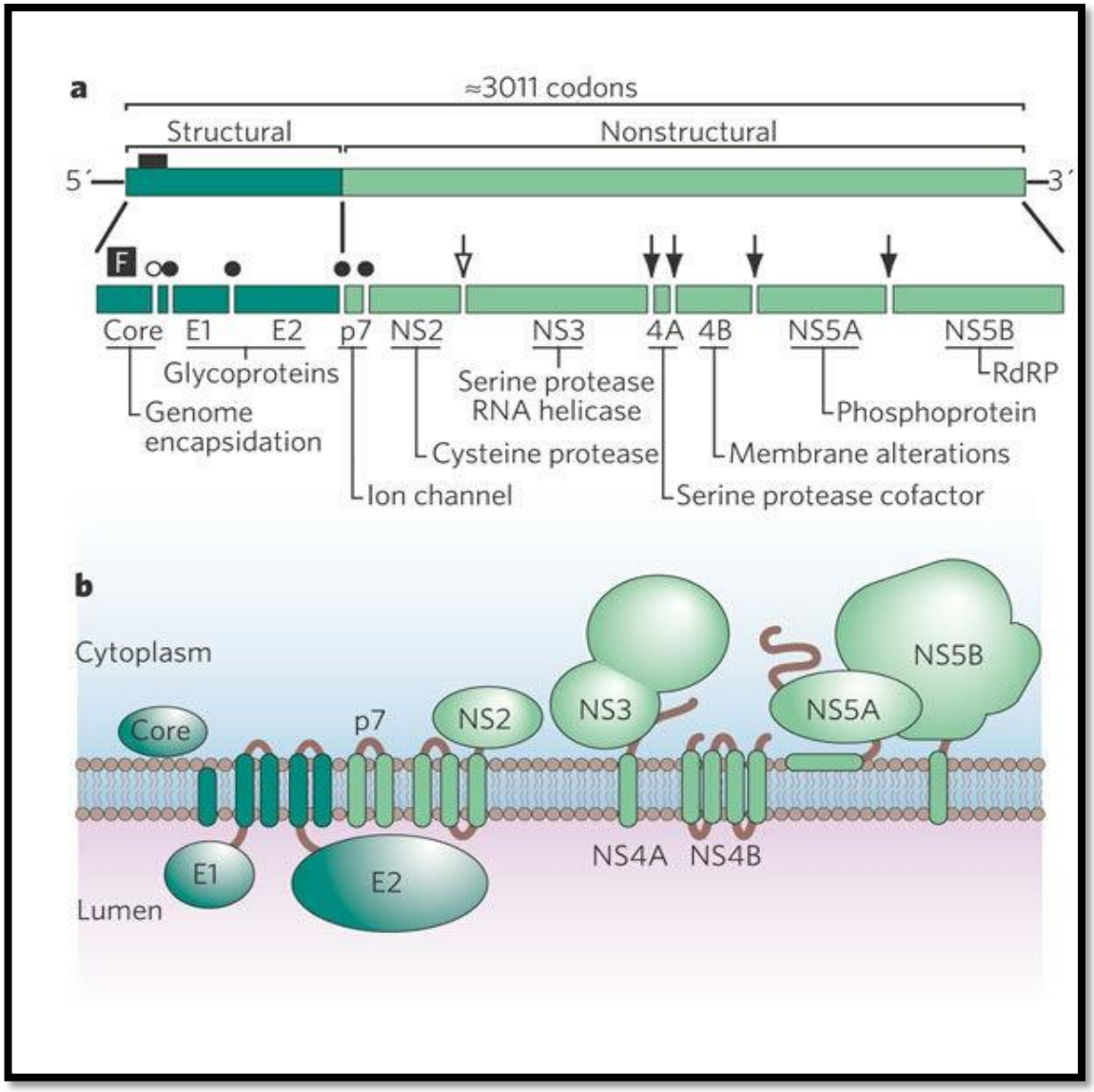


Figure 1.2: a) Structure of the HCV genome. The closed circles represent signal peptidase cleavage sites and the open circles represents signal peptide peptidase cleavage sites. b) The topology of HCV proteins with respect to the cellular membrane (Lindenbach and Rice, 2005a). Copyright permission requested from Springer Nature.

1.5.2.1 HCV Untranslated regions

For a nucleotide substitution to be considered as a viable mutation, it has to occur in a variable region of the HCV genome (Davis, 1999). The 5'UTR region is a highly-conserved region, which consists of about 341-344

nucleotides (Francesco, 1999). Thus nucleotide substitution in this region cannot be considered as a viable mutation (Davis, 1999).

The 5'UTR region is known to be the slowest evolving region within the HCV genome. Hence, genotype determination of HCV based on the 5′UTR region is regarded accurate for most genotypes (Smith et al., 1995). However, the 5'UTR region does not contain sufficient information to distinguish subtypes. Analysis showed NS5B, core / E1 sub-genomic regions contain adequate phylogenetic information to accurately classify HCV subtypes (Simmonds et al., 1994).

Located inside the 5'UTR is the internal ribosome entry site (IRES). The site essential for internal ribosomal entry is located between bases 125-323 of the 5'UTR (Brown et al., 1992, Wang et al., 1993). The IRES is responsible for initiating translation of the viral polyprotein. It initiates translation through the internal ribosome-binding mechanism by forming a stable complex with the 40S ribosomal subunit (Wang et al., 1993).

The 5'UTR region as well as the 3'UTR region are very crucial for HCV viral replication (Honda et al., 1996). Like the 5'UTR region, the 3'UTR region is a highly-conserved region. This region is made up of three regions namely; 1. A non-conserved variable region of 42-287 nt. 2. A polypyrimidine sequence made up of 20-200 nt, and 3. The highly conserved X-region characterized by stem loops. The X-region is made up of 98 nt (Wang et al., 2005).

1.5.2.2 Translation and post translation of the HCV genome

Once the coding ORF is translated it gives rise to a polyprotein of approximately 3000 amino acids (Figure 1.2.). The translated polyprotein

will therefore undergo co-translation and post-translational proteolytic processing within the cytoplasm or the endoplasmic reticulum (ER). The host and viral proteases are responsible for proteolytic processing of the polypeptide (Atoom et al., 2014). After proteolytic processing the polypeptide will give rise to 3 structural proteins and 7 non-structural proteins (Grakoui et al., 1993). The structural proteins are responsible for the formation of the viral particle while the non-structural proteins are believed to be involved in replication (Francesco, 1999, Takamizawa et al., 1991).

1.5.2.3 Structural proteins

The structural proteins include the core, and envelope glycoproteins, E1 and E2 (Grakoui et al., 1993).

1.5.2.3.1 Core protein

The first structural protein to be cleaved by host and viral proteases is the core protein (also known as the capsid protein) of molecular weight 21 kDa (Grakoui et al., 1993). The core protein of HCV is associated with viral nucleocapsid formation. In addition, the core protein is responsible for several factors including nucleocytoplasmic localization, regulation of cellular and unrelated viral promoters in *in-vitro* studies, suppression of programmed cell death, physical association with apolipoprotein II and cytoplasmic tail of the lymphotoxin b-receptor, promotion of normal cells to a transformed phenotype, and also transactivation of suppression of cell growth (Francesco, 1999).

1.5.2.3.2 Envelope proteins

Envelope glycoproteins E1 and E2 are highly glycosylated proteins of molecular weight 31 kDa and 70 kDa, respectively. These proteins are embedded in the lipid membrane (Ashfaq et al., 2011, Grakoui et al.,

1993). Envelope glycoproteins are characterised by two regions, the heavily glycosylated N-terminal ectodomain and a C-terminal transmembrane domain. The N-terminal ectodomain is responsible for the attachment of the virus to viral receptors while the C-terminal transmembrane domain functions in supporting each glycoprotein in a lipid bilayer (Goffard et al., 2005). E1 and E2 are very crucial in facilitating HCV viral entry into the cell by forming the E1E2 noncovalent heterodimer complex (Vieyres et al., 2010)

1.5.2.4 Non-structural proteins

The non-structural proteins include p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (RNA dependent RNA polymerase)(Figure 1.2), which function in the replication of the viral genome (Sharma, 2010). p7, which consists of 63 amino acids, is a membrane-spanning protein that resides in the ER (Atoom et al., 2014, Griffin et al., 2003). It is made up of two transmembrane domains, which are connected by a short cytoplasmic loop (Atoom et al., 2014). p7 functions in enhancing virus particle assembly and also releases the infectious virions (Steinmann et al., 2007, Griffin et al., 2003).

Interaction of p7 with the NS2 is believed to support late viral assembly and envelopment (Gentzsch et al., 2013, Atoom et al., 2014). NS2 is a transmembrane protein of 21-23 kDa (Ashfaq et al., 2011). Together with the NS3, NS2 is responsible for the maturation of the non-structural region (Rice, 1996). Apart from being involved in non-structural region maturation, NS3, a 67 kDa protein, has catalytic activity. This catalytic activity is produced by the amino acids His-1083, Asp-1107 and Ser-1165 found within the NS3 region. It has an N-terminal, which possesses a serine protease activity and helicase/NTPase activity found in the C-terminal (Gallinari et al., 1998).

NS4A plays an important role in the cleavage of NS3-NS4A, NS4A-NS4B and NS4B-NS5A sites. This NS3 serine type proteinase cofactor is made up of 54 amino acids in total. It functions in promoting the cleavage between NS5A and NS5B (Wolk et al., 2000, Bartenschlager et al., 1995). NS4B (27 kDa) is localised in the ER and during viral replication it aids in assembling of the replication complex. The protein is hydrophobic in nature and contains 4 transmembrane domains (Egger et al., 2002). NS5A is a 56-58 kDa hydrophilic protein. It is believed to have a crucial role in the replication of the virus (Reed et al., 1997, Neddermann et al., 1999). The NS5A region contains the interferon-sensitivity-determining-region. This region binds to the serine-threonine protein kinase during the HCV infection, repress and inhibit its antiviral function (Gale et al., 1998).

The NS5B of HCV possesses an RNA-dependent RNA polymerase(RdRp) activity (Behrens et al., 1996). HCV NS5B RdRp can initiate HCV RNA synthesis in a primer dependent and primer independent way. A study carried out by Lohmann and colleagues has shown that initiation of RNA synthesis is dependent on a DNA or RNA primer (Lohmann et al., 1997). The 3' end of the template RNA can be used as a primer during primer dependent pathway. It can fold itself against a complementary sequence in the same molecule forming a loop; this can be extended by the RdRp activity resulting in the formation of a hairpin. HCV RNA can also be synthesized without a primer by allowing the RNA template to copy itself back (Lohmann et al., 1997, Behrens et al., 1996, Cheney et al., 2002). The NS5B RdRp lacks repair and proof reading activity, which may lead to high HCV genetic diversity (Sabahi, 2009).

1.7 HCV lifecycle

HCV enters the hepatocytes through binding of the envelope glycoproteins E1 and E2 onto the surface of the hepatocytes (Figure 1.3). The host proteins responsible for mediating HCV internalization include 1. Low density lipoprotein receptors, which mediate initial low-affinity cell binding (Agnello et al., 1999), 2. tetraspanin CD81 (Pileri et al., 1998), 3. Human scavenger receptor class B type I, the scavenger receptor is responsible for binding E3 to human hepatic cells (Scarselli et al., 2002) and 4. Asialoglycoprotein receptor (Saunier et al., 2003).

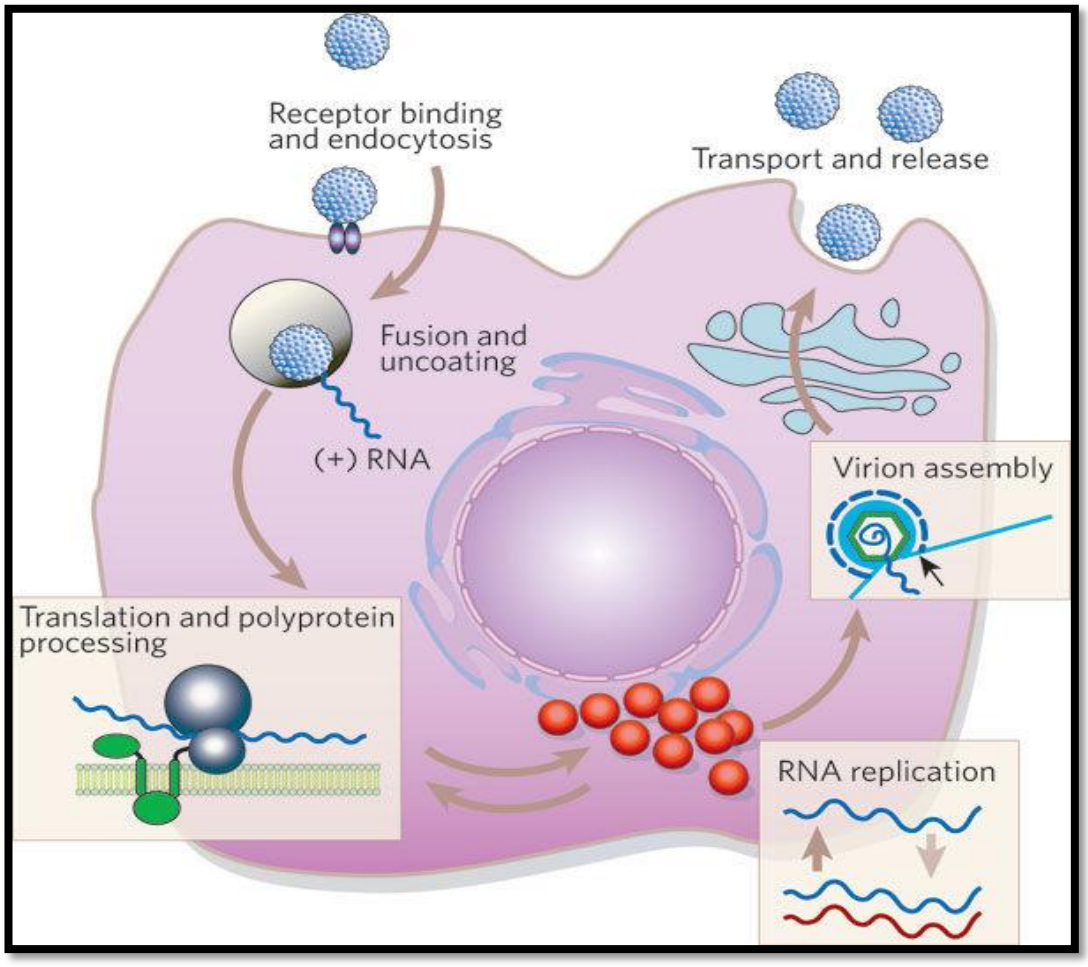


Figure 1.3: HCV lifecycle. Schematic diagram shows the lifecycle of HCV from binding to the receptors to RNA replication, viral assembly and

release (Lindenbach and Rice, 2005b). Copyright permission requested from Springer Nature

The HCV will be taken up by the hepatocytes in a pH dependent clathrin mediated endocytic manner (Sharma et al., 2011, Blanchard et al., 2006). Once the virus is internalized, the nucleocapsid is released into the cytoplasm (Ashfaq et al., 2011). It is in the cytoplasm of the hepatocytes where the viral replication complex initiates replication. The replication complex will replicate the positive sense ssRNA producing many negative ssRNAs. After replication, the viral particles are packaged and secreted and will infect other cells. Studies suggest that replication of the virus is enhanced by binding of the liver specific micro-RNAs to the 5'UTR of HCV (Sabahi, 2009).

1.8 HCV genotypes

For genotyping purposes the NS5 region is the most suitable region to use as it is sufficiently variable and it can allow differentiation between isolates of HCV. Initially Simmonds *et al,* observed three levels of sequence diversity in the NS5 region constructed phylogenetic tree (Simmonds et al., 1993). The phylogenetic tree showed clustering of samples into six different major groupings, which are less divergent from each other (Simmonds et al., 1993)

Currently HCV is classified into seven phylogenetic clades - designated as genotypes (numerically 1 to 7) and 67 subtypes - named in alphabetical order from a to z (Figure 1.4) (Sharma, 2010, Smith et al., 2014). Classification of HCV genotype is defined by greater than 30 % nucleotide inter-genotype divergence, and 20 % divergence for subtype (Simmonds et al., 1994).

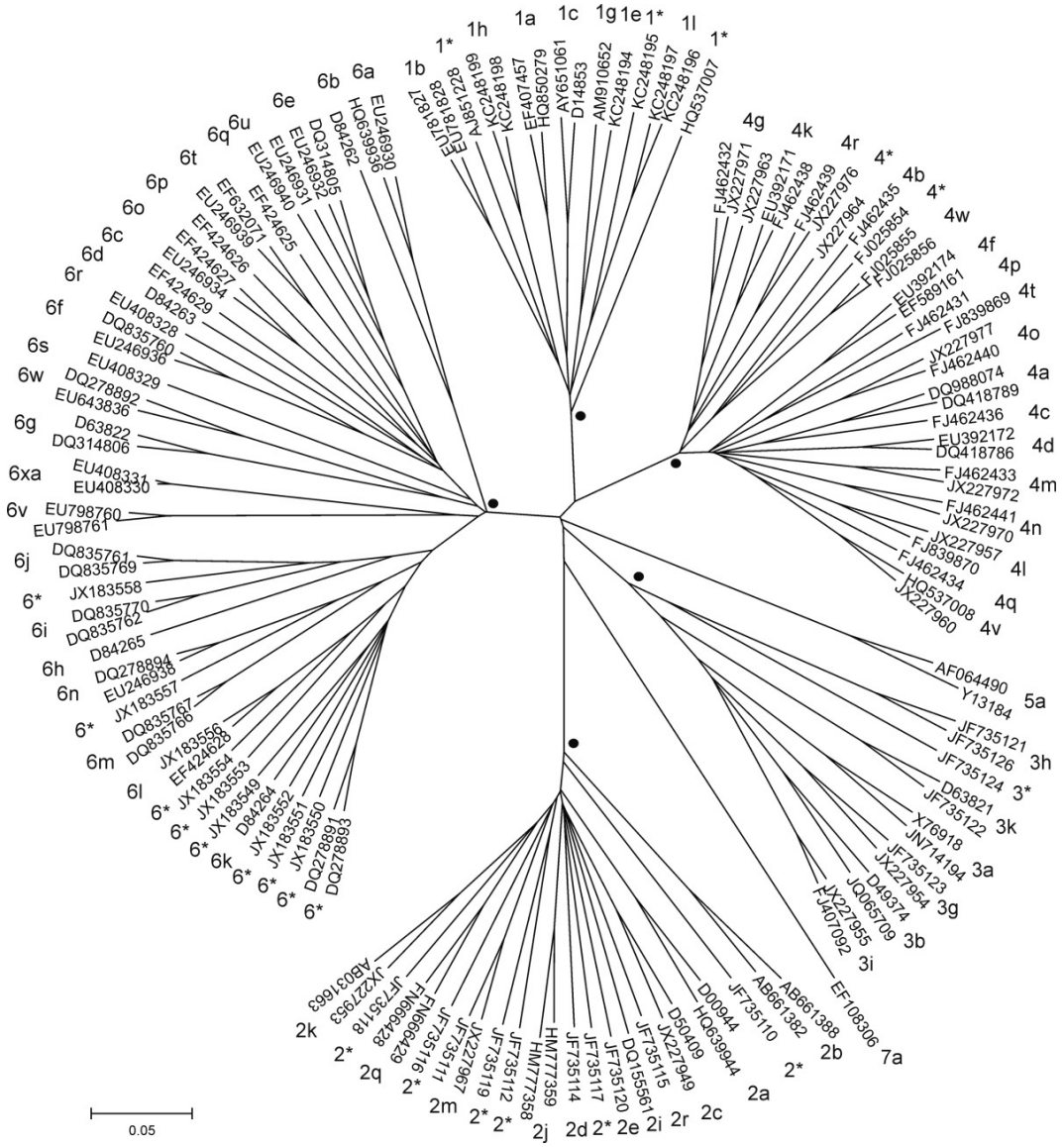


Figure 1.4: Phylogenetic tree showing the different HCV genotypes and subtypes. Unassigned subtypes are indicated by (*) while branches that are supported by 100% bootstraps are indicated by (.). (Smith, et al., 2014). Copyright permission requested from John Wiley and Sons.

The HCV genotypes have distinct geographical distribution globally. Genotypes 1, 2 and 3 are known to have a worldwide distribution (

*Figure **1.5***) (Messina et al., 2015). A study was conducted in Germany where 17 of the patients were Africans from Sudan and Egypt, this study detected an HCV infection with subtype 1c and 4a (Feucht et al., 2004). Though genotype 4 of HCV can be found in other parts of the world, it is

believed to have originated from Central Africa (Iles et al., 2014). In Sudan, previous studies have found that genotype 4 was the predominant genotype isolated, with subtypes, 4e and 4c/4d predominating (Mudawi et al., 2007b). Although data indicating the prevalence of genotypes 1 and 4 in Sudan, the knowledge on genotype distribution in Sudan is still weak (Messina et al., 2015).

