DETECTION AND GENOTYPING OF HEPATITIS C VIRUS AMONGST BLOOD DONORS AND PREGNANT WOMEN IN PARTS OF NORTH CENTRAL NIGERIA

BY

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DETECTION AND GENOTYPING OF HEPATITIS C VIRUS AMONGST BLOOD DONORS AND PREGNANT WOMEN IN PARTS OF NORTH CENTRAL NIGERIA

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DECLARATION

I declare that the work in this thesis titled "Detection and Genotyping of Hepatitis C Virus Amongst Blood Donors and Pregnant Women in some parts of North Central Nigeria" has been performed by me in the Department of Microbiology. The information derived from the literatures has been duly acknowledged in the text and a list of references provided. No part of the thesis was previously presented for another degree or diploma at any university

Emmanuel Isa BIGWAN	………………………	………………….
Name of student	Signature	Date

CERTIFICATION

This thesis titled DETECTION AND GENOTYPING OF HEPATITIS C VIRUS AMONGST BLOOD DONORS AND PREGNANT WOMEN IN SOME PARTS OF NORTH CENTRAL NIGERIA' by Emmanuel Isa BIGWAN meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Hepatitis C virus has been known to be a menace globally causing chronic liver diseases. The advent of different immunoassays for the detection of specific markers for the diagnosis of the infection since the discovery of the virus is a positive development, but their varied degrees of sensitivity and specificity is of public health concern. The study aimed at determining the seroprevalence and genotypes of hepatitis C virus among blood donors and pregnant women in the study area. Blood samples were collected from 1511 participants (710 blood donors and 801 pregnant women). A third generation ELISA kit was used to analyse all the samples, while two immunochromatographic test kits were used to compare 500 samples analysed using the ELISA Kit. Polymerase Chain Reaction was carried out on all the seropositive HCV samples and sequences/phylogenetic analysis were carried out on all the PCR positive amplicon products. Of the 1511 samples analysed, 5.2% were seropositive for HCV. Of the blood donors examined, 7.0 % were seropositive for HCV. Of the pregnant women examined, 3.6% were seropositive for HCV. Nasarawa State had the highest seroprevalence of 7.0% among blood donors, while Benue State had the least with 5.2% ($p = 0.023$). Of the pregnant women examined, Plateau State had the highest with 4.4%, while Benue State had the least with 2.9% ($p = 0.644$). Males had 7.1%, while the females had 6.7%. Male blood donors of the age group 41- 50 years had the highest with 11.9%, age groups 51- ≥ 61 had the least with 0.0% ($p = 0.583$). The female blood donors of the age group 41-50 years had 10.9%, while the age groups ≥ 51 years had 0.0% ($p = 0.326$). Pregnant women of the age group ≤ 20 had the highest with 6.3% while the age group 21- 30 had the least with 3.2% ($p = 0.528$). Among blood donors, the separated had 33.3%, while the divorced and widowed had 0.0% ($p = 0.123$). Among pregnant women, the married had the highest with 8.9%, while the divorced and the separated had 0.0% ($p = 0.930$). Blood donors with tertiary educational status had 8.4% while those with primary had 3.2% ($p = 0.392$). The pregnant

women with non-formal education had the highest with 4.9%, while those with primary had the least with 2.4% (p= 0.789). The unemployed blood donors had 8.5% while farmers had the least with 0.0% (p= 0.301). Pregnant women that were farmers had the highest with 6.8%, while health workers had the least with 0.0% (p= 0.580). Polygamous blood donors had 12.5%, while the monogamous had 4.9% (p = 0.344). Polygamous pregnant women had 3.4%, while monogamous had 3.0% (p = 0.887). Blood donors engaged in sexual activities with multiple partners without condoms had 7.8%, while those who used condoms had 4.1% (p = 0.4054). Pregnant women engaged in multiple sex activities with condoms had 4.1%, while those who did not use condoms had 0.7% (p = 0.3191). Non-alcoholic blood donors had 7.5% while the alcoholics had 5.4% (p = 0.382). Non-alcoholic pregnant women had 3.8% while the alcoholics had 1.4% (p = 0.296). Blood donors, non- cigarette smokers had 7.3% while the smokers had 3.6% (p = 0.290). Pregnant women, non- cigarette smokers had 3.6% while the smokers had 0.0% (p = 0.634). Of all the risk factors examined among blood donors, only those examined for the history of surgery and unsafe injections showed significant associations. Those with a history of surgery had 15.6%, while those without a history of surgery had 5.2% (p= 0.016; OR= 3.404). Those with a history of unsafe injections had 50%, while those without history of unsafe injections had 5.9% (p= 0.009; OR= 15.960). Pregnant women, with a history of blood transfusion showed a significant association (p= 0.042; OR= 2.716). Of the 500 samples used for comparative evaluation of the three kits, 15.80% were anti- HCV positive by ELISA, 11.80% tested positive by Wondfo kit, whereas only 9.00% by Global strip kit. This gave Wondfo Kit a sensitivity of 75.0%, specificity of 99.0%, overall accuracy of 95.2%, while Global Kit had a sensitivity of 57.0%, specificity of 100.0%, and overall accuracy of 93.2%. Of the 79 samples that were anti-HCV positive, 18.98% were positive for HCV RNA. Of the 9 samples that were

sequenced, the genotypes/ subtypes determined in the study area were: 1a, 1b, 2a/f, and 4a. The predominant HCV genotype in the study area was genotype 1b. The overall seroprevalence of 5.2% is of great public health concern, there is a need for a prompt intervention.