The high diversity of genotype 4 samples in other countries such as France can be explained by colonization, which took place in central Africa including immigration of individuals to different countries. Previous studies showed that genotype 1a is mostly prevalent in patients with history of intravenous drug use (Iles et al., 2014). Genotype 5 is mainly found in South Africa, whereas Genotype 6 is found mainly in East Asia (Messina, et al., 2015). Most recently, a newly discovered genotype was assigned as genotype 7 (Figure 1.4). It was identified in subjects originating from the Democratic Republic of Congo (DRC) (Murphy et al., 2014).

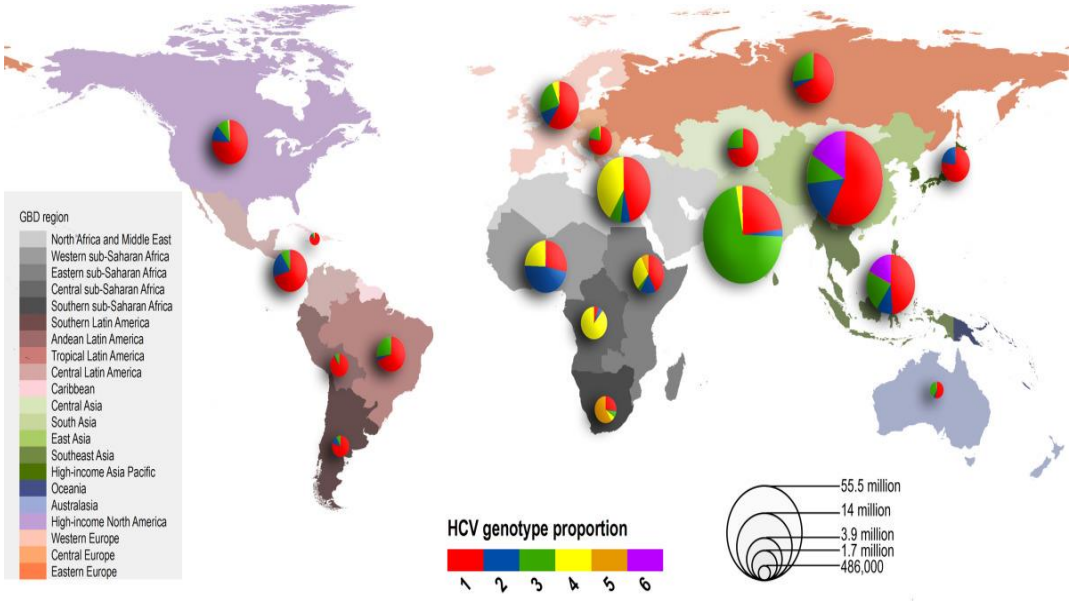


Figure 1.5: Global distribution of the HCV genotypes in different Global Burden of Disease regions. (Messina, et al., 2015). Copyright permission requested from John Wiley and Sons.

1.9 Relationship between HCV infection and its genotypes

There is not much certainty on the influence of HCV genotypes on disease progression. Researchers strongly believe HCV genotypes do play a role in disease progression, manifestation and treatment of the infection (Zein, 2000). Genotype 1b is associated with more severe liver disease and more aggressive course than other HCV genotypes (Nousbaum et al., 1995). It is more prevalent in patients with liver cirrhosis, and decompensated liver disease that need liver transplantation than those with chronic infected patients (Zane et al., 1996).

HCV genotype 3, prevailing on the Indian subcontinent, is known to be associated with hepatic steatosis (Tapper and Afdal, 2013). Although not much clinical data is available on the relationship between genotype 4 and HCC, epidemiological data suggest patients infected with genotype 4 are likely to be at high risk of developing HCC (Kamal and Nasser, 2008).

The high mutation rate of HCV has made it difficult to treat the infection. Different genotypes respond differently to treatment regimens. Historically, patients with genotype 1 infection have significant lower sustained virological response rate than patients infected with HCV genotype 2 and 3 when using interferon (Gharny et al., 2009). Studies have shown that treating genotype 4 which is the most prevalent genotype in Sudan is difficult, even with the use of pegylated interferon thus addition of ribavirin has shown improvement in controlling the HCV virus (Antaki et al., 2009). The different response of genotypes grew a need for new more efficient therapeutic treatments. Over the years there has been advancement of the treatment of HCV, from the use of interferon based therapy to the introduction of direct acting antiviral agents (DAAs) (Geddawy et al., 2017), which generally speaking have pan-genotypic activity.

The DAAs are designed to specifically target the different polyproteins in the HCV genome. These DAAs are divided into three main classes which includes; 1. Protease inhibitors (Boceprevir, Telaprevir, Simeprevir, Asunaprevir, Paritaprevir and Grazoprevir), 2. NS5A inhibitors (Daclatasvir, Ledipasvir, Ombitasvir, Elbasvir and Velpatasvir) and 3. Polymerase inhibitors (Sofosbuvir and Dasabuvir). To date there is no information available on the use of DAAs in Sudan. Despite the advancement on treatment for HCV, currently there is no HCV vaccine available on the market (Parfieniuk et al., 2007, Geddawy et al., 2017). Therefore, the spreading of infection can only be controlled by protective measures (Sharma, 2010).

1.10 End-Stage Renal Disease and haemodialysis

Kidneys are important organs responsible for cleaning out waste products in a human's body. Kidney function can sometimes deteriorate leading to chronic renal failure, and can also result in loss of kidney function known as end stage renal disease (ESRD). Kidney function is measured using the glomerular filtration rate, and when the glomerular filtration rate of a patient is <15 ml/min/1.73 m^2 then the patient is considered to have ESRD (Garg and Bakris, 2002).

Haemodialysis is a process whereby a machine equipped with an artificial kidney is connected to the body to clean out the blood and remove toxins and excess water from the body. Failure to adhere to all necessary precautions during the haemodialysis process can lead to transmission of various infections. Patients undergoing haemodialysis are at particularly high risk for the transmission of blood-borne pathogens because of prolonged vascular access, likely exposure to infected patients, cross-contaminated equipment supplies and surfaces within the same dialysis unit (El-Amin et al., 2007).

1.11 HCV and extra hepatic manifestations

HCV infection has been shown to be associated with many extra-hepatic manifestations. It is classified as a cause and complication for chronic renal diseases. HCV infection has been identified to be associated with an increased risk of developing chronic kidney disease, with almost two-fold increased risk of developing end-stage renal diasease. (Fabrizi et al., 2012, Molnar et al., 2015). This may be caused by cryoglobulins or noncryoglobulin-immune complexes deposition in the glomeruli (Agnello et al., 1992).

In addition, HCV-NS3 protein is responsible for forming an immune complex with IgG and IgM in patients with type II cryoglobulinemia. This type of systemic immune response to HCV infection can lead to kidney injury (Tsui et al., 2006). Chronic HCV infection is recognized to be extensively associated with risks of progressive deterioration of renal function, morbidity and mortality (Fabrizi et al., 2012, Molnar et al., 2015).

1.12 Study rationale

The aim of the study was to genotype and molecularly characterize HCV isolated from ESRD patients from Sudan. The study was set out to determine the prevalence of HCV in Sudanese ESRD patients. Genotyping and molecular characterization of the HCV virus in the haemodialysis population in Sudan will help in implementation of different treatment regimens such as DAAs as different HCV genotypes respond differently to treatment. The study also aimed to acquire information about the regional distribution of HCV genotypes in the haemodialysis population in Sudan. It is postulated that HCV genotypes may vary from region to region. There is limited information concerning

HCV genotype distribution in Sudan (Messina et al., 2015). The lack of detailed information about the prevalence and genotypes present in Sudanese patients with ESRD highlighted a need for this study to be conducted in these patients. The outcome of this study may also provide insight in determining whether HCV infection is responsible for kidney function deterioration.

There is substantial variability in the prevalence of anti-HCV and chronic HCV infection in dialysis units globally. Although it has been more than 3 decades since viral hepatitis has been found to be most prevalent in ESRD patients, this infection continues to be a burden. Moreira et al and others have reported an HCV prevalence of 10% to 40% in the ESRD population (Moreira et al., 2005, Fabrizi et al., 2002).

The knowledge on HCV prevalence and incident rates among haemodialysis patients in developed countries is sufficient. In contrast data of HCV epidemiology in dialysis patients from developing nations is limited, and generally based on single centre or region. It has been reported that strict adherence of recommended universal infection-control precautions can decrease prevalence and incidence of HCV in these patients (Scheeberger et al., 2000) and thus the present study can inform on whether these recommendations are being adhered to in Sudanese haemodialysis centres.

1.13 Aims and objectives

1.13.1 Study aim

To genotype and molecularly characterize HCV isolated from ESRD patients from Sudan.

1.13.2 Study objectives

- To genotype and molecularly characterize HCV isolates from anti-HCV-positive ESRD Sudanese patients undergoing haemodialysis.
- To provide information on whether haemodialysis units are settings for HCV transmission.
- To determine HCV viral loads in haemodialysis patients

Chapter 2 Materials and methods

2.1 Study design and population

2.1.1 Study design

A cross-sectional study was conducted.

2.1.2 Study population

2.1.2.1 Study area

Study participants were recruited and serum samples were collected for a period between Decemeber 2014 and January 2016 from nine haemodialysis units at Khartoum, Sudan: Soba, Salma, Elakadeeemi, Elturki, Lbn sena, Tropical hospital, Alnaw, Police hospital and Elshurta.

2.1.2.2 Study participants

A total of 548 haemodialysis patients were initially recruited and tested for anti-HCV, of which 93 patients were found to be anti-HCV positive. No follow up tests were performed to the 93 anti-HCV positive patients. Of the 93 patients, 2 were HBsAg-positive HBV/HCV co-infected. The remaining 91 HBsAg negative samples of which 8 were found to be HBV DNA-negative and therefore hepatitis C with occult HBV (Figure 2.1).

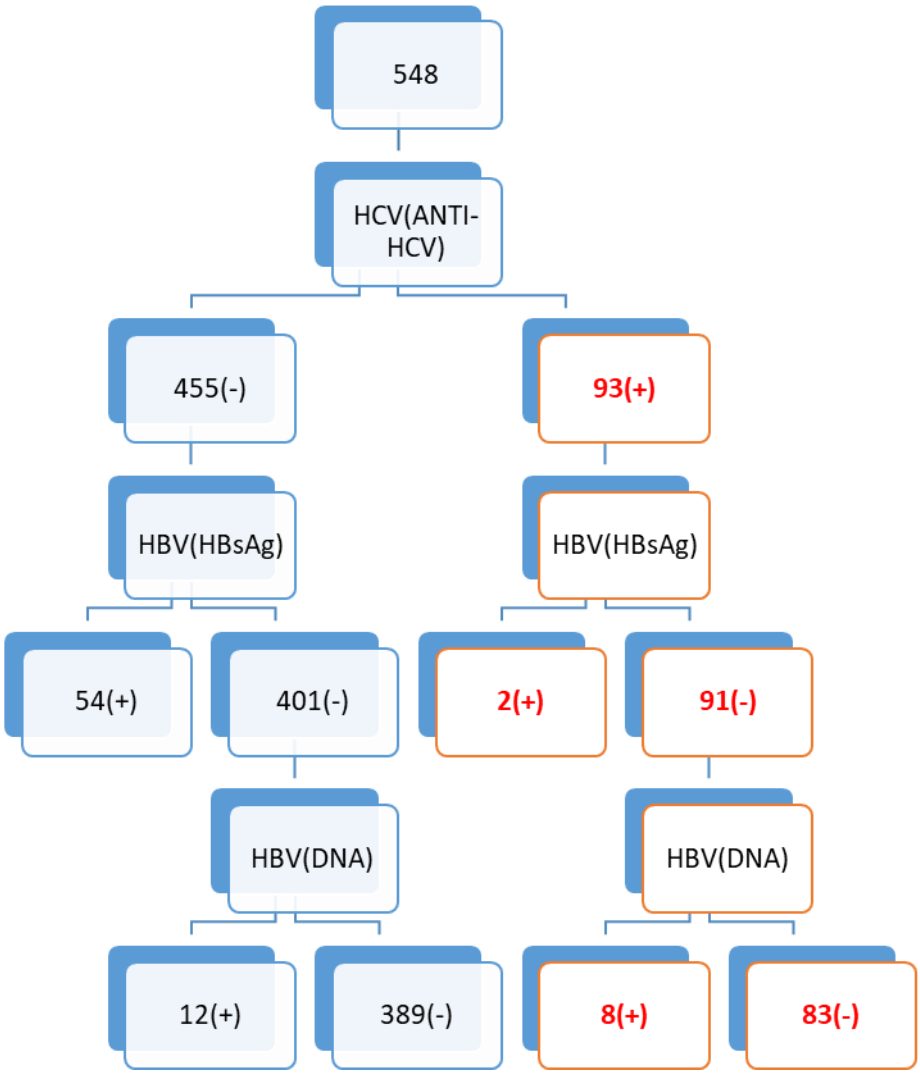


Figure 2.1: A flow diagram of specimen acquired from Khartoum, Sudan. Serum samples from a total of 91 patients were used in the present study, 83 were anti-HCV positive and 8 anti-HCV/ HBV co-infected.

2.2 Ethics clearance

Ethical approval (3-10-2016) to conduct the study was obtained from the National Health Research Ethics Committee, Federal Ministry of Health in Sudan by Professor Hatim Mohamed Yousif Mudawi (Appendix A). Ethical clearance certificate for the umbrella study “Phylogenetic Analysis of Hepatitis Viruses Found in Africa Relative to those Derived from other Geographic Regions of the World” was granted to Professor Anna Kramvis by the University of the Witwatersrand Human Research Ethics Committee with the (Medical) Clearance Certificate No. M150158. Ethical clearance for the present study was a sub-study under Primary Study M150158 (Appendix B). The study was approved by the University of the Witwatersrand Human Research Ethics Committee, with (Medical) Clearance Certificate No. M170165 (Appendix C).

2.3 Methodology

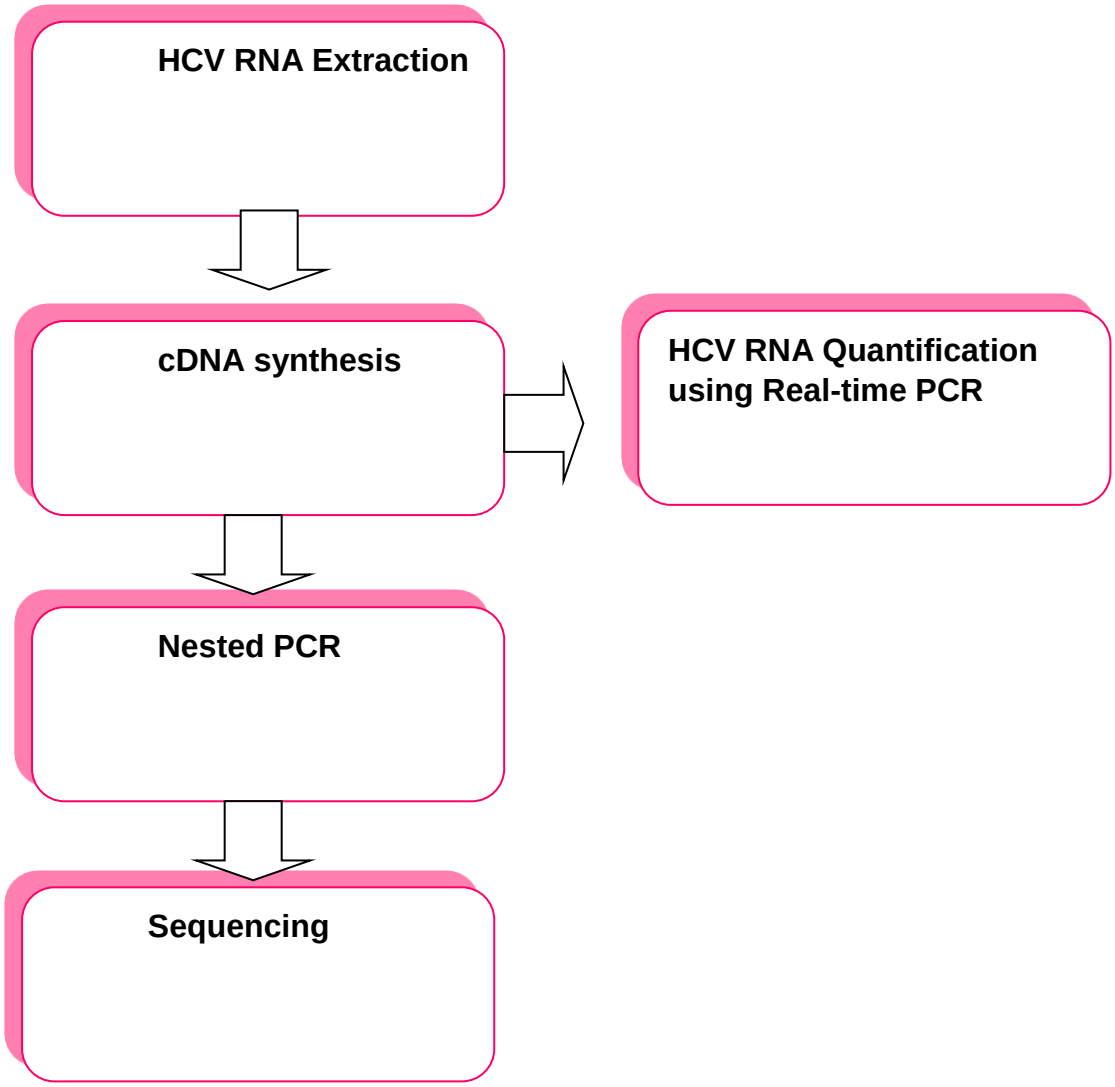


Figure 2.2: Overview of laboratory method used in this study

2.3.1 Sample collection, transportation and storage

Patient serum was collected in Sudan, where anti-HCV testing was performed. The samples were transported in a temperature controlled environment and stored in -70°C freezer upon arrival in South Africa to

prevent nucleic acid degradation. The samples were thawed on ice prior to HCV-RNA extraction.

2.3.2 HCV RNA Extraction

The QIAamp® Viral RNA Mini Kit (Qiagen, GmbH, Hilden, Germany) was used to extract HCV RNA according to manufacturer's instructions. Briefly an aliquot of 140 µl serum was added to 1.5 ml micro-centrifuge tube containing 560 µl Buffer AVL and 1 µg/µl carrier RNA. The tube was mixed by pulse vortex for 15 seconds and incubated at temperature between 15 to 20°C for 10 minutes.

Micro-centrifuge tube was briefly centrifuged to remove droplets from the lid. An aliquot of 560 µl (96-100%) ethanol was added to the mixture and pulse vortexed for 15 seconds followed by brief centrifugation. The mixture was loaded to the QIAamp Mini column and centrifuged at 8000 rpm for 1 minute. The collection tube was discarded and the column was placed in a new collection tube.

An aliquot of buffer AW1 was added to the QIAamp Mini column and centrifuged at 8000 rpm for 1 minute. The QIAamp Mini column was placed in a new clean collection tube. After the addition of 500 µl buffer AW2, the column was centrifuged at 8000 rpm for 3 minutes. In order to eliminate any possible residual buffer AW2 to carryover, the column was further centrifuged at 8000 rpm for 1 minute. The QIAamp Mini column was placed in a new clean collection tube before proceed to the RNA elution step.

A volume of 60 µl buffer AVE, pre-equilibrated to room temperature, was added to the column and incubated at room temperature for 1

minute. Following incubation, the column was centrifuged at 8000 rpm for 1 minute. Extracted HCV RNA was immediately processed for cDNA synthesis and the remaining aliquots were stored at -70 °C for later use. Known HCV-negative, HCV-positive sera, best quality water were included as controls for extraction.

2.3.3 Complementary DNA (cDNA) synthesis

Immediately post RNA extraction, complementary DNA (cDNA) was synthesized using the Superscript® III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Briefly, 8 µl aliquot of HCV RNA was mixed with 1µl of 50ng/µl random hexamer and 1 µl of 10 mM dNTP mix. The mixture was incubated at 65°C for 5 minutes, and placed on ice for at least 1 minute. cDNA synthesis mix was prepared using 2 µl of 10X reverse transcriptase (RT) buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of 40 U/µL RNaseOUT™ and 1 µl of 200 U/µL Superscript® III RT.

The prepared mixture was then added to the RNA/primer mixture, gently mixed, collected by brief centrifugation and incubated at 25°C for 10 minutes, 50°C for 50 minutes and terminated at 85°C for 5 minutes. Post-incubation, the reaction was chilled on ice and collected by brief centrifugation. To remove the RNA template, 1 µl of RNase H was added and incubated at 37°C for 20 minutes. Aliquoted cDNA was stored at -20 °C.