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DEDICATION

This thesis is dedicated to the Almighty God and also my father and in memory of my late mother.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the study

The hepatitis C virus (HCV) was described for the first time in 1989. However, it is still being transmitted today to persons of every age, gender and race in all regions of the world. The discovery of the Hepatitis C virus ended a period of intensive research aimed at finding the agent responsible for 80% of transfusion associated ("non-A, non-B") hepatitis cases (Vardas *et al*.,1999).

Hepatitis is inflammation of the liver. Viral hepatitis, on the other hand, is the infection/inflammation of the liver caused by one of the hepatotropic viruses namely hepatitis viruses A, B, C, D, E, G and recently the TT and SEN hepatitis viruses (Sule *et al.,* 2009).

Hepatitis C virus is a member of the *Flaviviridae* family and is a single, positive-stranded RNA virus of approximately 10,000 nucleotides. HCV is a blood-borne virus and percutaneous, per mucosa and perinatal routes of transmission are well documented but many patients report no recognisable source of infection. The prevalence of HCV infection varies throughout the world and has been reported with the highest incidence in Egypt. WHO estimates that up to 3.0 % of the world's population has been infected with HCV and worldwide there are more than 170 million chronic carriers of HCV (Armstrong *et al*., 2000; Frank *et al.*, 2000; Strader *et al*., 2004). Hepatitis C Virus is a major health problem around the globe. About 185 million people are living with HCV,

of which 80% are living in low-income and middle- income countries (Graham and Swan, 2015).

The HCV genome displays considerable sequence divergence. HCV has been classified into six genotypes, 1–6, with each genotype further subdivided into subtypes displaying different geographical distributions worldwide (Simmonds *et al*., 2005). HCV subtype 5a and all subtypes of genotype 6 are mainly found in South Africa and South East Asia, respectively (Simmonds *et al*., 1993), while subtypes 1a, 1b, 2b and 3a are distributed globally (Smith *et al*., 1997). A new genotype 7 has been registered in the HCV databases, but the full report on this isolate has not been published (Nakano *et al*., 2012).

The genotypes have a geographically distinct distribution which carries important implications such as treatment decisions, possible transmission route and vaccine development. For example, the response to therapy observed in patients infected with genotypes 1 and 4 is lower than those infected with genotypes 2 and 3 (*Kurbanov et al., 2003; Poynard et al., 2004).*

Hepatitis C virus is predominantly transmitted parentally and causes hepatic inflammation. Usually, acute HCV infection passes un-noticed and a majority of the persons remain asymptomatic or have mild symptoms. Individuals positive for HCV antibody are potentially infectious and 60-85% of them become chronically infected with the virus whereas 10-20% of them may develop cirrhosis and 1-5% may develop hepatocellular carcinoma in 20-30 years time (Attia *et al.,* 1996; Armstrong *et al.*, 2000; Davis *et al.*, 2003; Brown and Gaglio, 2003).

Hepatitis C virus (HCV) is mainly transmitted through contact with blood and blood products. The majority of HCV-seropositive individuals will have persistent viraemia. Chronic HCV infection often runs an asymptomatic course and only 25 to 30% of infected persons seek medical attention for symptoms attributable to HCV infection. Early detection is of key importance in order to prevent complications of the HCV-related liver disease (Slavenburg *et al.*, 2008).

The risk factors for the transmission of HCV include: intravenous drug use, dental care, previous abortion through dilation and curettage (D&C), previous surgery, tattooing, unprotected sexual exposures with multiple sexual partners, transfusion of blood and blood products, haemodialysis, employment in the health care field, birth to an HCV-infected mother (Lauer and Walker, 2001; Shepard *et al*., 2005; Kumar *et al.*, 2007).

A variety of technologies has been developed for HCV genotype determination. Most of these assays rely on the amplification of short HCV RNA regions from clinical specimens, followed by a type-specific assay, such as restriction fragment length polymorphism analysis (Buoro *et al*., 1999), line probe reverse hybridization (Stuyver *et al*., 1993; Stuyver *et al*., 1995), or sequence analysis (Simmonds *et al*., 1994; Weck, 1994). Sequence analysis of specific regions (NS5, core, E1 and 5'UTR) is considered to be the gold standard for genotyping (Ohno and Lau, 1996).

Current treatment for HCV infection is not highly effective and at least 90% of the patients who need treatment are unable to afford it. Immunisation for passive prophylaxis of the hepatitis infection is not readily available. Public health interventions, therefore, continue to be the only effective method of preventing HCV infection. These include

screening blood and blood products before transfusion, effective use of universal precautions and contraceptive barrier methods, use of disposable sharps and promotion of health education on HCV infection and its prevention. However, any strategy to prevent HCV infection must be based on accurate data (Vardas *et al*., 1999; Chukwurah *et al*., 2005).

1.2 Statement of Research Problem

Hepatitis C is a blood-borne virus infection with an estimated 180 million infected individuals worldwide. Hepatitis C virus infection may lead to liver failure and cancer of the liver (Kok *et al.*, 2007). The prevalence of HCV in population can be predicted by the risk factors associated with transmission of infection. These risk factors include blood products transfusion, occupational injury, surgery, injection and vertical transmission (Yen and Ahmed, 2003). Viral hepatitis during pregnancy is associated with high risk of maternal complications. It has a high risk of vertical transmission, and it has been reported as the leading cause of maternal death (Shaikh *et al., 2009*).

Hepatitis C virus is a major cause of chronic liver disease worldwide. The WHO estimated that 3% of the world's population is chronically infected with HCV and Sub-Saharan Africa is of great interest because it is reported to have the highest HCV prevalence of 5·3%. A review of published literatures showed a consistent evidence of high HCV prevalence in many African countries was established with an overall estimated prevalence of 3.0% in Sub-Saharan Africa. The central African region had the highest estimated prevalence of 6%, West Africa had an estimated prevalence of 2·4%, and southern and eastern Africa with the lowest estimated prevalence of 1·6% (Madhava *et al*., 2002; Mezban and Wakil, 2006).

HCV infection occurs frequently and is highly endemic in Nigeria. This high prevalence has been confirmed by various studies from different parts of Nigeria among selected groups. Alao *et al*. (2009) reported 5.4% among blood donors in Makurdi, Olokoba *et al*. (2009) reported 2.4% among blood donors in Yola; Isa *et al.* (2011) reported 1.8% among blood donors in ABUTH Kaduna while Ogunro *et al*. (2007) reported a prevalence of 9.2% among pregnant women in Osogbo; and Buseri *et al.* (2010) reported a prevalence of 0.5% HCV antibodies among pregnant women in Benin City.

1.3 Justification of the study

Currently, there are few data on HCV and genotyping in Nigeria as such there is a need for a wider study to cover the whole country of which this study seek to determine its position in the study area. To the best of my knowledge, there are no published works on genotypes and subtypes of HCV in the study area as such this work will serve as a base line data for future studies.

Conventional tests for antibody to Hepatitis C virus (HCV) and HCV RNA require considerable time before results are available, remain very expensive and are not adapted to many sub-Saharan African countries where HCV is endemic (Njouom *et al*., 2006). This study evaluated the accuracy of an algorithm consisting of two rapid anti-HCV test, and ELISA which served as the gold standard for anti-HCV detection. Early diagnosis and evaluation of HCV cases are very helpful for the management of the disease.

HCV infection patterns have not significantly changed in most parts of the world since 1997, when it was first analysed, this may be partly due to the lack of new and more accurate data. The ‘patchy’ epidemiological situation in some areas will continue to

complicate the task of the establishment of global, regional and national base line data (Lavanchy, 2011). The assessment of the prevalence of HCV finds a global prevalence of 2.35%, affecting 160 million chronically infected individuals. There is an urgent need for more accurate information on the costs and burden of HCV to society. Twenty-one years after the discovery of HCV, the assessment is far from being complete and little progress has been made in many countries. In some countries significant increases have been reported and this may also apply to countries where insufficient data exist. A safe and efficient vaccine against HCV is urgently needed (Lavanchy, 2011).

1.4 Research Questions

1. Is HCV infection a public health concern in the study area?
2. What is the current prevalence of HCV within the study populations/study area?
3. What are the likely risk factors associated with the transmission of HCV in the study area?
4. Are there any benefits of one method over the other on the diagnostic tools to be used?
5. What are the circulating HCV genotypes and sub-types in the study area?

1.5 Hypothesis

Null Hypothesis (Ho): There is no difference in the prevalence, risk factors, demographic data and genotypes/sub-types of HCV in the study area.

Alternate Hypothesis (Ha): There is a difference in the prevalence, risk factors, demographic data and genotypes/sub-types of HCV in the study area.

1.6 Aim

The aim of the study is to determine the seroprevalence of HCV and its genotypes among blood donors and pregnant women in some parts of North Central Nigeria.

1.7 Objectives

The specific objectives of the study are:

1. To determine the seroprevalence of HCV in blood donors and pregnant women in the study area.
2. To determine the seroprevalence of HCV in relation to some socio-demographic data in the study populations.
3. To determine risk factors associated with HCV infection in the study area.
4. To determine the sensitivity and specificity of two commonly used immunochromatographic diagnostic test kits in the study area.
5. To detect HCV RNA in the HCV antibody positive samples.
6. To determine the HCV genotypes/subtypes and the Phylogenetic relatedness of the HCV strains in the study area.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Background of HCV

Until 1975, only two hepatitis viruses had been identified, the "infectious hepatitis virus" (hepatitis A virus, HAV) and the "serum hepatitis virus" (hepatitis B virus, HBV). However, other viruses were exempted from being the cause of about 65% of post-transfusion hepatitis. Therefore, these hepatitis cases were called "non-A, non-B hepatitis" (NANBH) (Feinstone *et al.,* 1975).

In 1988, the hepatitis C virus was confirmed by Dr. Alter in the course of verifying its presence in a panel of NANBH specimens. In April 1989, the discovery of HCV was published in two articles in the journal *Science* by Choo *et al*. (1989) and Kuo *et al*. (1989). In 2000, Drs. Alter and Houghton were honoured with the Lasker Award for Clinical Medical Research for "pioneering work leading to the discovery of the virus that causes hepatitis C infection and the development of screening methods that reduced the risk of blood transfusion-associated hepatitis in the U.S. from 30% in 1970 to virtually zero in 2000" (Lasker Foundation, 2000).