2.3.4 Amplification of the HCV genome using nested polymerase chain reaction.

Post cDNA synthesis, the 5'UTR region for HCV (for genotyping) and NS5B (for HCV sub-typing) were amplified individually using nested polymerase chain reaction (PCR).

2.3.4.1 5'UTR region

Nested PCR of the 5'UTR region was firstly optimized as shown in Appendix F1. PCR was performed in the 5'UTR region using published primers (Chen and Weck, 2002; Murphy et al 2007). The first-round PCR cycle was carried out using Platinum SuperFi master mix (Invitrogen, Carlsbad, CA, USA) with 4 µl cDNA as template with forward and reverse primers (Table 2.1) at 1 µM final concentration in a final volume of 25 µl PCR mix. The cycling conditions were: an initial activation at 95°C for 45 seconds, followed by 40 cycles of denaturation at 98°C for 17 seconds, annealing at 51°C for 22 seconds, extension at 72°C for 1 minute 20 seconds, followed by final extension at 72°C for 5 minutes.

The second-round PCR was carried out using the same master mix as the first round with 5 µl of the first-round PCR product as a template with forward and reverse primers (Table 2.1) at 1 µM final concentration in a final volume of 50 µl PCR mix. The cycling conditions were: an initial activation at 98°C for 45 seconds, followed by 40 cycles of denaturation at 98°C for 17 seconds, annealing at 51°C for 22 seconds, extension at 72°C for 1 minute 20 seconds, followed by final extension at 72°C for 5 minutes.

Known HCV-negative, HCV-positive sera, best quality water, as well as full length HCV Strain pCV-H77 (genotype 1a), pCNJ4C6S (HCV

genotype 1b) and pJ6CF2a (HCV genotype 2a) HCV plasmid clones (obtained from Prof R. Purcell, NIH, USA) were included as controls for both rounds of PCR. Details of plasmids encoding the full-length HCV genome preparation and extraction are shown in Appendix E1-E6.

Table 2.1: 5'UTR Region primer set

Region in HCV genome	Primer Name	Primer Sequence[1]	Nucleotide position in the HCV genome[2]
5'UTR	HCV5UTR1F	5'-CTGTGAGGAACTACTGTCTT-3'	nt 45-64
	HCV5UTR2F	5'-TTCACGCAGAAAGCGTCTAG-3'	nt 63-82
	HCV5UTR3F	5'-CACTCCCCTGTGAGGAACT-3'	nt 38-56
	HCV5UTR3R	5 '-TGCACGGTCTACGAGAC-3'	nt 323-339
	HCV5UTR4F	5'-CTCCCCTGTGAGGAACT-3'	nt 40-56
	HCV5UTR4R	5 '-GCACGGTCTACGAGACCT-3'	nt 321-338

[1] Nested PCR primers were modified to successfully amplify the study samples.

[2]Nucleotide position of HCV genome pCV-H77C (Genbank accession number NC-004102)

2.3.4.2 NS5B region

Nested PCR of the NS5B region was firstly optimized as shown in Appendix F2. PCR amplification in the NS5B region was performed using published primers (Chen and Weck, 2002 and Murphy et al 2007). The first-round PCR cycle was carried out using Platinum SuperFi master mix (Invitrogen, Carlsbad, CA, USA) with 4 µl cDNA as template with forward and reverse primers (Table 2.2) at 1 µM final concentration in a final volume of 25 µl PCR mix. The cycling conditions were: an initial activation at 98°C for 45seconds, followed by 40 cycles of denaturation at 98°C for 17 seconds, annealing at 59°C for 22 seconds, extension at 72°C for 1 minute 20 seconds, followed by final extension at 72°C for 5 minutes.

Using the same master mix as in the first-round PCR, the second round was performed with 5 µl of the first-round PCR product as a template with forward and reverse primers (Table.2.2) at 1 µM final concentration in a final volume of 50 µl PCR mix. The cycling conditions were: an initial activation at 98°C for 45 seconds, followed by 40 cycles of denaturation at 98°C for 17 seconds, annealing at 53.5°C for 22 seconds, extension at 72°C for 1 minute 20 seconds, followed by final extension at 72°C for 5 minutes.

Known HCV-negative, HCV-positive sera best quality water, as well as full length HCV Strain H77 (genotype 1a), pCNJ4C6S (HCV genotype 1b) and pJ6CF2a (HCV genotype 2a) HCV plasmid clones (obtained from Prof R. Purcell, NIH, USA) were included as controls for PCR.

Table 2.2: NS5B region primer sets

Region in HCV genome	Primer Name	Primer Sequence[1]	Nucleotide position in the HCV genome[2]
NS5B	HCV7904F	5'-TGGGGTTCTCGTATGATACCC-3'	nt 8245-8265
	HCV8295R	5'-CCTGGTCATAGCCTCCGTGA-3'	nt 8616-8636
	HCV8284R	5'-CCTCCGTGAAGGCTCTCAG-3'	nt 8607-8625
	HCVN5BG4 - 1F	5'-ACCCGCTGYTTTGACTC-3'	nt 8262-8278
	HCVN5BG4 - 3F	5'-CCGCTGYTTTGACTC-3'	nt 8264-8278
	HCVN5BG4 - 2R	5'-GTCATAGCYTCCGTGAA-3'	nt 8616-8632
	HCVN5BG1/3 - 1F	5'-ACCCGYTGYTTTGACTC-3'	nt 8262-8278
	HCVN5BG1/3 - 1R	5'-TGGTCATAGCYTCCGTGA-3'	nt 8617-8634
	HCVN5BG1/3 - 3F	5'- CCGYTGYTTTGACTC-3'	nt 8264-8278

[1] Nested PCR primers were modified to successfully amplify the study samples.

[2]Nucleotide position of HCV genome pCV-H77C (Genbank accession number NC-004102)

2.4 Detection of amplified PCR product

Amplified PCR products were detected by agarose gel electrophoresis. Amplicons were separated by molecular size in a 1% agarose TBE gel containing 1 µg/µl ethidium bromide (Appendix D4, D5). An aliquot of 5 µl second round PCR product was loaded in the gel alongside the 100 bp ladder (Promega, Madison, WI, USA). The gel was electrophoresed at 100 volts for 2 hour. Gel were visualised using Syngene G: Box-Imaging, Vacutec (Syngene, Cambridge, UK).

2.5 Gel purification

Some of the PCR products showed double bands in the agarose gel. Therefore, Zymoclean™ Gel DNA Recovery Kit (Zymoresearch, CA, USA) was used to extract DNA from the excised agarose gel fragment.

PCR products were electrophoresed in 1% agarose TBE gel without ethidium bromide. The DNA fragment was excised from the agarose gel and weighed. Three volumes of ADB solution was added to each volume of the excised gel and incubated at 55°C for 10 minutes until the gel slice is completely dissolved. The reaction was centrifuged at 3000 rpm for 1 minute at room temperature and the flow through was discarded. A volume of 200µl wash buffer was added and centrifuged at 3000 rpm for 30 seconds at room temperature. Finally, 10µl of best quality water was added to the column and centrifuged at 13 000 rpm for 1 minute at room temperature. The gel purified PCR product was sent out for Sanger sequencing.

2.6 HCV RNA Quantification using Real-time PCR

Quantification of HCV RNA using real-time PCR was performed in a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with in-house designed primers and TAQMAN TAMRA probe sets in the 5'UTR region of the HCV genome (Table.2.3).

Table 2.3: Real-Time PCR primers/probe set

Primers and probe name	Sequence	Nucleotide position on the HCV genome
HCVQ1F	5'-AGCGTCTAGCCATGGCGT-3'	74-91
HCVQ1R	5'-ATTCCGGTGTACTCACCGGT-3'	155-174
HCVQ1TAQ	5'-FAM-CCCTCCCGGGAGAGCCATAG TGGTC-TAMRA-3'	124-148

Nucleotide position of HCV genome pCV-H77C Genbank accession number NC-004102.

2.6.1 Preparation of the biological standard

The HCV RNA was extracted from 140 µl of Acrometrix™ HCV High Control (Thermo Fisher Scientific, Carlsband, CA, USA) serum containing 3.8 x 10^6 copies/ml using QIAamp® viral RNA mini kit (Qiagen, GmbH, Hilden, Germany). RNA was eluted in 60 µl buffer AVE. HCV cDNA was synthesised using 8 µl of the extracted RNA together with the Superscript® III first strand synthesis system (Invitrogen, Carlsband, CA, USA) as previously described in 2.3.3.

2.6.2 Preparation of plasmid standard

The pCV-H77C genotype 1a plasmid (obtained from Prof R Purcell, NIH, USA) was prepared using using PureLink® HiPure Plasmid DNA Maxi prep kit (Invitrogen, Carlsbad, CA, USA). Details of plasmid encoding the full-length HCV genome preparation and extraction were shown in Appendix E1-E6.

Serial dilutions of the plasmid DNA encoding full length HCV genome ranging from 1.21×10^2 to 1.21×10^{11} copies/µl were used in triplicate to generate the standard curve.

2.6.3 Real-time PCR reaction

The in-house HCV real-time PCR was first optimized. Details of optimization are shown in Appendix F3. HCV viral load was quantified by real time PCR using Taqman Fast Advanced master mix (ThermoFisher Scientific, Carlsbad, CA, USA). Reaction mix was prepared in total volume of 25 µl containing 0.5 µl Taqman FAM/TAMRA probe with the final concentration of 250 nM, 1.8 µl each of in-house designed forward and reverse primers with final concentration of 900 nM, 2 µl each of known HCV-negative, HCV-positive sera, best quality water or HCV cDNA. The cycling conditions were: an initial cycle at 50°C for 2 minutes and 95°C for 20 seconds followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. The data was captured and analysed using the Bio-Rad CFX Manager Software version 2.1(Bio-Rad Laboratories, Hercules, CA, USA). The Acrometrix™ HCV High Control (the biological standard) was included as a positive control. The real-time PCR was performed in duplicates. The dynamic range of HCV real-time PCR was estimated to be 1.86×10^3 – 1.86×10^{12} IU/ml.

2.7 Sequencing

Amplicons were prepared for sequencing using the ABI v3.1 Big Dye kit (Applied Biosystems, Foster city, USA). Sanger sequencing was performed by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) using the ABI 350 XL genetic analyser (Applied Biosystems, Foster city, CA, USA). Specific second round forward primers (Table.2.4) were used in the sequencing reaction. The 5'UTR ad NS5B region were analysed in the forward direction of a single fragment.

Table 2.3: Sequencing primers

Region	Primer Name	Sequence	Nucleotide* position in the HCV genome
5UTR	HCV5UTR2F	5'-TTCACGCAGAAAGCGTCTAG-3'	nt 63-82
	HCV5UTR4F	5'-CTCCCCTGTGAGGAACT-3'	nt 40-56
NS5B	HCVN5BG4- 3F	5'-CCGCTGYTTTGACTC-3'	nt 8264-8278
	HCVN5BG1/3-3F	5'-CCGYTGYTTTGACTC-3'	nt 8264-8278

Nucleotide position of HCV genome pCV-H77C Genbank accession number NC_004102.

2.8 Data analysis

2.8.1 Statistical analysis

Statistical analysis was performed using the SPSS version 25.0 software (IBM, Armonk, North Castle, NY, USA). Student t test was used to compare continuous variables. Fischer's exact and Chi-square tests were used to determine the significant difference between categorical variables. A p-value of <0.05 was considered as statistically significant.

2.8.2 Phylogenetic analysis

Genotypes in this study were determined by comparing sequences obtained from GenBank. Sequences were aligned using CLUSTALW, MEGA software package, version 7.0. Phylogenetic tree and bootstrap analysis (1000 data sets) were determined using the MEGA software version 7.0 by using the maximum Likelihood method. Trees were constructed using the Tree Explorer in the MEGA programme. Bootstraps greater than 70% were considered significant.

Chapter 3 Results

Previously published primers (Chen and Weck, 2002 and Murphy et al., 2007) were modified to amplify samples obtained from five haemodialysis centers in Sudan. These primers were specifically designed and optimized to ensure successful amplification of the HCV genome (see Appendices). The NS5B and 5'UTR region of the HCV genome were amplified using these primers described in (Table 2-3) thereby producing PCR amplification products of about 251 bp (5'UTR) and 368 bp (NS5B).

3.1 Amplification using nested PCR.

3.1.1 Amplification of the 5'UTR region.

The 5'UTR region was amplified using first and second round nested PCR. The amplified samples were visualized using 1% agarose gel electrophoresis. Successful amplification of the PCR product showed the presence of 251 bp band (Figure 3.1).

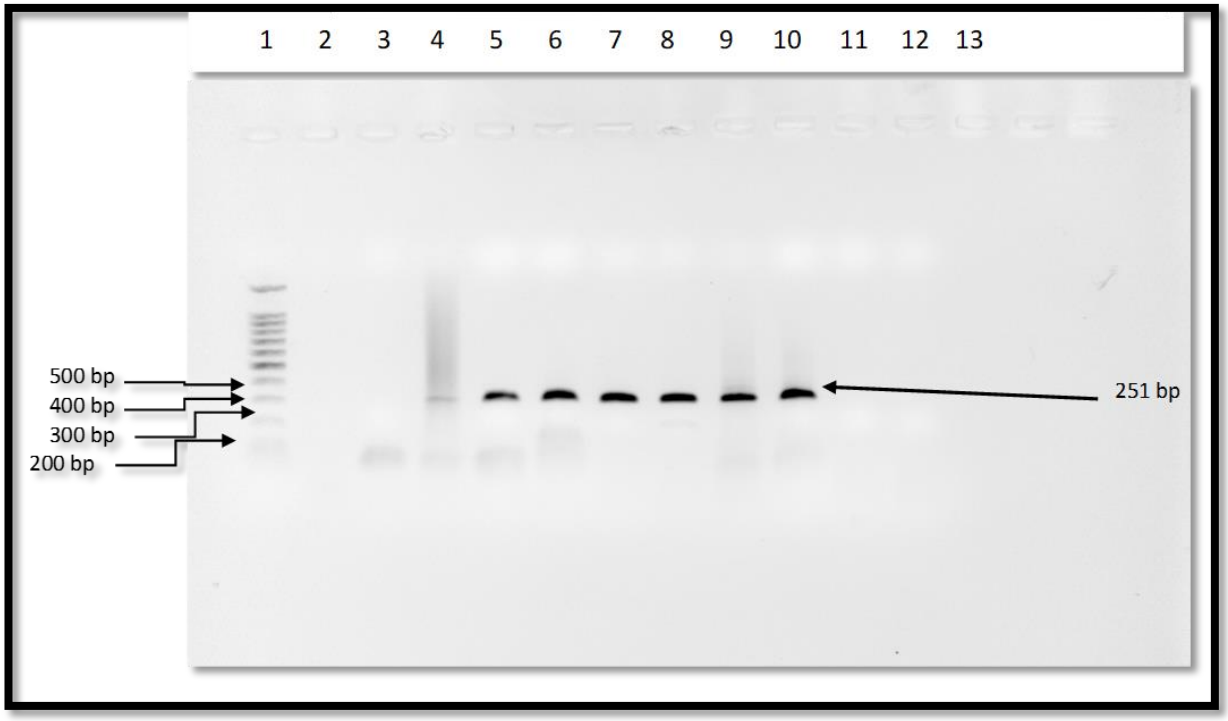


Figure 3.1: Detection of the amplified 5'UTR region PCR products. PCR products are 251 bp in length are visualised on an ethidium bromide stained 1 % agarose gel.

Lane 1 = 100 bp molecular weight marker (Promega, Madison, WI, USA)

Lane 2 = PCR Negative control

Lane 3-9 = Sudanese ESRD patients 5'UTR region products

Lane 10 = PCR positive control

Lane 11 = PCR negative control

3.1.2 Amplification of the NS5B region PCR

First and second rounds nested PCR cycles were performed to amplify the NS5B region. Not all samples amplified in the NS5B region, and of those that amplified successfully a 368 bp band were visualized during agarose gel electrophoresis (Figure 3.2).

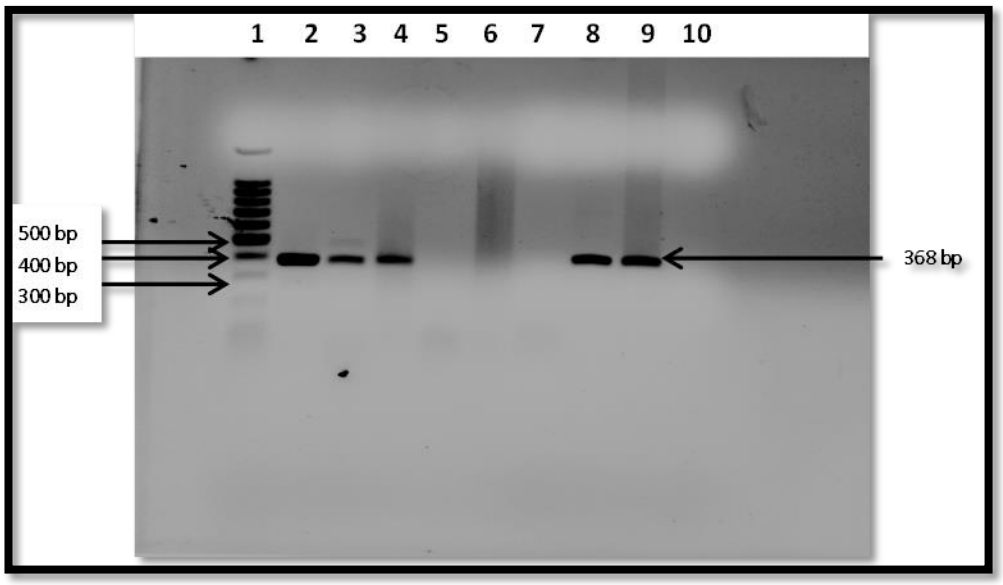


Figure 3.2: Detection of the amplified NS5B region PCR products. PCR products are 368 bp in length are visualised on an ethidium bromide stained 1 % agarose gel.

Lane 1 = 100 bp molecular weight marker (Promega, Madison, WI, USA)

Lane 2-8 = Sudanese ESRD patients NS5B region products

Lane 9 = PCR positive control

Lane 10 = PCR negative control

3.2 Amplification of HCV from Sudanese end-stage renal disease patients.

Amplification of the 5' UTR and the NS5B region was performed on 93 anti-HCV–positive sera from ESRD Sudanese patients. Of the 93 samples, 2 were HBsAg-positive HBV DNA positive, 8 were HBsAg-negative, HBV DNA positive and the remainder were negative for HBV. The HCV sero-prevalence in the study was estimated to be 17% (93/548). The total of 64/93 samples successfully amplified in the 5'UTR region and 44 in the NS5B region with only 43% (40/93) of the samples having amplified in both regions (Figure 3.3). The viremic rate was estimated to be 7% (40/548).

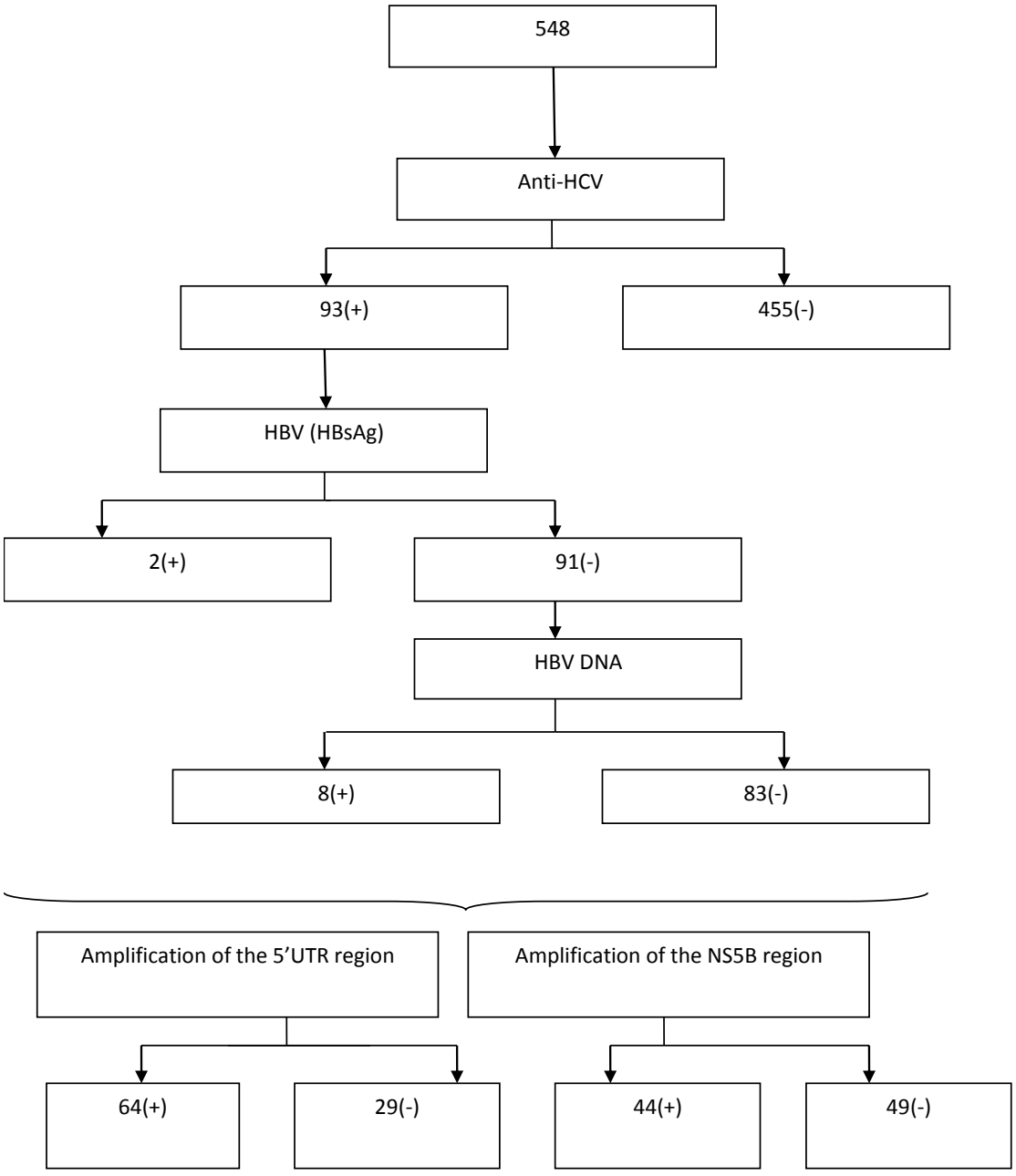


Figure 3.3: Overview of the amplification algorithm and results obtained from amplifying the 5'UTR and NS5B region

3.3 Genotyping and molecular characterization of the HCV isolates.

3.3.1 Genotyping of HCV using the 5'UTR region.

The PCR positive samples were sequenced and thereafter subjected to phylogenetic analysis. Bioinformatic analysis of the 5'UTR region was performed using the maximum likelihood based on the Kimura 2 parameter model with Gamma distribution. Clustering of the Sudan sequences with reference sequences obtained from Genbank showed the presence of genotypes 1 (Figure 3.4), 3 (Figure 3.5), 4 (Figure 3.6) and 5 (Figure 3.4) among the Sudanese haemodialysis population.

The genotype most frequently identified using amplification and sequencing of the 5'UTR of HCV isolated from Sudanese haemodialysis population belonged to genotype 4 (48%). The remainder of the isolates included genotype 1 (38%), genotype 3 (6%) and genotype 5 (5%) (Figure 3.7).

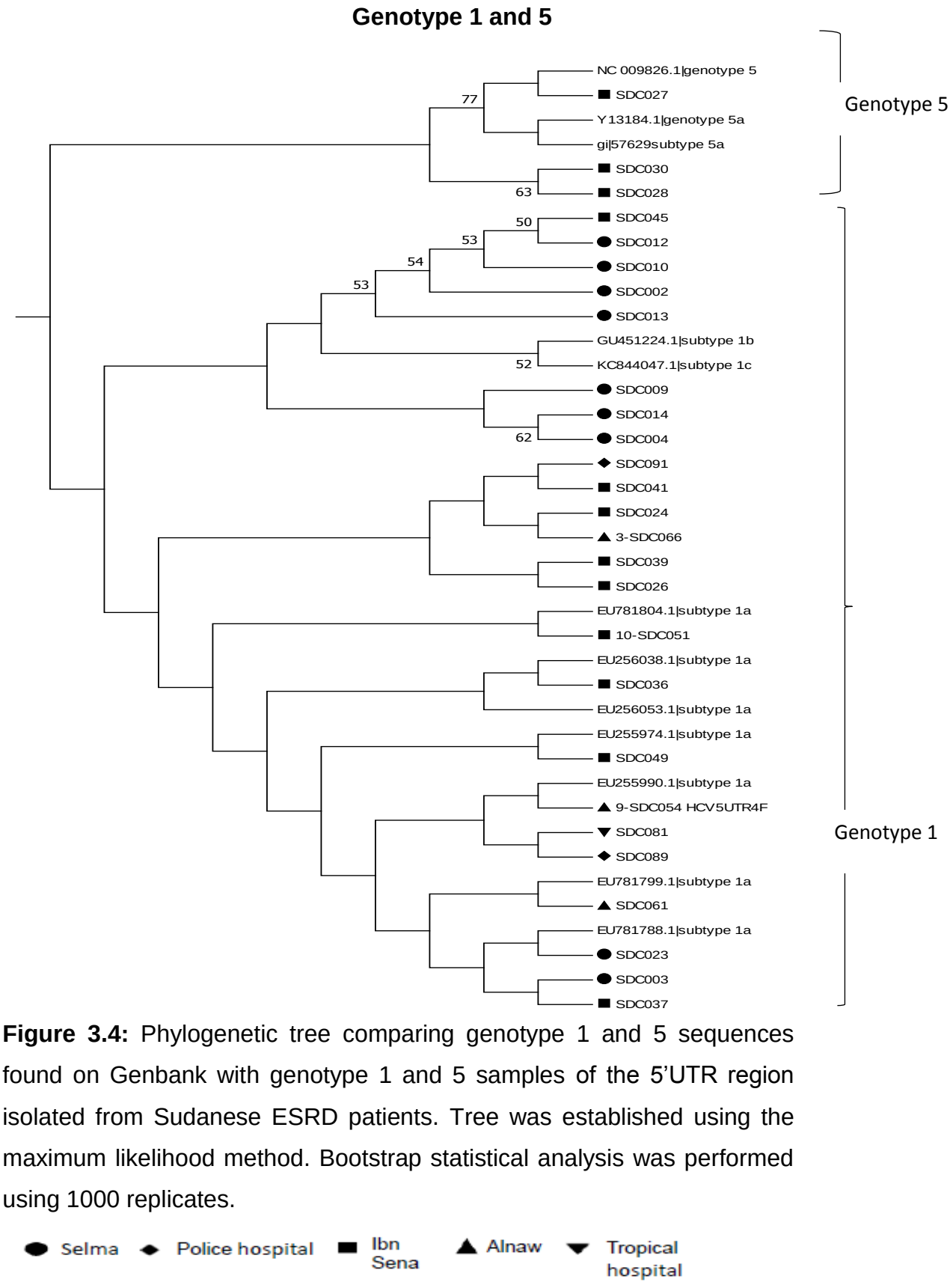


Figure 3.4: Phylogenetic tree comparing genotype 1 and 5 sequences found on Genbank with genotype 1 and 5 samples of the 5'UTR region isolated from Sudanese ESRD patients. Tree was established using the maximum likelihood method. Bootstrap statistical analysis was performed using 1000 replicates.

Genotype 3

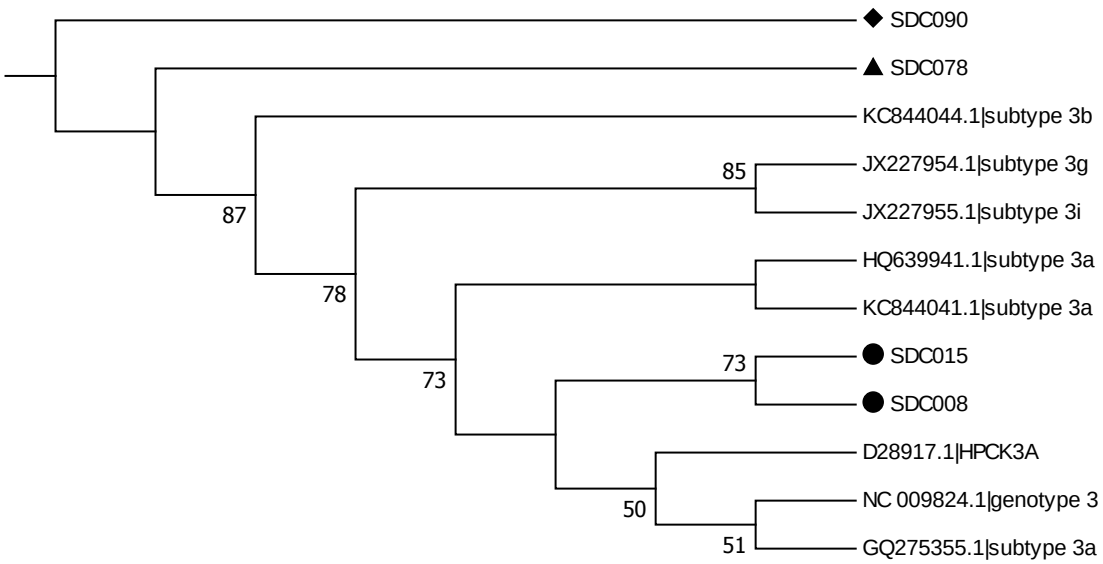
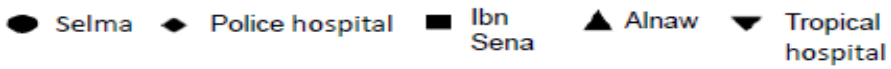


Figure 3.5: Phylogenetic tree comparing genotype 3 sequences found on Genbank with genotype 3 samples of the 5'UTR region isolated from Sudanese ESRD patients. Tree was established using the maximum likelihood method. Bootstrap statistical analysis was performed using 1000 replicates.



Genotype 4

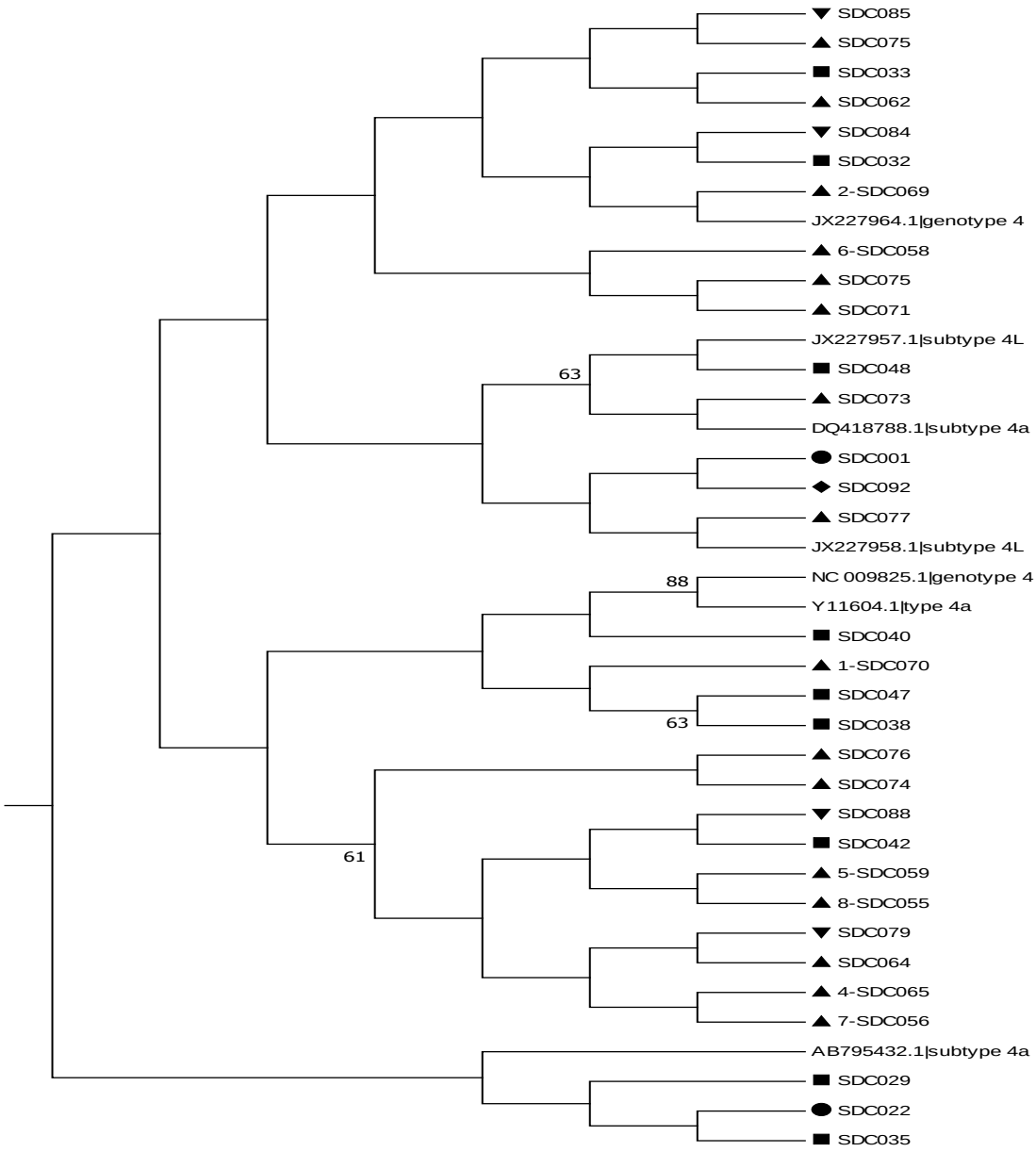


Figure 3.6: Phylogenetic tree comparing genotype 4 sequences found on Genbank with genotype 4 samples of the 5'UTR region isolated from Sudanese ESRD patients. Tree was established using the maximum likelihood method. Bootstrap statistical analysis was performed using 1000 replicates.

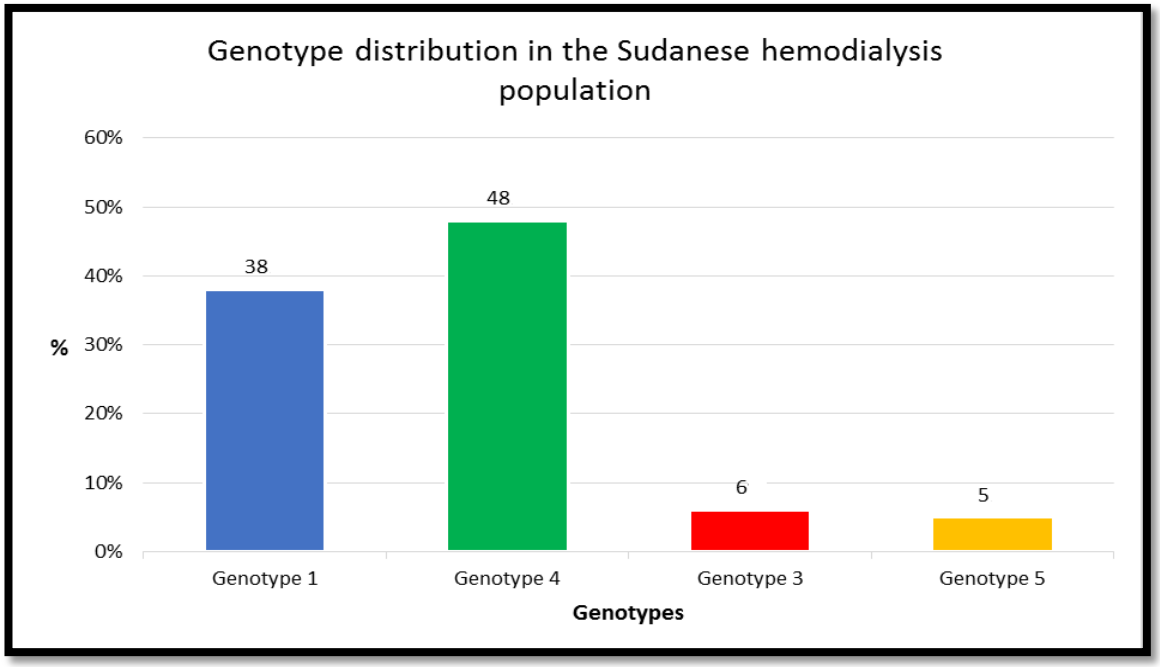


Figure 3.7: Frequency of genotypes as per 5'UTR region amplification and sequencing.

3.3.2 Distribution of genotypes across dialysis centers.

The HCV genotype distribution across all dialysis centers was analysed. A variety of genotypes was detected across dialysis centers (Figure 3.8). Genotypes 1 and 4 were were found in all centers. Patients that attended their haemodialysis treatment sessions in Selma were observed to have been infected with genotypes 1, 3 and 5. A total of 9 (69%) of these patients were infected with genotype 1 making this genotype the most prevalent genotype in Selma. Genotype 4 and genotype 3 were observed in lower percentages (15%) in this dialysis center.

Patients attending dialysis treatment in Ibn Sena were found to be infected with genotypes 1, 4 and 5. Similar percentages of genotypes 1 and 4 were observed in Ibn Sena. Only 3 (14%) patients were infected with genotype 5. Dialysis center Alnaw had the highest prevalence of genotype 4

observed in the entire study. Sixteen (80%) of the patients in this center were infected with genotype 4. Apart from the high prevalence of genotype 4 in Alnaw, genotypes 1 and 3 were observed but in lower percentages.

Unlike Alnaw, Ibn Sena and Selma, Tropical hospital and Police hospital had fewer patients found to be infected with HCV. Of these patients three genotypes (1, 4 and 3) were observed. Four patients from Tropical hospital were infected with genotype 4 while only 1 was infected with genotype 1. Police hospital had 2 patients infected with genotype 1 and another 2 patients infected with genotypes 4 and 3 each.

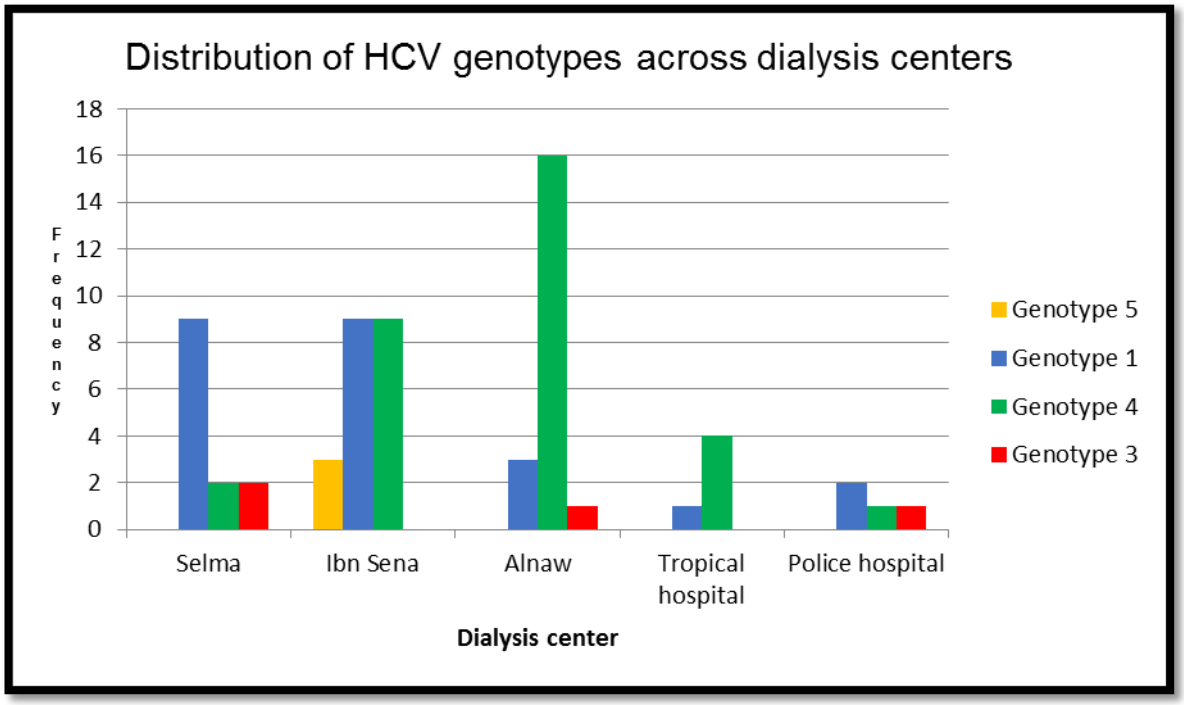


Figure 3.8: Distribution of HCV genotypes in the different dialysis centers.

3.3.3 The relationship between baseline characteristics of the participants and genotype distribution

The relationship between baseline characteristics of the participants and genotype distribution was assessed. Genotypes determined from the phylogenetic analysis of the 5'UTR region sequences were used. The majority of the participants were male N=42 (76%) across all genotypes, were diagnosed with HCV after they had started haemodialysis treatment and had been undergoing haemodialysis for more than a year.