The inoculation of chimpanzees with plasma or serum obtained from humans with NANB hepatitis led to persistent increases of serum alanine aminotransferase (ALT) indicating that an infectious agent was the cause of the disease (Alter *et al.,* 1978; Hollinger *et al.,* 1978). It was later discovered that the NANBH agent could be inactivated by chloroform (Feinstone *et al.,* 1983). Furthermore, it was reported that the infectious agent passed through 80 nm membrane filters (Bradley *et al.,* 1985). These earlier studies suggested that the NANBH causing agent could be a small virus

containing a lipid envelope. However, the lack of a suitable cell culture system for propagation of the NANBH agent and the limited availability of chimpanzees made a further characterization of the etiologic agent of NANBH impossible for several years. The genome of the major causative agent for NANBH was eventually characterised in 1989 using a newly developed cloning strategy for nucleic acids obtained from plasma of NANBH infected chimpanzees (Choo *et al.,* 1989). The cDNA clone 5-1-1 encoded immunological epitopes that interacted with sera from individuals with NANBH (Choo *et al.,* 1989; Kuo *et al.,* 1989). The corresponding infectious virus causing the majority of NANBH was later on called hepatitis C virus (Kupfer, 2013).

2.2 Virology of Hepatitis C Virus

2.2.1 Taxonomy of HCV

HCV is a small enveloped, single-stranded positive-sense RNA virus of approximately 9.6 kb. It is a member of the Flaviviridae family. This viral family contains four genera, flavivirus, pestivirus, hepacivirus, and the newly defined genus pegivirus (Stapleton *et al*., 2011). Novel hepaciviruses have been described from bats, rodents, horses, bank voles, primates and dogs enabling researchers to possibly develop new model systems for the analysis of the molecular biology and the pathogenesis of HCV (Drexler *et al*., 2013; Kapoor *et al*., 2013; Lauck *et al*., 2013).

Studies have shown that only two members of the hepacivirus genus have been identified, HCV and GB virus B (GBV-B), a virus that had been initially detected together with the then-unclassified virus GB virus A (GBV-A) in a surgeon with active hepatitis (Simons *et al*., 1995; Ohba *et al*., 1996; Thiel *et al*., 2005; Ray and Thomas ,2009; Rosen, 2011).

Nevertheless, the natural hosts for GBV-B and GBV-C seem to be monkeys of the Saguinus species (tamarins). Analyses of viral sequences and phylogenetic comparisons supported HCV's membership in a distinct genus from flavivirus or pestivirus (Choo *et al*., 1991). The population of HCV has been found to comprise a quasispecies that consists of a number of identical sequences and other closely related sequences. The sequence of this quasispecies population changes during infection. It has been found that the sequence of the hypervariable region I changes rapidly in infected individuals. It is possible that the quasispecies nature of HCV constitutes a mechanism by which HCV escapes immune surveillance and establishes a persistent infection in the host (Bukh *et al*., 1995).

2.2.2 HCV genotypes

Comparisons of HCV nucleotide sequences derived from individuals from different geographical regions showed the presence of six major HCV genotypes with a large number of subtypes within each genotype (Simmonds, 2004; Simmonds *et al*., 2005). An additional or seventh genotype was reported-genotype 7 (Nakano *et al*., 2012).

Hepatitis C Virus genotype 1 is the most prevalent genotype globally, accounting for 46.2% (83.4 million) cases, approximately one-third of which are in East Asia, genotype 3 is the second most prevalent globally accounting for 30.1% (54.3 million); genotypes 2, 4, and 6 make up 22.8% of all cases while genotype 5 comprises the remaining <1%. Genotypes 1 and 3 dominate are more prevalent in most countries irrespective of their economic status, while the largest proportions of genotypes 4 and 5 are in lower-income countries (Messina *et al*., 2015).

Sequence divergence of genotypes and subtypes was 20% and 30%, respectively as reported by Simmonds (1995). In the United States, about 70% of cases are caused by genotype 1, 20% caused by genotype 2, and about 1% by each of the other genotypes (Wilkins *et al.,* 2010). Genotype 1 is also the most common in South America and Europe (Rosen, 2011).

HCV genotype plays an important role in the initiation of anti-HCV treatment since the response of different genotypes varies significantly with regard to specific antiviral drug regimens, e.g., genotype 1 is most resistant to the combination therapy of pegylated interferon alpha and ribavirin than genotypes 2 and 3 (Manns *et al.,* 2001).

2.2.3 HCV structure

The attempt to clarify the structure of hepatitis C virus, employed an indirect immunogold electron microscopic study on two plasma samples with high HCV RNA titres using polyclonal and monoclonal antibodies specific to the putative HCV envelope protein. The study revealed a spherical virus-like particle, 55 to 65 nm in diameter with spike-like projections. This is the first known report in which the morphology of the HCV particle is clearly shown (Kaito *et al*., 1994).

Several studies reported that different forms of HCV virions appear to exist in the blood of infected individuals: virions bound to very low density lipoproteins (VLDL), virions bound to low density lipoproteins (LDL), virions complexed with immunoglobulins, and free circulating virions (Bradley *et al*., 1991; Thomssen *et al*., 1992; Thomssen *et al*., 1993; Agnello *et al*., 1999; Andre *et al*., 2002). It is speculated that the association with LDL and/or VLDL protects the virus against neutralisation by Hepatitis C Virus specific