As indicated in (Table 3.1) there was a statistically significant relationship between genotypes and duration of haemodialysis treatment. Ninety-four percent (94%) of patients infected with genotype 4 were observed to have been on haemodialysis treatment for less than a year while majority of the patients infected with genotype 1 had been on haemodialysis treatment for more than a year. The likelihood ratio of duration of haemodialysis treatment having an influence in the genotypes observed was found to be 13.668, p=0.034. Phi and Crammer's V tests were used to determine the level of association (Cramer, 1946, Liebetrau, 1983). Duration of haemodialysis has been observed to have a small to medium effect on influencing the possible genotypes.

Another statistically significant relationship was observed between the genotypes and diaysis centers. The frequency of genotype 4 was statistically significantly higher (50%, $p<0.05$) in Alnaw as compared to the other centers. There was no statistically significant relationship between gender, age, number of blood transfusions, the diagnosis of chronic HCV (before or after dialysis), number of centers attended, ALT levels and HCV viral loads.

Table 3.1: Baseline characteristics of ESRD patients relative to HCV genotypes

	Genotype 1 (n=26)	**Genotype 4 (n=31)**	**Genotype 3 (n=4)**	**Genotype 5 (n=3)**	***P* value**	**Degree of effect**
Age (years)	52.4 (±11.2)	50.2 (±15.6)	57.2 (±10.8)	42.7 (±9.2)	0.117	
Gender					0.721	
Male	19 (73%)	19 (61%)	2 (50%)	2 (67%)		
Female	7 (27%)	12 (39%)	2 (50%)	1 (33%)		
Dialysis centers					0.01**	0.02
Selma	9 (38%)	2 (6%)	2 (50%)			
Alnaw	3 (12%)	16 (48%)	1 (25%)			
Ibn Sena	9 (38%)	9 (29%)		3 (100%)		
Tropical hospital	1 (4%)	4 (13%)				
Police hospital	2 (8%)	1 (3%)	1 (25%)			
Years on haemodialysis treatment					0.034**	0.056
<1	1 (4%)	29 (94%)	4 (100%)	2 (67%)		
1-10	15 (57%)	2 (6%)		1 (33%)		
>10	10 (38%)					

No. of dialysis centers attended					0.340
1	15 (58%)	11 (35%)	2 (50%)	2 (67%)	
>1	11 (42%)	20 (65%)	2 (50%)	1 (33%)	
No. of blood transfusions received					0.766
None	8 (31%)	10 (32%)	1 (25%)		
1	7 (26%)	11 (35%)	1 (25%)	1 (33%)	
>1	11 (42%)	10 (32%)	2 (50%)	2 (67%)	
Diagnosis of Chronic HCV					0.197
Before dialysis	2 (8%)	4 (13%)	2 (50%)	1 (33%)	
After dialysis	24 (98%)	27 (87%)	2 (50%)	2 (67%)	
ALT LEVELS (U/L)	20.9 (11-125.6)	20.9 (8.1-25.8)	25.2 (19.9-30.5)	27.4 (22.9-30)	0.165
Viral load (log copies per µl)	2.40	2.39	2.40	2.39	0.201

***p*-value <0.05, statistically significant.

3.3.4 Subtyping using the NS5B region

To determine subtypes of HCV present in the Sudanese haemodialysis population, the highly variable NS5B region (Sabahi, 2009) was amplified and phylogenetic analysis of the region was performed using MEGA7 (Figure 3.9). The genetically diverse NS5B region showed high variability in subtypes observed. HCV subtypes 1a, 1b, 1e, 3a, 4a, HCV isolates present, the majority belonged to subtype 1a (36.36%) followed by subtype 4a (29.55%). One sample that was classified as genotype 5 in the 5'UTR region was found to fall under genotype 1a in the NS5B region.

Six (11%) of the sequences observed in the study could not be categorized in terms of subtypes. This group did not cluster with any known subtypes following phylogenetic analysis. Interesting enough this group of sequences had a bootstrap value of 100 between them. Subtypes 1b, 1e, 3a, 4b, 4v, 4o and 4t were found at a lower frequency compared to subtypes 1a, 4a and the unspecified subtypes (Figure 3.13).

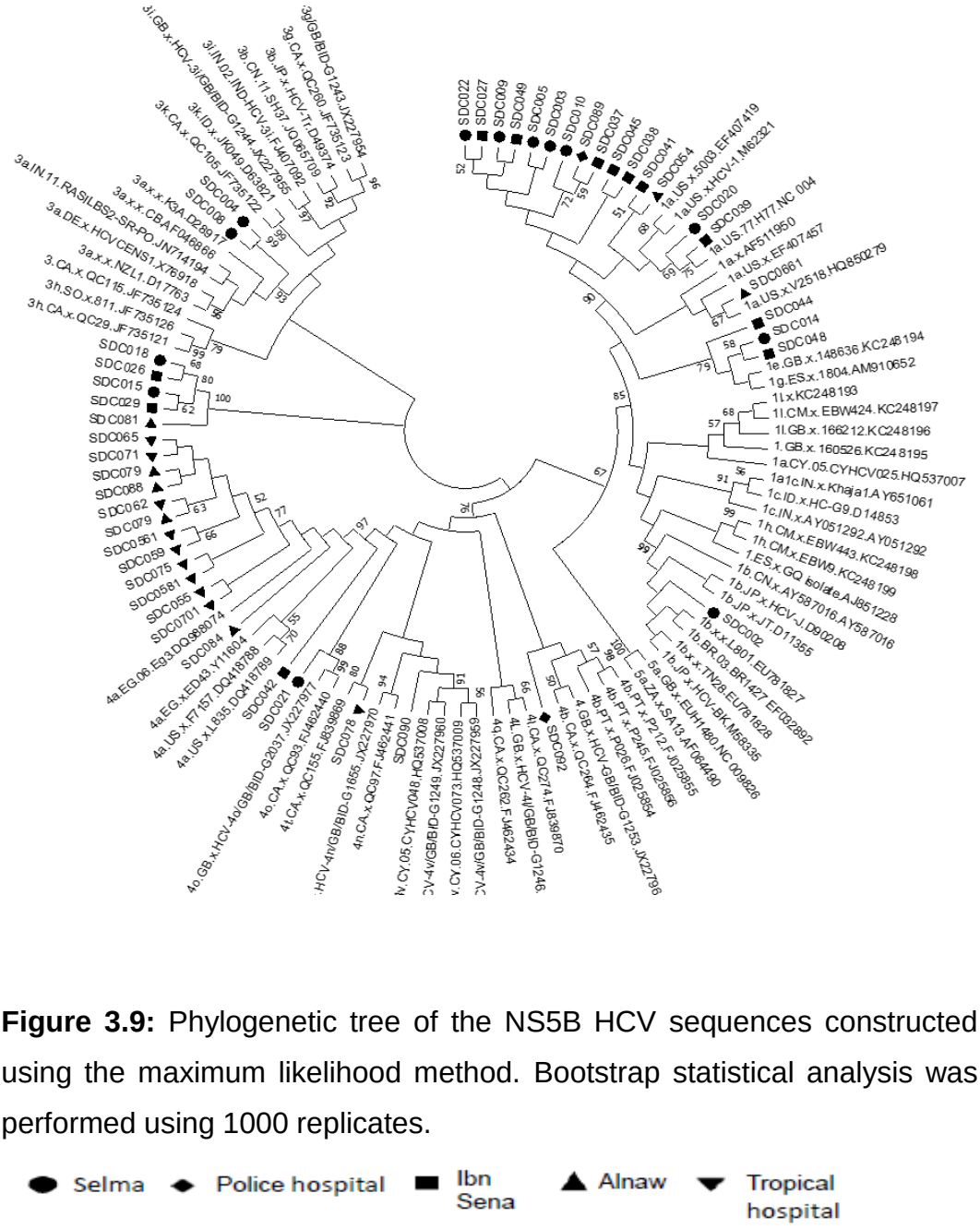


Figure 3.9: Phylogenetic tree of the NS5B HCV sequences constructed using the maximum likelihood method. Bootstrap statistical analysis was performed using 1000 replicates.

Genotype 1

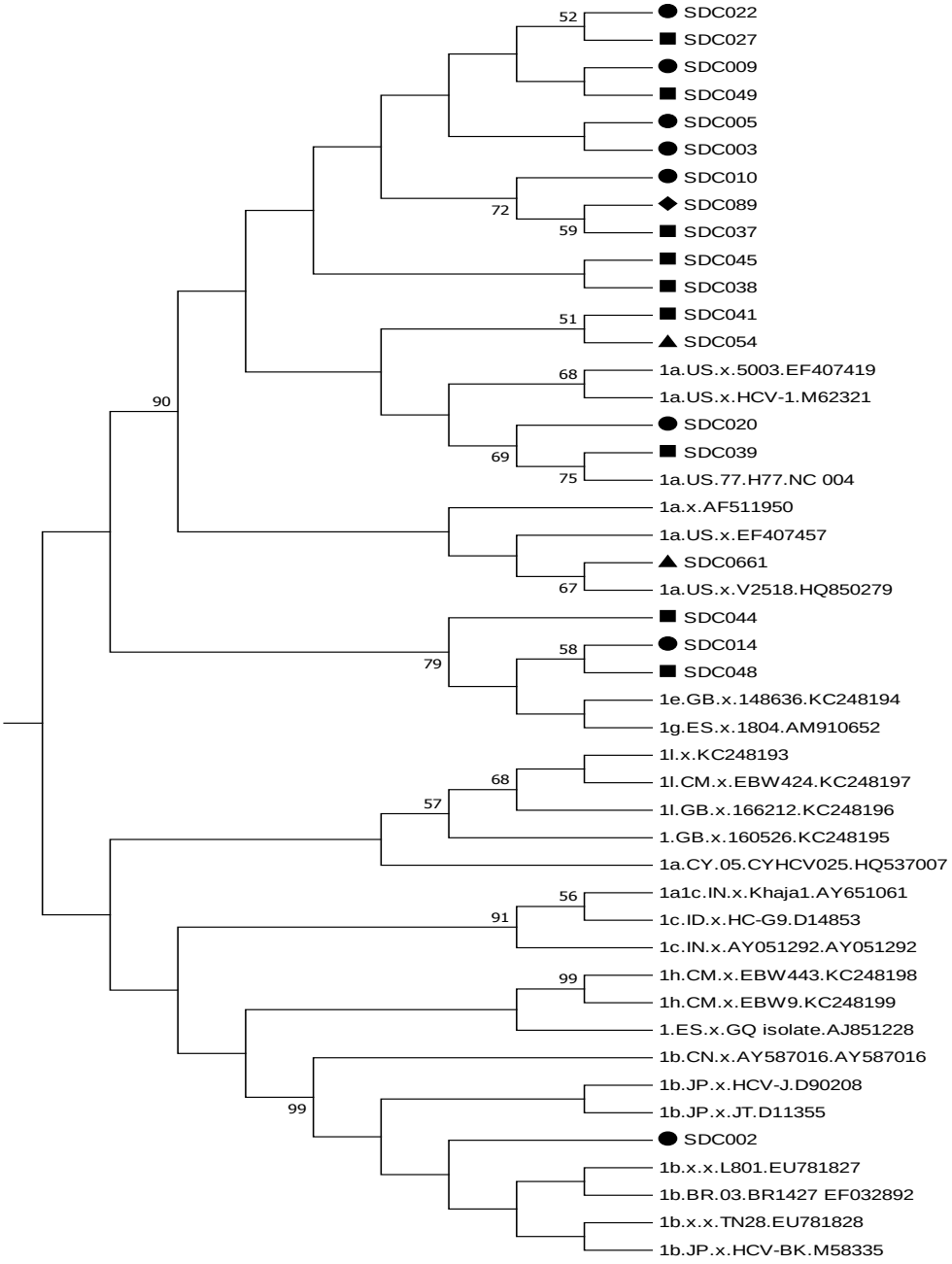


Figure 3.10: Phylogenetic tree of the NS5B HCV sequences comparing genotype 1 strains isolated from Sudanese ESRD patients to genotype 1 sequences deposited in Genbank. Tree was established using the maximum likelihood method. Bootstrap statistical analysis was performed using 1000 replicates.

Genotype 3

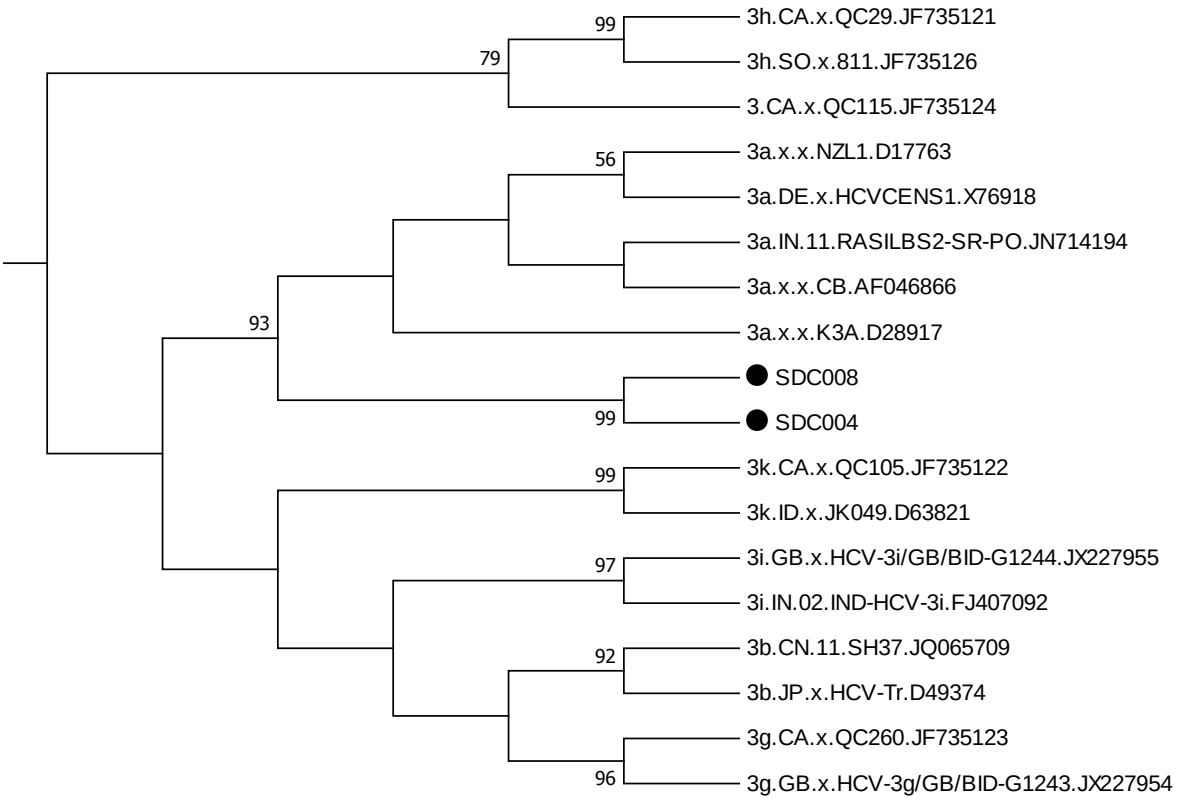


Figure 3.11: Phylogenetic tree of the NS5B HCV sequences comparing genotype 3 strains isolated from Sudanese ESRD patients to genotype 3 sequences deposited in Genbank. Tree was established using the maximum likelihood method. Bootstrap statistical analysis was performed using 1000 replicates.

● Selma

Genotype 4

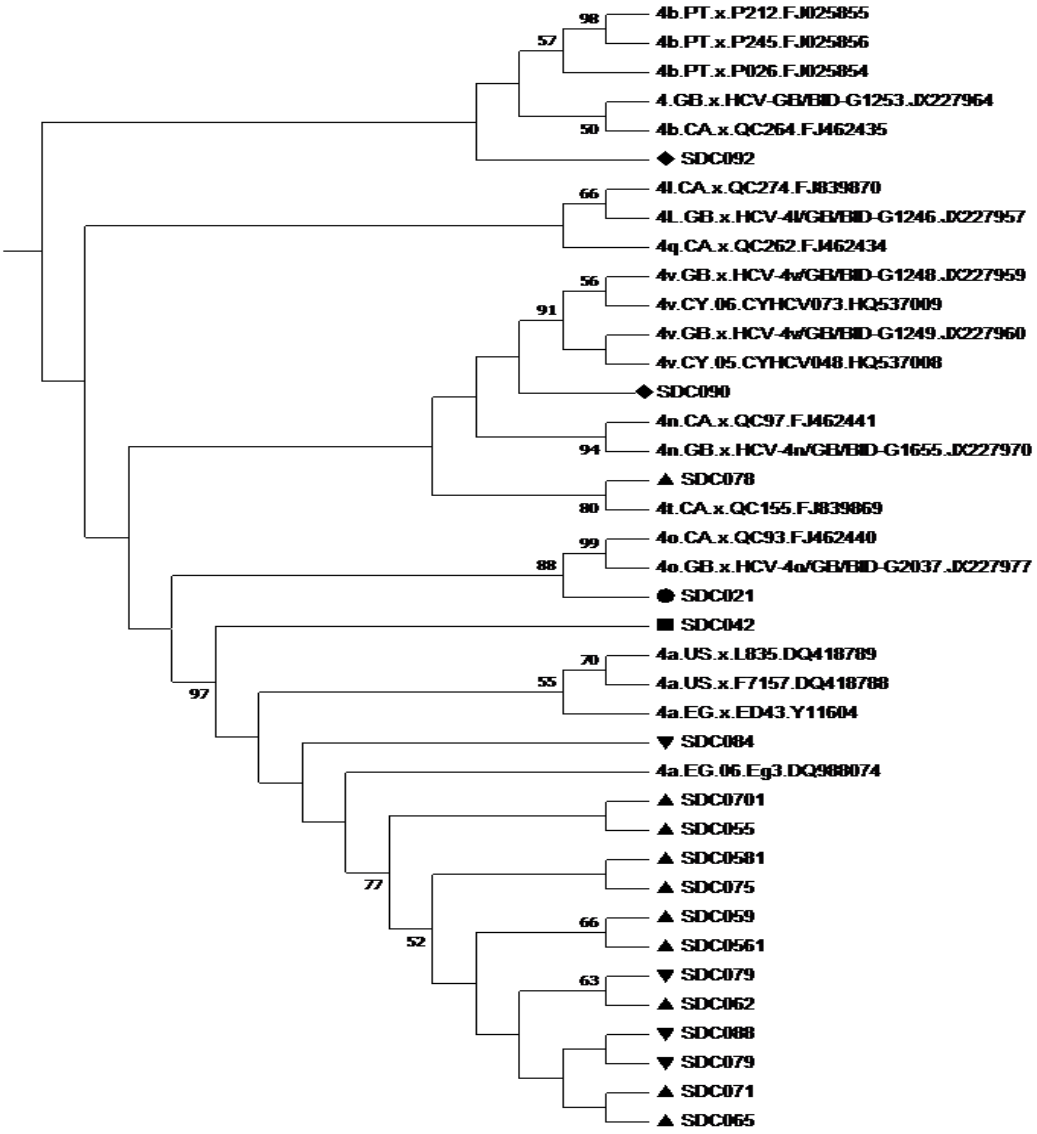


Figure 3.12: Phylogenetic tree of the NS5B HCV sequences comparing genotype 4 strains isolated from Sudanese ESRD patients to genotype 4 sequences deposited in Genbank. Tree was established using the maximum likelihood method. Bootstrap statistical analysis was performed using 1000 replicates.

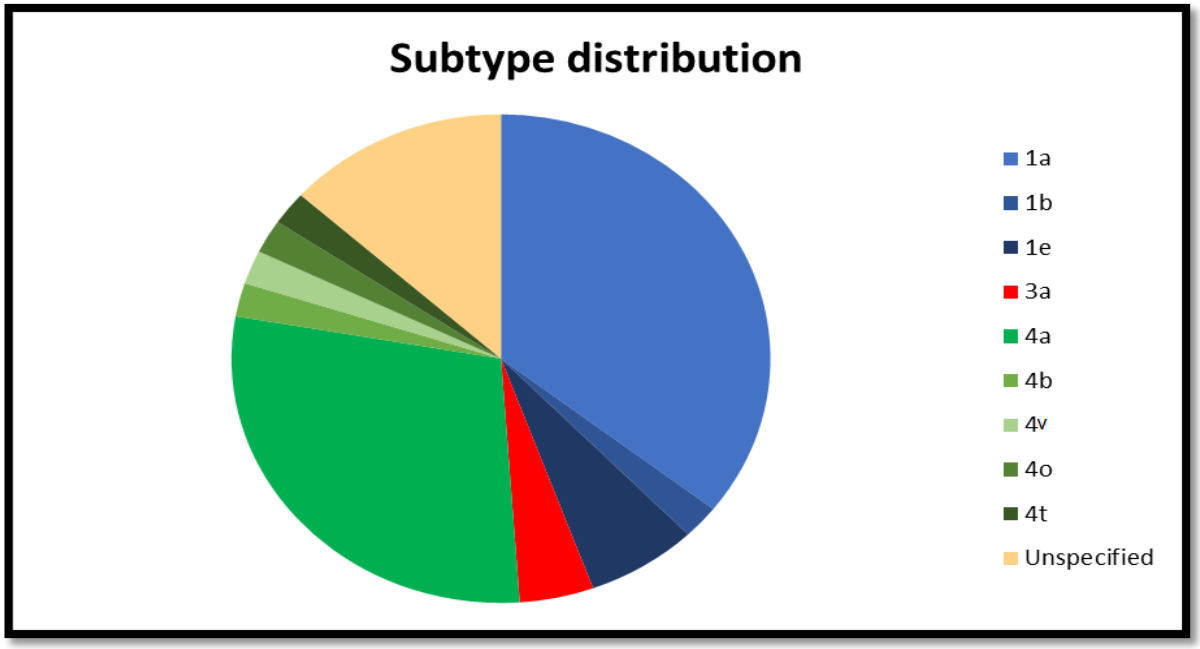


Figure 3.13: Subtype distribution in the haemodialysis population (n = 44).