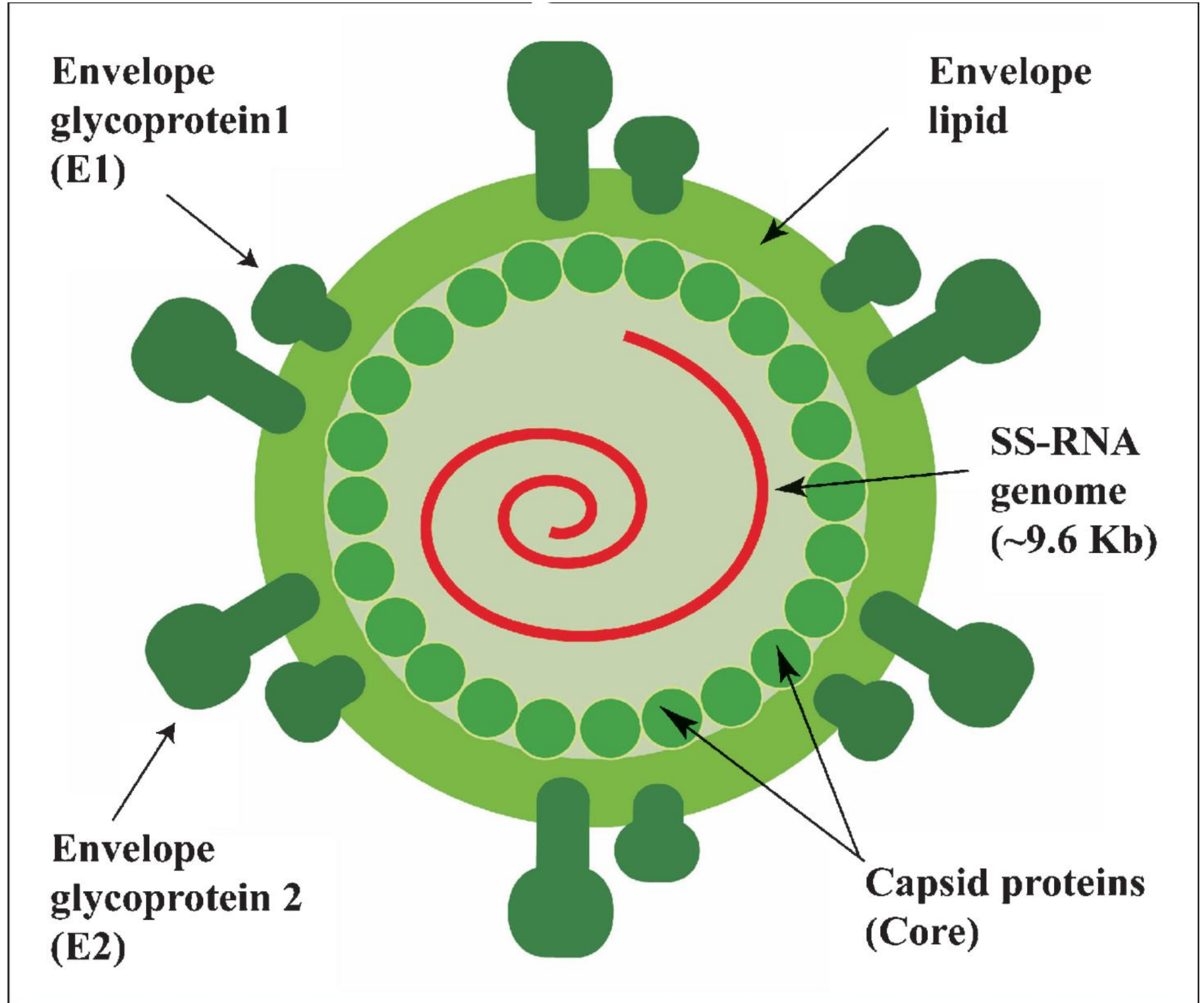

Fig. 2.1: Hepatitis C virus particle structure (Sharma, 2010).

antibodies. The design and optimization of genomic and subgenomic HCV replicons in the human hepatoma cell line Huh7 offered for the first time the possibility to investigate HCV RNA replication in a standardised manner (Lohmann *et al*., 1999; Ikeda *et al*., 2002; Blight *et al*., 2002).

Despite the high level of HCV gene expression, no infectious viral particles are actually produced. Therefore, it cannot be used for structural analysis of free virions. Infectious HCV particles have been achieved in cell culture by using recombinant systems (Heller, 2005; Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005; Yu *et al*., 2007). However, even in these *in vitro* systems, the limited production of viral particles prevents 3D structural analysis (Yu *et al*., 2007). It was shown by cryo-electron microscopy (cryoEM) and negative stain transmission electron microscopy that HCV virions isolated from cell culture have a spherical shape with a diameter of approximately 50 to 55 nm (Heller *et al*., 2005; Wakita *et al*., 2005; Yu *et al*., 2007) confirming earlier findings that measured the size of putative native HCV particles from the serum of infected individuals (Prince *et al*., 1996).

In a study on the biochemical and ultrastructural properties of hepatitis C virus particles produced in cell culture. Negative stain electron microscopy revealed that the particles were spherical (~40 to ~75 nm diameter) and pleomorphic and that some of them contain HCV E2 protein and apolipoprotein E on their surfaces. Electron cryomicroscopy showed two major particle populations of ~60 and ~45 nm in diameter. The ~60-nm particles were characterised by a membrane bilayer (presumably an envelope) that is spatially separated from an internal structure (presumably a capsid) (Gastaminza *et al*., 2010). Figure 2.1 shows a schematic structure of HCV.