3.3.5 Distribution of HCV subtypes in haemodialyis centers (NS5B region)

Across the haemodialysis centers a variability of subtypes was observed. Dialysis center Alnaw had majority of the genotypes 4a observed in the study, followed by Tropical hospital. To a lesser extent subtypes 1a and 4t were also observed in dialysis center Alnaw. Subtype 1a was more prevalent in Ibn Sena and Selma with Ibn Sena having the highest percentage (44%) of subtype 1a. As mentioned earlier, the frequency of HCV positive patients in Tropical hospital and Police hospital was very low. Even though this may be the case, three subtypes (1a, 4b and 4v) were observed in Police hospital.

As compared to the other haemodialysis centers, Selma showed a high degree of variability in the distribution of HCV subtypes. Subtypes 1a, 1b, 1e, 3a, 4o and unspecified subtype were observed in Selma. The majority of the sequences in this center were found to be related to subtype 1a. The unspecified subtypes in the study were distributed across three haemodialysis centers. Four patients, 2 (SDC026 and SDC029) in Ibn Sena and 2 (SDC015 and SDC018) in Selma were infected with the unspecified genotype. Another patient infected with the unspecified genotype was receiving haemodialysis treatment in Tropical hospital.

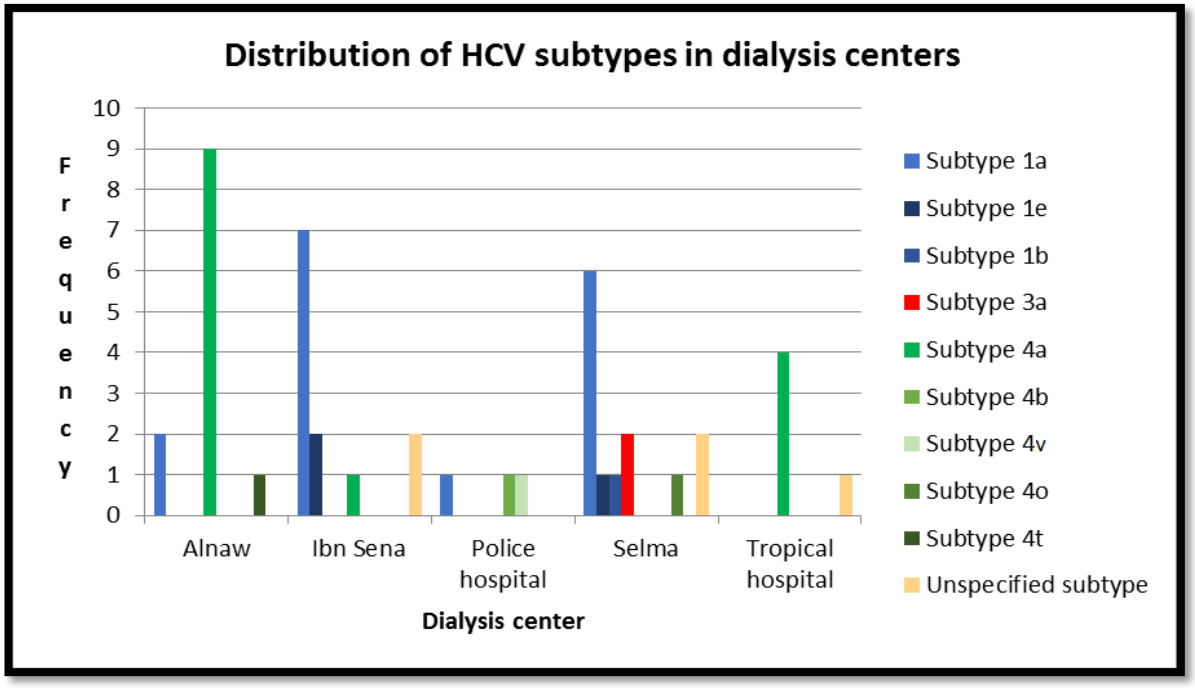


Figure 3.14: Subtype distribution across the haemodialysis centers.

3.4 Quantification of HCV using real-time PCR

The viral load of patients in the haemodialysis units in Sudan was determined using real-time PCR. Statistical analysis to define the level of association between viral loads and the dialysis centers attended, participant age, gender, time spent on dialysis treatment, frequency of blood transfusions, diagnosis of chronic HCV and ALT levels was performed using Chi-square analysis. Variables with p-value less than 0.05 were considered statistically significant. Two variables (diagnosis of chronic HCV and ALT levels) could not comply with Chi-square analysis assumptions thus Fisher's exact test was used for the analysis.

Viral loads could be determined using real-time PCR for 66% of the samples tested (61/93). However, the viral loads for 24/61 fell below the assay's detection limit and were thus excluded from further analysis. The

total of 37/93 (40%) samples had HCV viral loads ranging from 2.95 x 10^3-4.78 x 10^6 IU/ml, which fell within the linear quantification range of the in house assay. Average viral loads between dialysis centers were found to be 3.71 x 10^4 IU/ml in Selma, 2.75 x 10^4 IU/ml in Alnaw, 2.63 x 10^4 IU/ml in Ibn Sena, 3.02 x 10^4 IU/ml in Tropical hospital and 1.48 x 10^4 IU/ml in Police hospital. Low viral loads were detected in the current study (Table 3.2). Viral loads between genotypes were analysed. Genotype 1 samples had an average of 2.95 x 10^4 IU/ml while genotype 4 samples were found with an average of 2.63 x 10^4 IU/ml, $p>0.05$. There was no statistically significant relationship between viral loads and the dialysis centers attended, age, gender, time on haemodialysis, when HCV was diagnosed and ALT levels.

Table 3.2: Summary of the viral loads observed in dialysis centres and genotypes.

	N	Viral load (IU/ml)*
Dialysis centre		
Selma	8	3.71 x 10^4 ± 8.71
Alnaw	13	2.75 x 10^4 ± 5.89
Ibn sena	9	2.63 x 10^4 ± 5.13
Tropical hospital	4	3.02 x 10^4 ± 15.14
Police hospital	3	1.48 x 10^4 ± 1.17
Genotypes		
Genotype 1	14	2.95 x 10^4 ± 6.17
Genotype 4	18	2.63 x 10^4 ± 4.47

*average ± standard deviation

A statistically significant relationship was observed between HCV viral loads and the number of centers a patient attended to receive

haemodialysis treatment. High viral loads were associated with receiving haemodialysis treatment from more than one center while low viral loads were observed in patients who only attended one center for haemodialysis treatment. Another statistically significant relationship ($p<0.05$) was observed between the number of blood transfusions received and HCV viral loads in the study. Low viral loads were observed in patients who have had more than 1 blood transfusion as compared to patients who only received blood transfusion once (Table 3.3).

Table 3.3: Baseline characteristics of HCV viral loads in haemodialysis centers

	Chi-square	**Degree of freedom**	***P* value**
Dialysis center	3.578	4	0.466
Age	0.884	2	0.643
Gender	0.208	1	0.648
Time on haemodialysis	1.807	2	0.405
Number of centers attended	3.613	1	0.057**
Frequency of blood transfusions	10.165	2	0.006**
Diagnosis of chronic HCV*			0.705
ALT levels*			0.594

- *cells did not comply with Chi-square assumptions thus Fishers exact test was used. ***p*-value <0.05, statistically significant.

Chapter 4 : Discussion

Despite good clinical practices employed in dialysis centers to eliminate nosocomial infections, the prevalence of HCV in these centers continues to be high. On a global scale, HCV prevalence in haemodialysis populations can vary from 6.1-23.7% (Hammad et al., 2016b, Gasim et al., 2012, El-Amin et al., 2007). In the present study, ninety-three (17%) of the 548 patients tested were reactive for anti-HCV. A few studies have been conducted to determine the prevalence of HCV in haemodialysis populations in Sudan. The 17% seroprevalence of HCV observed in the current study was lower than that previously found by an earlier study (23.7%) (El-Amin et al., 2007) and higher than that observed by a subsequent one (8.5%) (Gasim et al., 2012). These three studies demonstrate a high prevalence of HCV sero-positivity in haemodialysis centers in Khartoum.

Approximately 1.5% of the study population had HCV with occult HBV. Occult HBV infection is defined as the presence of HBV DNA with the absence of HBsAg in serum (Said, 2011, Raimondo et al., 2008). Occult HBV in HCV infected patients is one of the many factors that needs attention as it may have an effect on disease progression. It may play a role in accelerating the development of cirrhosis in HCV/Occult HBV co-infected patients and leading to the development of hepatocellular carcinoma (Cacciola et al., 1999).

RNA, from HCV antibody positive sera collected between December 2014 and January 2016 in 6 haemodialysis centres in Sudan, was extracted and subjected to reverse transcription and nested PCR. The DNA was amplified in the 5’UTR and NS5B regions. Amplification of the 5’UTR and NS5B region produced different results. More positives were detected in

the 5'UTR region than the NS5B region. The highly conserved nature of the 5'UTR compared to the more variable NS5B region (Han et al., 1991) could be the reason for the difference in the nested PCR amplification results. In addition, the NS5B region is closer to the 3' end of HCV RNA, which is more prone to RNA degradation. These could be the two contributing factors for the poor amplification of the NS5B region. Although, the NS5B is a relatively difficult region to amplify, it enabled us to determine the HCV subtypes circulating in the haemodialysis centers in Sudan.

Only 28/40 (70%) of the analysed sequences had identical genotype results in both the NS5B and 5'UTR. Phylogenetic analysis of the two regions using the maximum likelihood detected the presence of genotypes 1, 3, 4 and 5. As expected the presence of genotype 3 was found in lower frequencies (Messina et al., 2015). Previous studies have documented the presence of genotype 3 in Egypt and some parts of Central Africa (Karoney and Siika, 2013). Similar results were observed in a study conducted in the Middle East and North Africa where the presence of genotypes 1, 2, 3 and 4 in the haemodialysis population was observed (Harfouche et al., 2017). The high prevalence of genotype 1 in the current study agrees with previous results that genotype 1 predominates in dialysis patients in other regions of the world (Khedmat et al., 2014).

In the current study, following phylogenetic analysis of the 5'UTR region, the presence of genotype 5a was observed. Genotype 5a predominates in South Africa (Smuts and Kannemeyer, 1995) and it is unlikely that this genotype was observed in the Sudanese population and the presence of genotype 5 in this population can be explained by the following possibilities. Firstly, the introduction of genotype 5a in the Sudanese population may have been brought about by infected travellers coming

from South Africa to Sudan. Secondly, the genotype observed as genotype 5a might not be in fact genotype 5a since following phylogenetic analysis of the NS5B region the isolate that was genotype 5a in the 5'UTR region clustered with genotype 1a samples in the NS5B region.

The conserved nature of the 5'UTR region may have a role to play in regard to the two regions showing different genotypes when the sample is isolated. The 5'UTR region have previously been documented to have 95-100% similarity across all genotypes and 96.2-96.5% between genotype 1a and 5a (Cha et al., 1992). Lastly, there is a possibility of a recombinant or mixed infection. Researchers have previously documented recombinants during sequencing of the 5'UTR and NS5B regions (Morel et al., 2011).

There are several risk factors associated with the transmission of HCV in dialysis centres. These factors include: the time spent on dialysis (Otedo et al., 2003, Mohamed, 2010), dialysis in multiple centres (Carneiro et al., 2001), blood transfusion (Abacioglu et al., 2000), patients undergoing dialysis on the same shift and inability of the nurses to change gloves in emergency situations (Abacioglu et al., 2000).

The current analysis displayed that majority of the patients may have contracted HCV after starting haemodialysis. Previous studies have demonstrated that detecting the presence of the virus using HCV antibody and liver enzymes may produce negative results in the presence of the virus (Fissell et al., 2004, Hanuka et al., 2002, Seelig et al., 1994). Thus this is a possible explanation for not detecting HCV infection. Thus, routine testing for HCV is recommended, especially the use of nucleic acid testing, which differentiates active from inactive HCV infection. None of the

patients who were positive for HCV had a history of intravenous drug abuse and other modes of transmission were not implicated.

There was a significant difference between HCV genotypes observed with the duration of haemodialysis. The number of years spent on haemodialysis is a risk factor for HCV transmission (Otedo et al., 2003, Mohamed, 2010). Patients who stay long in haemodialysis treatment are found to have a high risk in contracting HCV as compared to patients who only have been on haemodialysis for a short period of time (Fissell et al., 2004, Mohamed, 2010).

The current analysis demonstrated a significant relationship between the dialysis centre attended and genotypes present. The uneven distribution of genotypes in the dialysis centres exhibits a sign for a possible nosocomial infection. Genotypes 1 and 4 were detected in all the haemodialysis centers but none of the centers had an even distribution of the two genotypes. Dialysis unit Selma had more genotype 1 variants while dialysis centre Alnaw had highest frequency of genotype 4. The former factors display a great need for stringent measures to be put in place to eliminate the transmission of HCV in haemodialysis centers. The genotypes detected in the study were similar to those previously found in the general population. Previously the predominance of HCV genotype 4 was detected in the Sudanese general population (Mudawi et al., 2007a).

Subtyping using the NS5B region showed the prevalence of subtypes 1a, 1b, 1g, 3a, 4a, 4b, 4v, 4o and 4t. Another set of subtypes produced by phylogenetic analysis of the NS5B region did not cluster with any known genotypes. Bootstraps analysis (1000 replicates) of these sequences showed a bootstrap value as high as 100 between these sequences.

Patients which had “unspecified” genotypes were found to be patients who had more than 4 years’ duration on haemodialysis treatment. At the time of sample collection, the patients had attended more than 1 dialysis centre, had received HBV vaccination in the last 2 years and they were from similar original residence area. The above observation calls for control measures to be put in place as dialysis units may be the source of HCV transmission.

We cannot confirm if the group of HCV isolates, which could not cluster into any known genotypes belong to a new HCV clade. The unspecified samples do not comply with all assumptions to be labelled as a new genotype. For sequences to be labelled as a new genotype then the sequence needs to be fully coding and cover the complete genome, isolated from at least three epidemiologically distinct individuals and must not be a recombinant (Simmonds et al., 2005).

In our case the sequences were only amplified in the NS5B region and were all from the same area in Sudan, this may bring us back to the possibilities of nosocomial infection. Another assumption for a sequence to be considered a new subtype is that the sequence should form a different clade away from all the known available sequences and that it should be different by at least 15% (Simmonds et al., 2005). Sequence similarities test was executed against consensus sequences using the Bio-Edit software. The unspecified genotypes were 40% related to the H77 genotype 1a and 87-99% related to each other. The possibilities of these groups of patients to be infecting each other with the same strain of HCV were high.

Low viral loads were detected during quantification using real-time PCR assay. Haemodialysis patients are often found to have decreased levels of HCV RNA. The HCV particles found in the blood of haemodialysis patients are unable to pass through the filter membrane thus they are adsorbed onto the inner surface of the filter membrane. Some of the particles that cannot pass through the membrane are destroyed by the pressure applied to the blood during haemodialysis. Although this may be the case, the dialysis treatment does not clear the virus but can result in decreased HCV RNA levels detected (Fabrizi et al., 2000, Furusyo et al., 2000, Khedmat et al., 2014)

There was no significant difference between HCV viral load and ALT levels. Our results were in agreement to that of others in that the ALT levels of patients in haemodialysis treatment were normal (Furusyo et al., 2000, Silini et al., 1993). The frequency of blood transfusions received and number of centers attended for the haemodialysis treatment were statistically significant when measured against viral loads detected in the study. Patients who attended only 1 center for their dialysis treatment were found with significantly high viral loads as compared to patients who attended more than 1 center during their treatment period. This could be due to different viral replication efficiency between different genotypes as patients who attended more than one dialysis center were predominantly found to be genotype 4. For example, patients infected with genotype 4 usually have lower viral loads than genotype 1 (Mellor et al., 1999). Another significant relationship was observed between viral loads and frequency of blood transfusions. Low viral loads were detected in patients who have had blood transfusions more than 5 times.

There are a number of limitations in the current study: 24 patients had HCV cDNA detected with the value below the lower quantification limit.

This could be due to the in house real-time PCR assay was not sensitive enough to detect the viral loads below 1.86 x 10^3 IU/ml and poor storage and handling of samples during specimen collection and/or transportation may have contributed to RNA degradation.

Chapter 5 : Conclusion

In conclusion, the study demonstrated a relatively high prevalence of HCV in the Sudanese haemodialysis population, which was higher than the prevalence in the general population. The high prevalence of HCV observed in this population made further analysis necessary to determine whether there was a possible nosocomial infection.

The study had various factors that showed evidence of a nosocomial infection in these haemodialysis treatment centers. The genotype distributions differed across the centers. Alnaw had the highest frequency of genotypes 4 while majority of the genotype 1 strains were coming from Selma. The uneven distribution of the genotypes indicates that the patients may be infecting each other with the same strains of HCV. An interesting observation made in the study was the association between years spent on haemodialysis and the genotypes detected. The majority of the patients who have been on haemodialysis for less than a year were infected with genotype 4 while those who have been on haemodialysis for more than a year were mainly infected with genotype 1.

The phylogenetic trees constructed showed sequences of patients coming from the same haemodialysis treatment centres clustered together. These analyses demonstrated a possibility of a nosocomial infection. Apart from the sequences, which were related to genotypes 1 and 4, we observed the presence of genotype 3, which is commonly found in South Asia (Messina et al., 2015). Moreover, the two samples, which were infected with subtype 3a had a high (99) bootstrap support. These two patients were attending haemodialysis treatment at the same center and thus this may be a sign of a nosocomial infection. We were unable to classify one group of sequences into any known genotypes. Although we cannot confirm the possibility of a new genotype detected, we can highlight a possibility of a nosocomial infection and/or recombination.

Since currently there is no vaccine for HCV, stringent measures are needed to be put in place to prevent the transmission of HCV in haemodialysis centers. We recommend before starting haemodialysis all patients should be tested for HCV and constant testing should be done thereafter. For patients who may have been found to be infected with HCV we recommend that they be isolated during haemodialysis treatment. Genotyping of HCV in patients already infected in the haemodialysis population is also recommended. This will enable efficient treatment with DAAs in the population. Early diagnosis of the HCV infection in the haemodialysis population and treating these patients with DAAs is recommended as this may limit the spread of nosocomial infections.

There may be an underestimation of HCV in haemodialysis populations. This may be caused by the low viral loads detected in the population. Thus we recommend that when testing for HCV, HCV RNA detection test should also be included as testing for antibody cannot detect active infection.

In order to differentiate between mixed genotype infection or HCV recombinant, one would require the amplification of full HCV genome and cloning of the amplicon. The full length HCV genome will be sequenced using Sanger‘s sequencing method. Sequences will be analysed using SimPlot to detect recombinant strains. Full genome sequencing may also be able to characterize the isolates that were classified as “unspecified” genotypes in the present study.

Chapter 6 : Appendices

Appendix A: Republic of Sudan ethical clearance certificate

Republic of Sudan
Federal Ministry of Health

HEALTH RESEARCH COUNCIL

NATIONAL RESEARCH ETHICS REVIEW COMMITTEE

Date: 19 12/ 2017

Ethical Clearance Certificate

This is to certify that the proposal (No. 3 -10-2016) entitled (Characterization of hepatitis B virus and hepatitis C virus genotypes in Sudan) submitted by Prof. Hatim Mohamed Yousif Mudawi from Faculty of Medicine, University of Khartoum has been approved by the National Health Research Ethics Committee, Federal Ministry of Health to be conducted in Khartoum State - Sudan.