2.2.4 Genome organisation of HCV

The genome of the Hepatitis C virus consists of one 9.6 kb single-stranded RNA molecule with positive polarity. Similar to other positive-strand RNA viruses, the genomic RNA of hepatitis C virus serves as messenger RNA (mRNA) for the translation of viral proteins. The linear molecule contains a single open reading frame (ORF) coding for a precursor polyprotein of approximately 3000 amino acid residues. During viral replication, the polyprotein is cleaved by viral as well as host enzymes into three structural proteins (core, E1, E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B). An additional protein (termed F [frame shift] or ARF [alternate reading frame]) is predicted as a result of ribosomal frame shifting during translation within the core region of the genomic RNA (Xu *et al*., 2001; Walewski *et al*., 2001; Varaklioti *et al*., 2002; Branch *et al*., 2005).

The structural genes encoding the viral core protein and the viral envelope proteins E1 and E2 are located at the 5' terminus of the open reading frame followed downstream by the coding regions for the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Figure 2.2). The structural proteins are essential components of the HCV virions, whereas the non-structural proteins are not associated with virions but are involved in RNA replication and virion morphogenesis. The ORF is flanked by 5' and 3' nontranslated regions (NTR; also called untranslated regions, UTR or noncoding regions, NCR) containing nucleotide sequences relevant for the regulation of viral replication. Both NTRs harbour highly conserved regions compared to the protein encoding regions of the HCV genome. The high grade of conservation of the NTRs makes them candidates: (i) for improved molecular diagnostics, (ii) as targets for antiviral

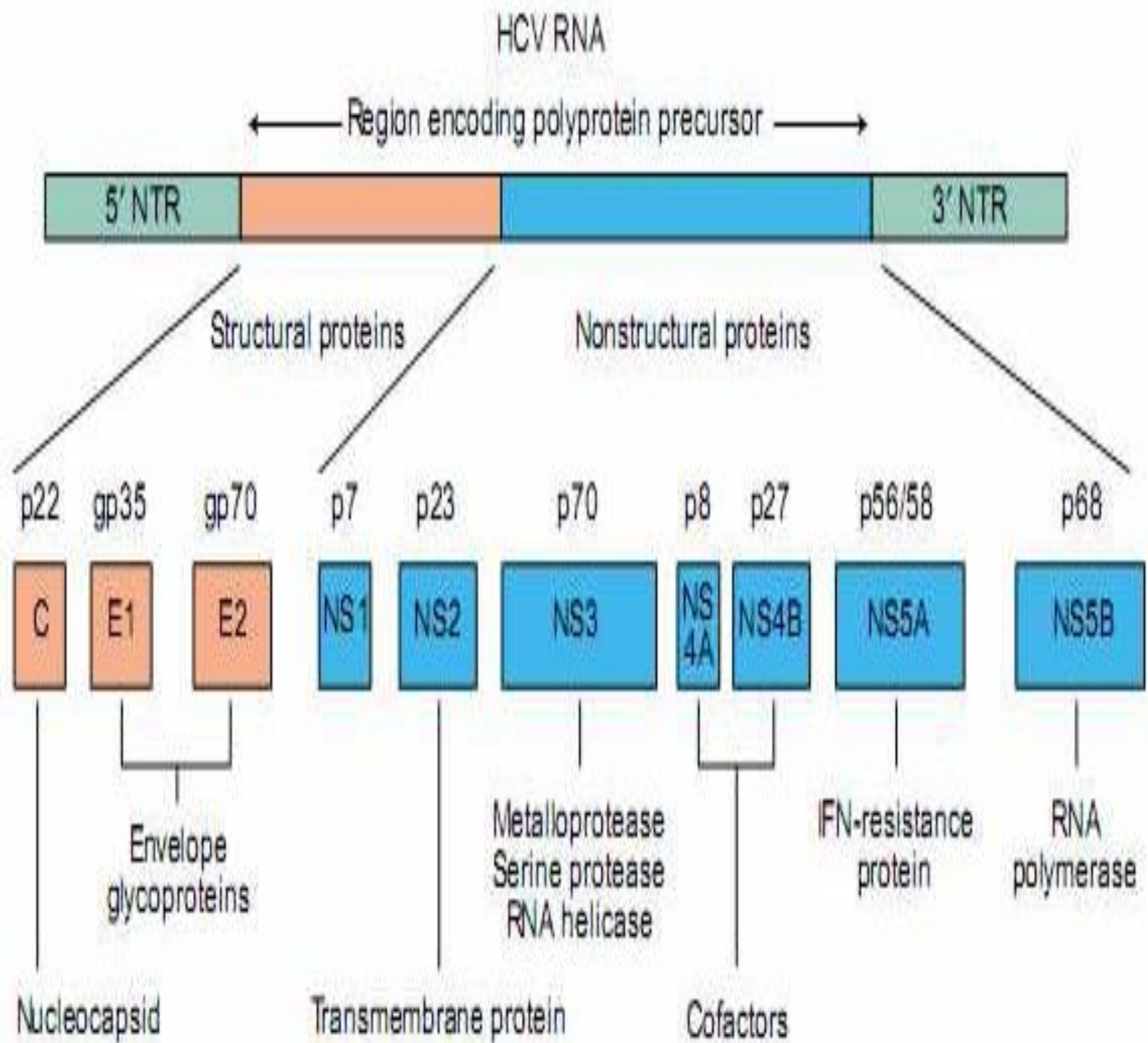

Fig. 2.2: Model structure of HCV genome organization (Anzola and Burgos, 2003)

therapeutics, and (iii) as targets for an anti-HCV vaccine. The 5'NTR is approximately 341 nucleotides long with a complex secondary structure of four distinct domains (I-IV) (Fukushi *et al*., 1994; Honda *et al*., 1999).

The 3'NTR consists of three functionally distinct regions: a variable region, a poly U/UC tract of variable length, and the highly conserved X tail at the 3' terminus of the HCV genome (Tanaka *et al*., 1995; Kolykhalov *et al*., 1996; Blight and Rice, 1997 and Friebe *et al*., 2002). The variable region of approximately 40 nucleotides is not essential for RNA replication. However, deletion of this sequence led to significantly decreased replication efficiency (Yanagi *et al*., 1999; Friebe and Bartenschlager, 2002). The length of the poly U/UC region varies in different HCV strains ranging from 30 to 80 nucleotides (Kolykhalov *et al*., 1996). The minimal length of that region for active RNA replication has been reported to be 26 homouridine nucleotides in cell culture (Friebe and Bartenschlager, 2002). The highly conserved 98 nucleotide X tail consists of three stem-loops (SL1-SL3) (Tanaka *et al*., 1996; Ito and Lai, 1997; Blight and Rice, 1997) and deletions or nucleotide substitutions within that region are most often lethal (Yanagi *et al*., 1999; Kolykhalov *et al*., 2000; Friebe and Bartenschlager, 2002; Yi and Lemon, 2003). Another so-called "kissing-loop" interaction of the 3'X tail SL2 and a complementary portion of the NS5B encoding region has been described (Friebe *et al*., 2005). This interaction induces a tertiary RNA structure of the HCV genome that is essential for HCV replication in cell culture systems (Friebe *et al*., 2005; You and Rice, 2008). Both NTRs appear to work together in a long-range RNA-RNA interaction possibly resulting in temporary genome circularisation (Song *et al*., 2006).

2.2.4.1 Genes and proteins

The translation of the HCV polyprotein is initiated through the involvement of some domains in NTRs of the genomic HCV RNA. The resulting polyprotein consists of ten proteins that are co-translationally or post-translationally cleaved from the polyprotein. The N-terminal proteins C, E1, E2, and p7 are processed by a cellular signal peptidase (SP) (Hijikata *et al*., 1991). The resulting immature core protein still contains the E1 signal sequence at its C-terminus. Subsequent cleavage of this sequence by a signal peptide peptidase (SPP) leads to the mature core protein (McLauchlan *et al*., 2002). The non-structural proteins NS2 to NS5B of the HCV polyprotein are processed by two virus-encoded proteases (NS2-NS3 and NS3) with the NS2-NS3 cysteine protease cleaving at the junction of NS2-NS3 (Santolini *et al*., 1995) and the NS3 serine protease cleaving the remaining functional proteins (Bartenschlager *et al*., 1993; Eckart *et al*., 1993; Grakoui *et al*., 1993a; Tomei *et al*., 1993).

2.2.4.2 Core

The core-encoding sequence starts at codon AUG at nt position 342 of the H77 genome, the start codon for translation of the entire HCV polyprotein. During translation, the polyprotein is transferred to the endoplasmic reticulum (ER) where the core protein (191 aa) is excised by a cellular signal peptidase (SP). The C-terminus of the resulting core precursor still contains the signal sequence for ER membrane translocation of the E1 ectodomain (aa 174-191). This protein region is further processed by the cellular intramembrane signal peptide peptidase (SPP) leading to the removal of the E1 signal peptide sequence (Hüssy *et al*., 1996; McLauchlan *et al*., 2002; Weihofen *et al*., 2002). The multifunctional core protein has a molecular weight of 21-kilo dalton (kd). *In vivo*,

the mature core molecules are believed to form homo-multimers located mainly in the ER membrane (Matsumoto *et al*., 1996).

The core protein has a structural function since they form the viral capsid that contains the HCV genome. In addition, they have regulatory functions including particle assembly, viral RNA binding, and regulation of RNA translation (Santolini *et al*., 1994; Ait-Goughoulte *et al*., 2006). The HCV core protein has pro- and anti-apoptotic functions (Kountouras *et al.,* 2003; Chou *et al.,* 2005; Meyer *et al.,* 2005), which stimulates hepatocyte growth in Huh-7 cell line by transcriptional up-regulation of growth-related genes (Fukutomi *et al.,* 2005), and has been implicated in tissue injury and fibrosis progression (Nunez *et al.,* 2004).

2.2.4.3 *E1 and E2*

Downstream of the core coding region of HCV RNA genome two envelope glycoproteins are encoded, E1 (gp35; 192 aa) and E2 (gp70; 363 aa). During translation at the ER, both proteins are cleaved from the precursor polyprotein by a cellular SP. Inside the lumen of the ER both polypeptides experience N-linked glycosylation post-translationally (Duvet *et al*., 2002). Both glycoproteins E1 and E2 harbour 5 and 11 putative N-glycosylation sites, respectively. E1 and E2 are type I transmembrane proteins with a large hydrophilic ectodomain of approximately 160 and 334 aa and a short transmembrane domain (TMD) of 30 aa. The TMD are responsible for the anchoring of the envelope proteins in the membrane of the ER and ER retention (Cocquerel *et al*., 1998; Duvet *et al*., 1998; Cocquerel *et al*., 1999; Cocquerel *et al*., 2001). Moreover, the same domains have been reported to contribute to the formation of E1-E2 heterodimers (Op de Beeck *et al*., 2000).