Dr. Iman Abdalla Mustafa
Reporter of the
National Research Ethics Review Committee

Appendix B: Human research ethics committee (Medical) clearance certificate no. M150158

R14/49 Professor Anna Kramvis

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150158

NAME: (Principal Investigator)	Professor Anna Kramvis
DEPARTMENT:	Internal Medicine Medical School
PROJECT TITLE:	Phylogenetic Analyses of Hepatits Viruses Found in Africa Relative to those Derived from other Geographic Regions of the World (Previously 050321) (Previoulsy M10471)
DATE CONSIDERED:	30/01/2015
DECISION:	Approved unconditionally
CONDITIONS:	Renewal Approved for the Period01 Jan 2015 -31 December 2020
SUPERVISOR:	Prof M Kew

APPROVED BY: Professor P Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 30/09/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C: Human research ethics committee (Medical) clearance certificate No. M170165

R14/49 Trodia Zitha

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170165

NAME: (Principal Investigator)	Trodia Zitha
DEPARTMENT:	Internal Medicine Hepatitis Virus Diversity Research Unit
PROJECT TITLE:	Molecular Characterization and Genotyping of Hepatitis C Virus from Sudanese End-Stage Renal Disease Patients
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	A Sub-study under Primary Study M150158 Prof A. Kramvis
SUPERVISOR:	Prof Anna Kramvis
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	15/02/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in January and will therefore be due in the month of January each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix D: Solutions and Composition of reactions

Reagents	Composition

D1. Luria-Bertini broth Agar plates with ampicilin

15 g agar

10 g Tryptone

5 g yeast extract

10 g NaCl

1 ml (50mg/ml) ampicillin

15 g of agar was added to the LB medium and finally adjusted to 1L. LB agar solution was autoclaved and allowed to cool down to 50°C to avoid agar to solidify. 1 ml of 50 mg/ml ampicillin was added to the liquid. The liquid was poured into sterile petri-dishes and allowed to solidify. Once the agar has solidified the plates were inverted and covered in foil to avoid light contact and stored in 4°C before usage.

D2. Luria-Bertini broth Liquid medium

10 g Tryptone

5 g Yeast Extract

10 g NaCl

900 ml distilled water

10 g Tryptone, 5 g Yeast extract and 10 g NaCl was dissolved in 900 ml distilled water and adjusted to final volume of 1000 ml. The liquid was autoclaved for 30 minute, cool down and was stored at 4°C.

D3. Ampicillin (50mg/ml)

1 g ampicillin

20 ml distilled water

1 g ampicillin was added to 20 ml distilled water. The solution was filter sterilized and stored at -20 °C.

D4. 1 % Agarose gel

1.5 g agarose

150 ml 1X TBE buffer

22.5 µl ethidium bromide

1.5 g agarose gel was dissolved in 150 ml 1X TBE buffer and the solution was heated in a microwave for the agarose to dissolve. The solution could cool down and 22.5 µl ethidium bromide was added. The solution was poured into gel trays and allowed to solidify.

D5. 10X TBE buffer

108 g Tris base

55 g Boric acid

9.3 g EDTA

After adding 108 g Tris base, 55 g Boric acid and 9.3 g EDTA, the volume was adjusted to 1000 ml by the addition of distilled water.

Appendix E: Protocols

E1 Bacterial Transformation

Before the chemical transformation can proceed, the heat block was set to 42°C, and S.O.C medium (Invitrogen, Carlsbad, CA, USA) warmed to room temperature. The LB agar plates containing 100mg/ml ampicillin (Appendix D1) were pre-warmed in a 37°C incubator for 30 minutes.

An aliquot 5 µl of the plasmid DNA encoding full length HCV genome pCV-H77 (HCV genotype 1a), pCNJ4C6S (HCV genotype 1b) and pJ6CF2a (HCV genotype 2a) (obtained from Prof R. Purcell, NIH, USA) was added directly to 50 µl of One Shot® cells (Invitrogen, Carlsbad, CA, USA). The reaction was gently mixed by tapping the vials, incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds, and placed on ice before the addition of S.O.C medium. An aliquot of 250 µl of the pre-warmed S.O.C medium was added to the vial, and placed on a shaker at a speed of 225 rpm for 1 hour at 37°C. Each of 50 µl and 200 µl recovered bacteria in SOC medium were spread on LB agar ampicillin plates individually and incubated at 37 °C overnight.

E2 Bacterial Culture

Briefly, a master mix 50 ml of LB medium (Appendix D2) containing 50 µl of 100 µg/ml ampicillin (Appendix D3) was prepared.

Each of the bacterial colonies containing the full-length HCV genome of genotype 1a (pCV- H77C) was picked from LB agar ampicillin plate and inoculated to a 50 ml tube containing 7 ml of LB medium with ampicillin. A tube without bacteria inoculation with LB and ampicillin was also included as negative control. Bacteria cultures were incubated at 37°C overnight in a shaking incubator (170 rpm).

When the bacteria reached mid-log phase, bacterial glycerol stock was prepared by adding 0.15 ml sterile glycerol to 0.85 ml of the bacterial culture. The mixture was gently vortexed and aliquoted into tubes of single use and stored at – 80°C for future usage.

E3 Primary bacterial culture

Primary bacterial culture was prepared by inoculating 50 µl of bacterial glycerol stock to the LB medium with ampicillin supplemented at 100 µg/ml. Bacterial culture was incubated at 37°C with vigorous shaking (170 rpm) until mid-log phase.

E4 Secondary bacterial culture

In a 1 000-ml flask, an aliquot of 2 ml from the primary bacterial culture was inoculated to 200 ml LB with ampicillin supplemented at 100 µg/ml. Bacterial culture was incubated overnight at 37°C with vigorous shaking (170 rpm).

E5 Mini Plasmid DNA extraction

Mini plasmid DNA extraction was performed using the QIAprep® Miniprep kit (Qiagen GmbH, Hilden, Germany). Bacterial cells harvested from the 7 ml overnight bacterial culture by centrifugation at 8000 rpm in a conventional, table-top micro-centrifuge for 3 minutes at 24°C. The supernatant was removed and pelleted bacterial cells were suspended in 250 µl buffer P1 and transferred into a micro-centrifuge. Buffer P2 (250 µl) was added and mixed thoroughly by inverting the tube 6 times.

A volume of 350 µl of Buffer N3 was added, mixed by inverting the tube 6 times and centrifuged for 10 minutes at 13 000 rpm. Supernatant (800 µl) was added into the QIAprep 2.0 spin column and centrifuged for 60 seconds. The QIAprep 2.0

spin column was washed by adding 0.5 ml Buffer PB and centrifuged for 60 seconds. The spin column was further washed by adding 0.75 ml Buffer PE and centrifuged for 60 seconds. Plasmid DNA was eluted by adding 50 µl Buffer EB to the QIAprep 2.0 spin column and allowed 1 minute incubation before centrifugation at 1 3000 rpm for 1 minute.

Plasmid DNA was confirmed and detected using agarose gel electrophoresis. An aliquote of plasmid DNA were loaded to a 1 % agarose Tris-Borate-EDTA (TBE) gel containing 1 µg/µl ethidium bromide (Appendix D4, D5). In addition, 1kb DNA ladder was also included (Promega, Madison, WI, USA). The gel was electrophoresed at 100 volts for 2 hour. DNA gel were visualised using Syngene G: Box-Imaging, Vacutec (Syngene, Cambridge, UK). Plasmid DNA was quantified using the Nanodrop 1000 UV/VIS version 3.7 software (Thermo Fischer Scientific, Waltham, MA, USA).

E6 Maxi Plasmid DNA extraction

Maxi plasmid DNA extraction was performed using PureLink® HiPure Plasmid DNA Maxiprep kit (Invitrogen, Carlsbad, CA, USA). Bacterial was harvested from 200 ml overnight culture by centrifugation at 6444 rpm for 10 minutes, 4°C. The supernatant was removed before bacteria pellet was resuspended in 10 ml resuspension buffer containing RNase. A volume of 10 ml of the lysis buffer was added to the resuspended bacteria, mixed thoroughly and incubated at room temperature for 5 minutes. Plasmid DNA was precipitated by adding 10 ml of the precipitation buffer, followed by gently mixing.

The precipitated lysate was loaded to the equilibrated HiPure filter maxi column. Lysate was passed the filter by gravity flow, and maxi column was washed using 50 ml wash buffer.

A sterile 50 ml centrifuge tube was placed under the HiPure filter maxi column and 15 ml elution buffer was added to elute plasmid DNA. A volume of 10.5 ml isopropanol was added to the DNA and the tube was centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant was removed and discarded. Plasmid DNA was precipitated by adding 5 ml of 70-100% ethanol to the pellet and the mixture was centrifuged at 3000 rpm for 5 minutes at 4°C. The resulting pellet was air dried for 10 minutes and DNA pellet was suspended in 500 µl Tris-EDTA buffer provided with the kit.

Plasmid DNA was confirmed and detected using agarose gel electrophoresis. An aliquote of plasmid DNA were loaded to a 1 % agarose TBE gel containing 1 µg/µl ethidium bromide (Appendix D4, D5). In addition, 1kb DNA ladder was also included (Promega, Madison, WI, USA). The gel was electrophoresed at 100 volts for 2 hour. DNA gel were visualised using Syngene G: Box-Imaging, Vacutec (Syngene, Cambridge, UK). Plasmid DNA was quantified using the Nanodrop 1000 UV/VIS version 3.7 software (Thermo Fischer Scientific, Waltham, MA, USA).

Appendix F: Optimization

During PCR amplification, some of the sample failed amplification. Therefore, alternative primer sets were designed in attempt to amplify the amplicons. The Primers (in house designed) were synthesized by DNA bio-synthesis services From University of Cape Town using the applied bio systems 394 DNA bio-synthesizer (Applied Biosystems, Foster city, CA, USA). The synthesized primers were used to optimize the PCR using Eppendorf PCR machine (Eppendorf, Hamburg, Germany). The PCR reaction mix was calculated and shown Table 6.1. A total of four sets of primers were optimized individually. To determine the annealing temperature optimal for the primers, the pCV-H77C plasmid was used as template. After amplification, PCR products were resolved using 1 % agarose gel electrophoresis in 1X TBE buffer. The PCR products were included alongside the 100bp DNA ladder (Promega, Madison, WI, USA).

Table 6.1: PCR reaction master mix preparation

	Final concentration	per 25 µl reaction mix
Platinum SuperFi master mix (2X)- Platinum SuperFi master mix (Invitrogen, Carlsbad, CA, USA)	1X	25 µl
Forward Primer (20 µM)	0.5 nM	0.625 µl
Reverse Primer (20 µM)	0.5 nM	0.625 µl
Template DNA	Adjusted accordingly	4 µl

Nuclease free water	-	7.25 µl

F1 Optimization of 5'UTR PCR

The first region to be amplified was the 5'UTR region because the 5'UTR region is highly conserved and less prone to degradation. Thus, it is easier to amplify than the NS5B region. In order to increase the number of PCR positive samples, primers used to amplify the 5'UTR region were further optimized to be more genotype specific. The PCR reaction mix was prepared for seven tubes per set of primers with the annealing temperatures ranged from 47.5°C to 60°C. The optimal annealing temperature was found to be 52°C for both the first round and second round primer sets (Figure 6.1).

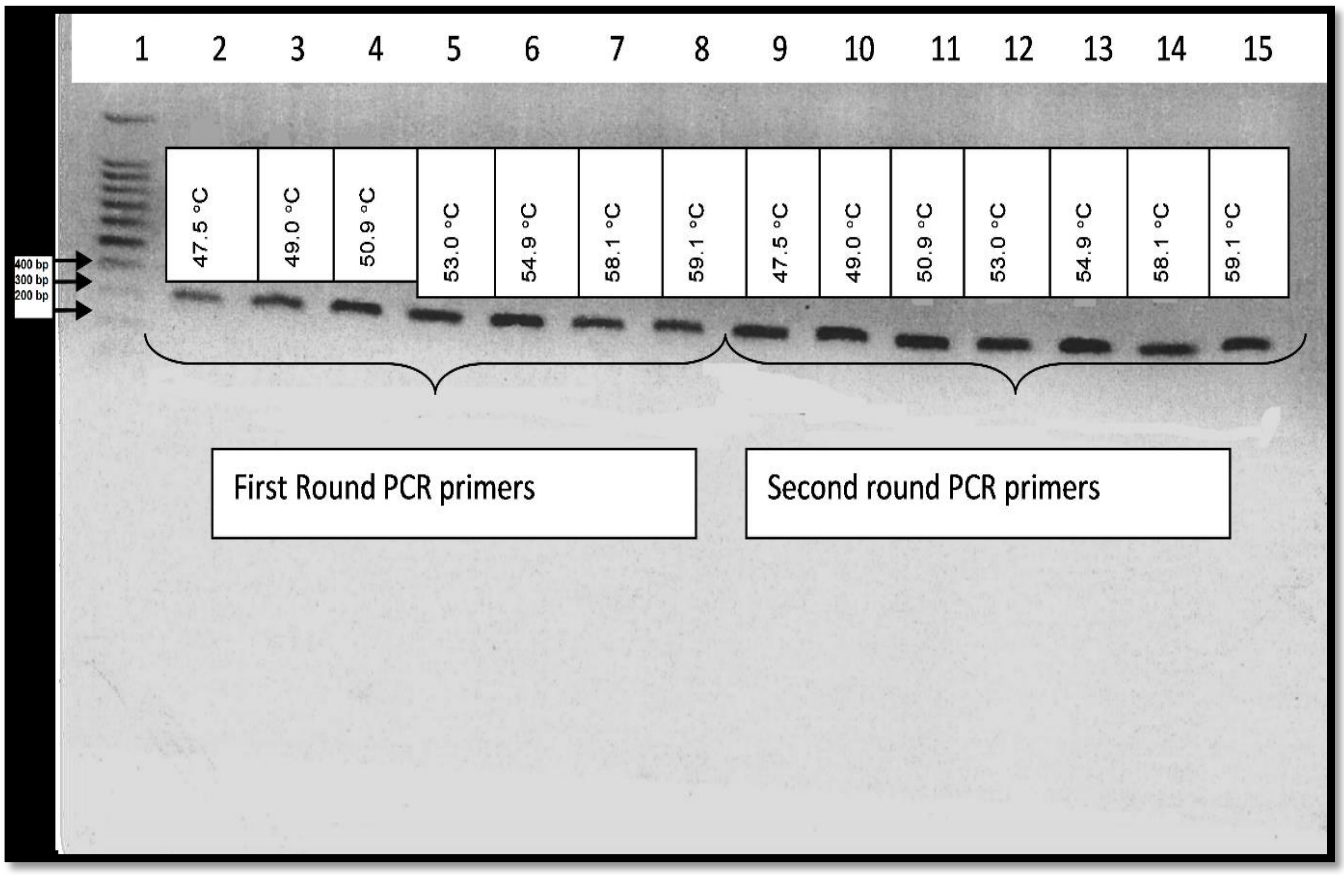


Figure 6.1: Agarose gel showing establishment of the optimal annealing temperature for the 5'UTR region

F2 Optimization of NS5B PCR

The NS5B region is not as highly conserved when compared to 5'UTR region, and is closer to the 3' end. Therefore, it is more prone to degradation, and making this region difficult to be amplified. Genotype specific primers were designed and optimized to amplify the NS5B region. The first set of primer to be optimized was specific to genotype 4. The PCR reaction mix was prepared for six tubes, and the annealing temperatures ranged from 53.4°C to 66.8°C. The optimal annealing

temperature was found to be 53°C for both the first round and second round primer sets (Figure 6.2).

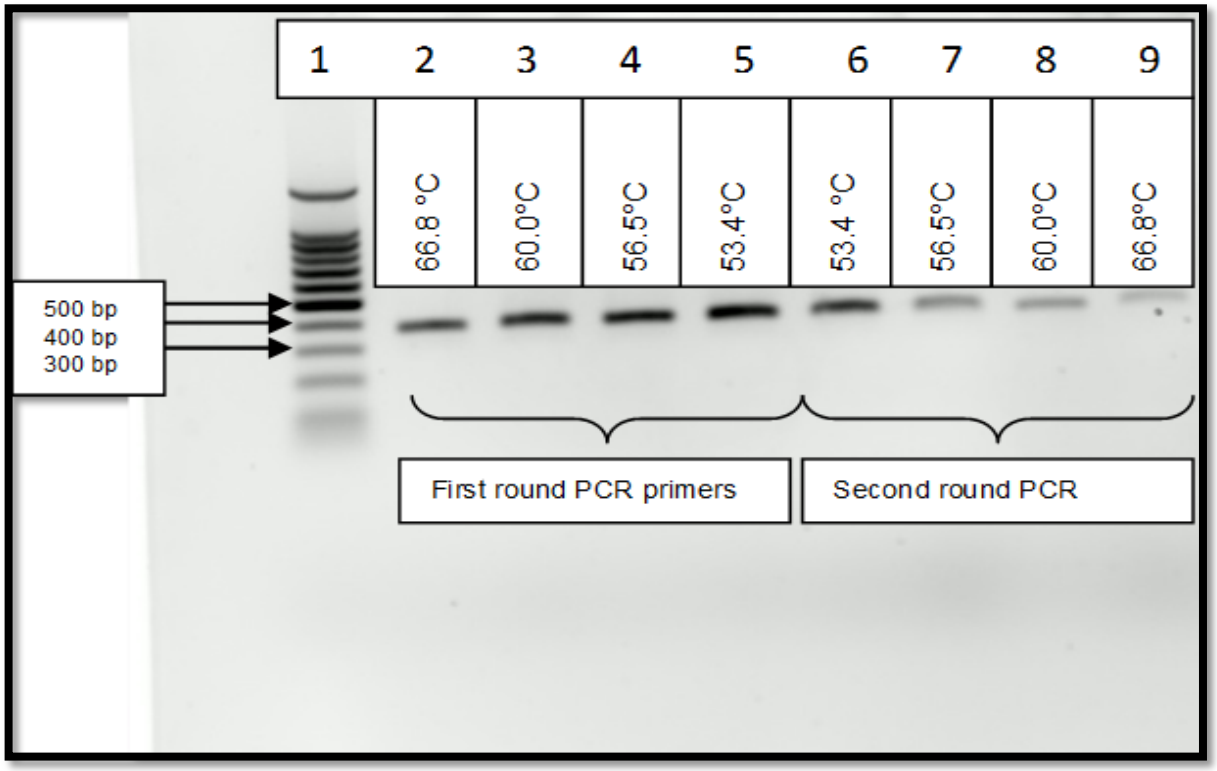


Figure 6.2: Agarose gel showing establishment of the optimal annealing temperature for the NS5B region

F3 Real-time PCR optimization

F3.1 Primer concentration optimization

The primers to be used for PCR reactions were first diluted and optimized to obtain the best optimal concentration. Real-Time PCR reactions were prepared as presented in Table 6.3 with concentrations ranging from 50 to 900 nM. The cycling conditions were as follows: An initial cycle at 50°C for 2 minutes and 95°C for 20 seconds followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. The data was analysed using the Bio-Rad CFX Manager Software version 2.1 (Bio-Rad Laboratories, Hercules, CA, USA). The optimal concentration for both the forward and reverse real-time PCR primers was found to be at 900 nM or 1.8 µl per primer per reaction, shown in (Figure 6.3).

Table 6.2: Primers concentration optimization

Real-Time PCR Reagents	Concentration (nM)[1]								
	50F/50R	**50F/300R**	**50F/900R**	**300F/50R**	**300F/300R**	**300F/900R**	**900F/50R**	**900F/300R**	**900F/900R**
Master mix	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
Probe[2]	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl
Forward Primer	0.1 µl	0.1 µl	0.1 µl	0.6 µl	0.6 µl	0.6 µl	1.8 µl	1.8 µl	1.8 µl
Reverse Primer	0.1 µl	0.6 µl	1.8 µl	0.1 µl	0.6 µl	1.8 µl	0.1 µl	0.6 µl	1.8 µl
Water	7.3 µl	6.8 µl	5.6 µl	6.8 µl	6.3 µl	5.1 µl	5.6 µl	5.1 µl	3.9 µl
DNA	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl

[1] Stock forward and reverse primers were prepared at 20 µM

[2] Stock HCVQ1Taq probe was prepared at 100 µM

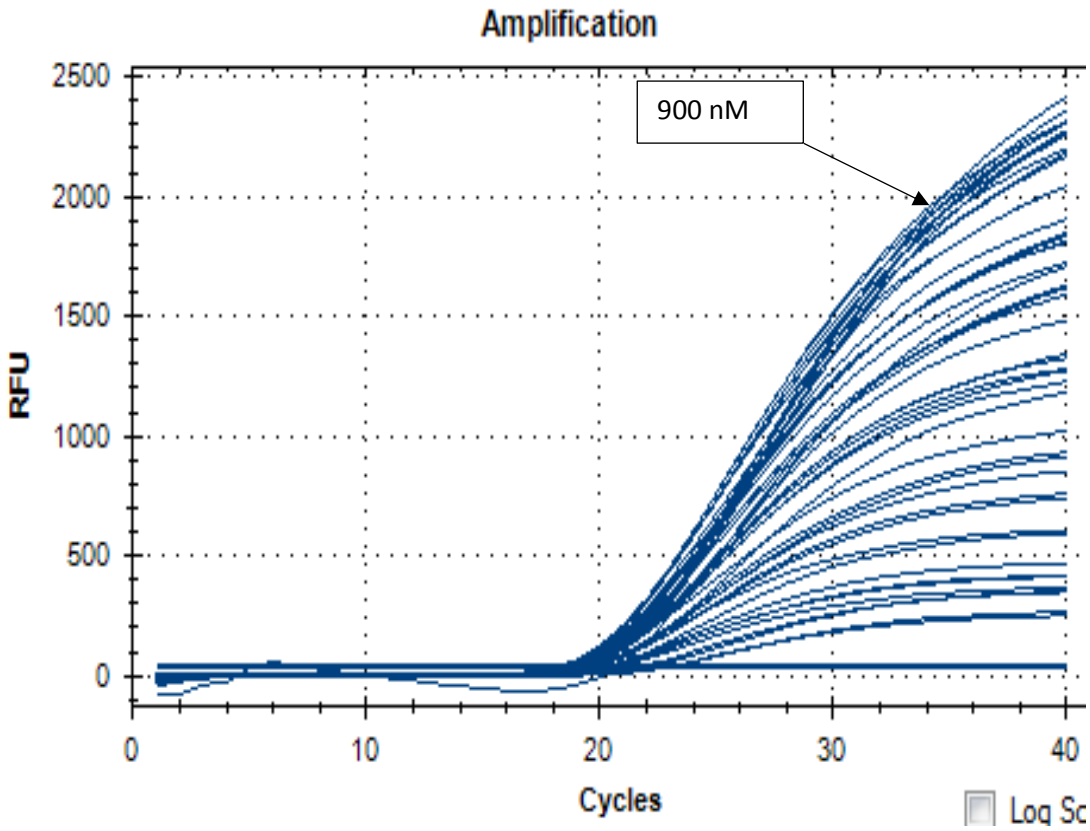


Figure 6.3: Amplification curve of the primer concentration optimization

F3.2 Probe concentration optimization

The Taqman probe concentration was optimized and PCR reactions were prepared as shown in (Table 6.3). The cycling conditions were the same as previously used in primer optimization. The data was analysed using the Bio-Rad CFX Manager Software version 2.1 (Bio-Rad Laboratories, Hercules, CA, USA). The optimal concentration for the probe was found to be 250 nM (Figure 6.4).

Table 6.3: PCR reaction mix

Concentration	**50 nM**	**100 nM**	**250nM**
Master mix (2X)	10 µl	10 µl	10 µl
Probe	0.1 µl	0.2 µl	0.5 µl
Forward Primer	1.8 µl	1.8 µl	1.8 µl
Reverse Primer	1.8 µl	1.8 µl	1.8 µl
Water	4.3 µl	4.2 µl	3.9 µl

Preparation of HCV Taqman probe

Probe name: HCV Q1TAQ PROBE

Probe Sequence: 5’-FAM-CCCTCCCGGGAGAGCCATAGTGGTC-TAMRA-3’

20 000 pmol * 1/1 000 000 p mol

= 0.02 µl

Therefore, 0.02 µmol/X = 100 µmol/L

X = (0.02 µmol/L) / 100 µmol

= 0.0002 L

Therefore, to obtain a working stock concentration of 100 µM, a lypholized primer of 20 nmol was reconstituted with 0.2 ml of nuclease free water.

$C_1V_1 = C_2V_2$

(100 µmol/1 000 000 µl) (200 µl) = (10 µmol/1 000 000 µl)V_2

V_2 = 2 000 µl

To achieve a final probe volume of 2 000 µl, 1800 µl of nuclease free water was added to 200 µl of the 100 µM Taqman probe solution.

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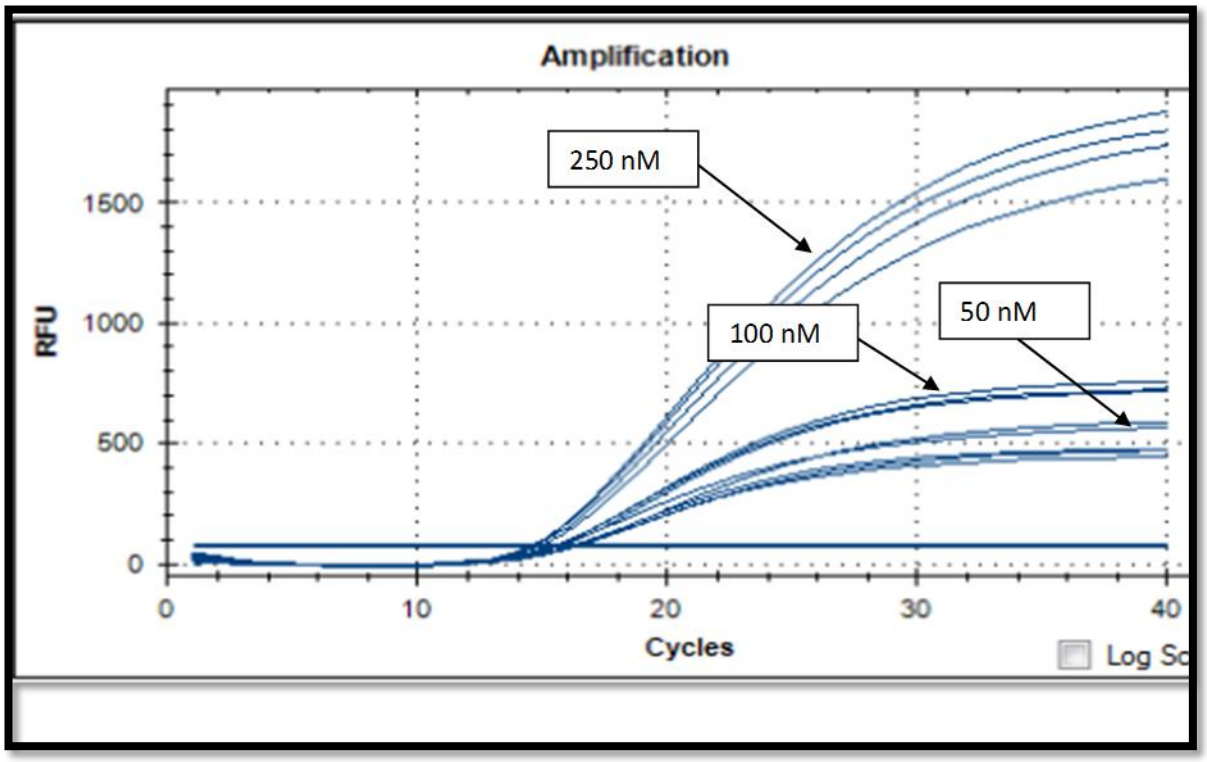

Figure 6.4: Amplification curve of the probe concentration optimization.

F3.3 HCV real time PCR standard curve optimization

Plasmid was serial diluted ranging from 1.21 x 10^{11} copies / µl to 1.21 x 10^{2} copies / µl (Appendix F3.4). The HCV standard curve was constructed using the pCV-H77C plasmid. For the standard curve to be considered as reliable, the PCR was repeated three times with replicates in each round of the Real-time PCR cycles. Amplification plots of the serially diluted HCV plasmid with or without the Acrometrix™ HCV High Control were shown in Figure 6.5 and Figure 6.7. Standard curve generated for the quantification

of HCV with or without the Acrometrix™ HCV High Control were shown in Figure 6.6 and Figure 6.8.

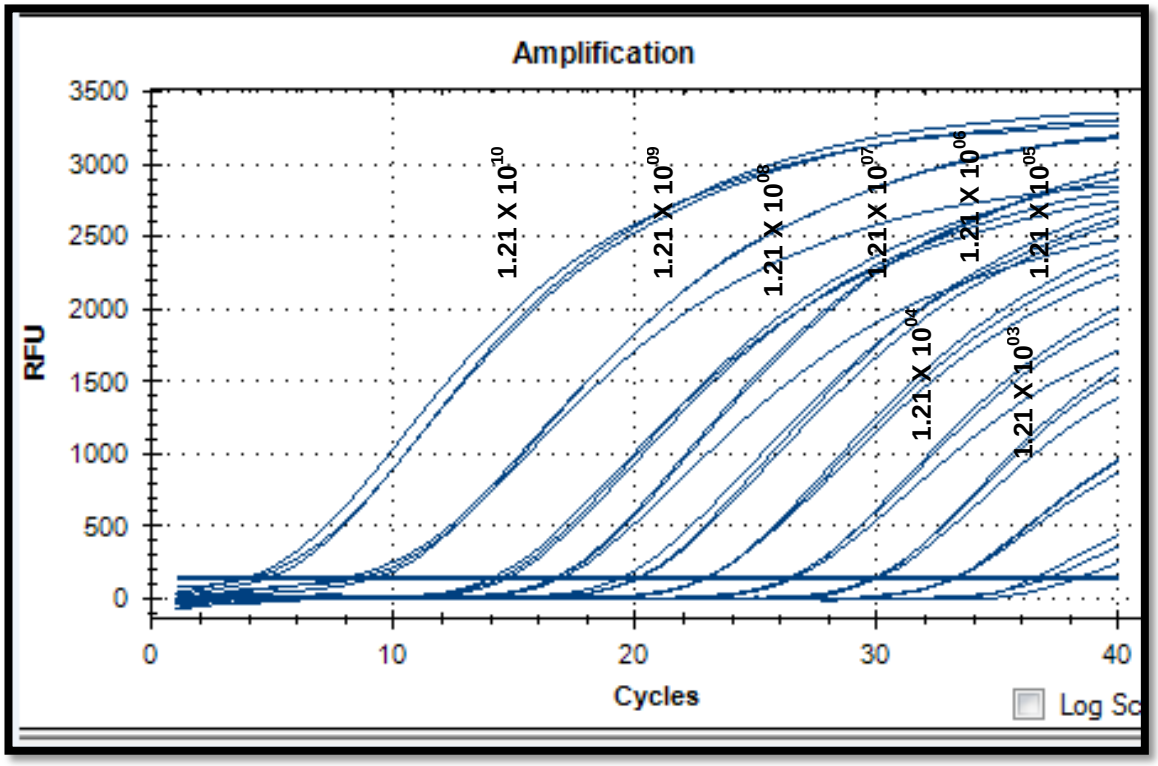

Figure 6.5: Amplification plot of the serially diluted HCV plasmid

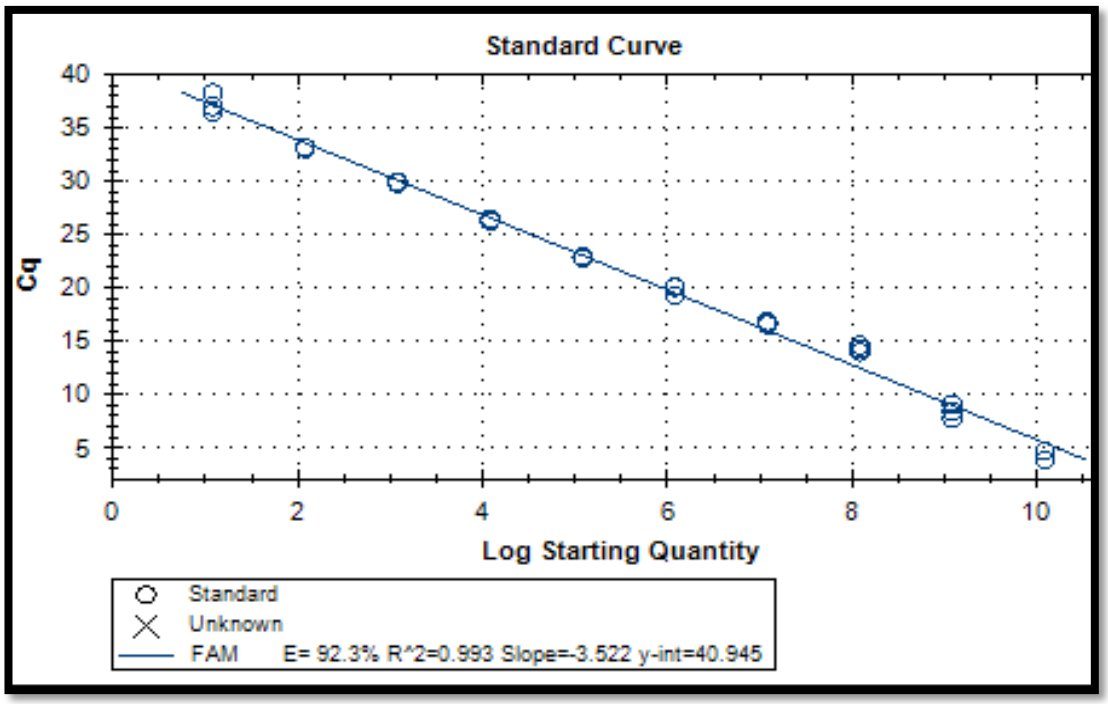

Figure 6.6: Standard curve generated for the quantification of HCV.

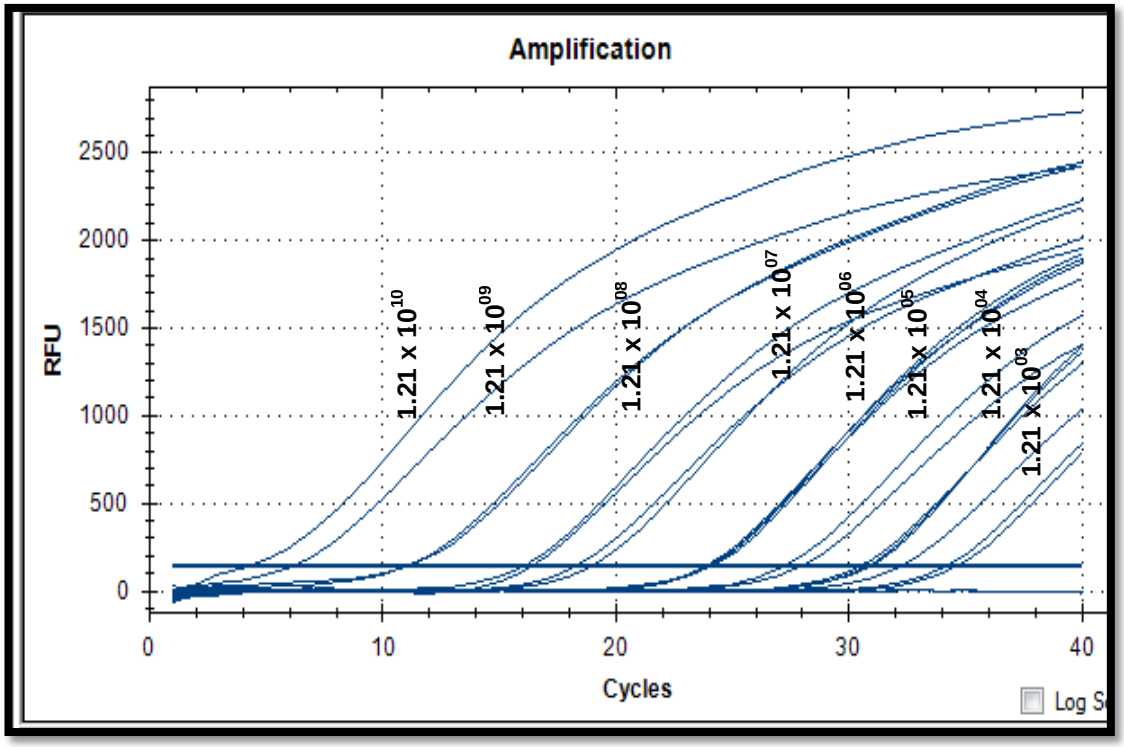

Figure 6.7: Amplification plot of the serially diluted HCV plasmid with the Acrometrix™ HCV High Control

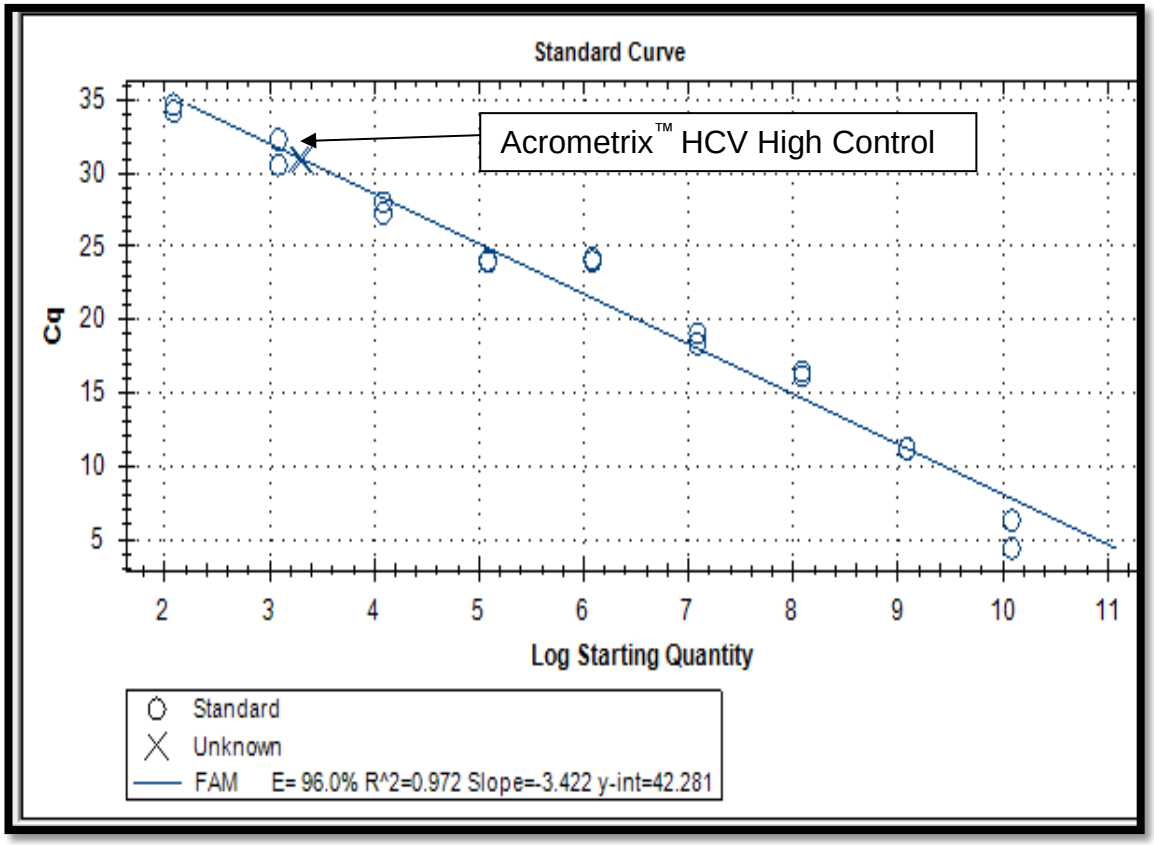

Figure 6.8: Standard curve generated for the quantification of HCV with the Acrometrix™ HCV High Control

F3.4 Calculating copies/ml for the generation of a standard curve

1 copy = 1 plasmid

HCV plasmid size = 13585.32 base pairs

1358.32 base pairs x 660 Da/base = 8.966×10^{6} Da

1 Da = 1 g/mol

1 mol = 6.02214×10^{23} units

1 plasmid copy = 8.966×10^{6} Da / 6.02214×10^{23} units = 1.48×10^{-11}

Optical reading = 1.7966 µg / µl

1.7966 µg / µl / 1.48 $x10^{-11}$ = 1.21×10^{11} copies / µl = 1.21×10^{8} copies / ml

F3.5 Converting iu per ml to copies per ml of the Acrometrix™ HCV High Control (Thermo Fisher Scientific, CA, USA)

The stock concentration Acrometrix™ HCV High Control (Thermo Fisher Scientific, CA, USA) is 4.62×10^{5} iu/ml

1 iu equals 4.3 copies (Leckie et al., 2004)

4.62×10^{5} iu/ml x 4.3

= 1 986 600 copies per ml

Convert to copies per µl

1 986 600 copies per ml ÷ 1000

= 1 986.6 copies per µl

An aliquot of 140 µl of the Acrometrix™ HCV High Control (Thermo Fisher Scientific, CA, USA) was used for extraction and elution was performed at 60 µl using TBE buffer.

1 986.6 copies per µl x (140 µl / 60 µl)

= 4 635.4 copies per µl

To perform real-time PCR, 2 µl of the Acrometrix™ HCV High Control (Thermo Fisher Scientific, CA, USA) was used

4 635.4 µl x 2

= 9.27 x 10^3 copies per µl

Therefore, the final concentration of the Acrometrix™ HCV High Control (Thermo Fisher Scientific, CA, USA) used for real-time PCR was 9.27 x 10^3 copies per µl

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