These envelope glycoproteins E1 and E2 are localised in endoplasmic reticulum compartment and have multiple functions. These functions include membrane anchoring, endoplasmic reticulum localisation and heterodimer assembly (Jeulin *et al*., 2013).

However, the precise mechanism of host cell entry is still not understood completely. Several other host factors have been identified to be involved in viral entry. These candidates include the scavenger receptor B type I (Scarselli *et al*., 2002; Kapadia *et al*., 2007), the tight junction proteins claudin-1 (Evans *et al*., 2007) and occludin (Ploss *et al*., 2009), the C-type lectins L-SIGN and DC-SIGN (Gardner *et al*., 2003; Lozach *et al*., 2003; Pöhlmann *et al*., 2003) and heparan sulfate (Barth *et al*., 2003). Two hypervariable regions have been identified within the coding region of E2. These regions termed hypervariable region 1 (HVR1) and 2 (HVR2) differ by up to 80% in their amino acid sequence (Weiner *et al*., 1991; Kato, 2001). The first 27 aa of the E2 ectodomain represents HVR1, while the HVR2 is formed by a stretch of seven amino acids (position 91-97). The high variability of the HVRs is an evidence of exposure of these domains to HCV-specific antibodies. The E2-HVR1 has been shown to be the most important target for neutralising antibodies (Farci *et al*., 1996; Shimizu *et al*., 1996). Nevertheless, the combination of the mutation of the viral genome with the selective pressure of the humoral immune response results in viral escape via epitope alterations. This makes the development of vaccines that induce neutralising antibodies a challenging task (Kupfer, 2013).

2.2.4.4 The p7 protein

The small p7 protein (63 aa) is situated between the E2 and NS2 regions of the polyprotein precursor. During the process of translation, the cellular SP cleaves with the E2-p7 as well as the p7-NS2 junction. The functional p7 is a membrane protein which is localised in the endoplasmic reticulum where it forms an ion channel (Griffin *et al*., 2003; Pavlovic *et al*., 2003; Haqshenas *et al*., 2007). Studies showed that the p7 protein is not essential for RNA replication since replicons without the p7 gene can replicate efficiently (Lohmann *et al*., 1999; Blight *et al*., 2000; Meredith *et al*., 2013). However, it has been suggested that p7 plays an important role in the formation of infectious virions (Sakai *et al*., 2003; Haqshenas *et al*., 2007). P7 has been shown to be essential for virus particle assembly and release of infectious virions in a genotype specific manner (Steinmann *et al*., 2007).

2.2.4.5 NS2

The non-structural protein 2 (p21; 217 aa) together with the N-terminal portion of the NS3 protein, form the NS2-3 cysteine protease which catalyses cleavage of the polyprotein precursor between NS2 and NS3 (Grakoui *et al*., 1993b; Santolini *et al*., 1995). The crystal structure of the C-terminal domain of NS2 reveals a dimeric protease containing two composite active sites (Lorenz *et al*., 2006). The N- terminus of the functional NS2 arises from the cleavage of the p7-NS2 junction by the cellular SP. Moreover, after cleavage from the NS3 the protease domain of NS2 seems to play an essential role in the early stage of virion morphogenesis (Jones *et al*., 2007). NS2 is a non-glycosylated transmembrane protein. It is a protein that has the tendency of losing its protease activity after self-cleavage from NS3. Protein kinase casein kinases 2 degrade

NS2 through a phosphorylation-dependent process and the NS2 is essential for the complete replication of the virus to occur (Suzuki *et al*., 2013).

2.2.4.6 NS3

The non-structural protein 3 (p70; 631 aa) is cleaved at its N- terminus by the NS2-NS3 protease. The N terminus (189 aa) of the NS3 protein has a serine protease activity. However, in order to develop full activity of the protease the NS3 protease domain requires a portion of NS4A (Faila *et al*., 1994; Bartenschlager *et al*., 1995; Lin *et al*., 1995; Tanji *et al*., 1995; Tomei *et al*., 1996). NS3 together with the NS4A cofactor are responsible for cleavage of the remaining downstream cleavages of the HCV polyprotein precursor. Since the NS3 protease function is essential for viral infectivity it is a promising target for the design of antiviral treatments. The C-terminal portion of NS3 (442 aa) has ATPase/helicase activity, i.e., it catalyses the binding and unwinding of the viral RNA genome during viral replication (Jin and Peterson, 1995; Kim *et al*., 1995). Not surprisingly, the NS3-NS4A protease is one of the most popular viral targets for anti-HCV therapeutics (Pawlotsky and McHutchison, 2004; Pawlotsky, 2006; Rosenberg *et al*., 2014).

2.2.4.7 NS4A

The HCV nonstructural protein 4A (p4) is a 54 amino acid polypeptide that acts as a cofactor of the NS3 serine protease (Faila, 1994; Bartenschlager *et al*., 1995; Lin *et al*., 1995; Tanji *et al*., 1995; Tomei *et al*., 1996). Moreover, this small protein is involved in the targeting of NS3 to the endoplasmic reticulum resulting in a significant increase of NS3 stability (Wölk *et al*., 2000).

2.2.4.8 NS4B

The NS4B (p27) consists of 217 amino acids. It is an integral membrane protein localised in the endoplasmic reticulum. The N-terminal domain of the NS4B has an amphipathic character that targets the protein to the ER. This domain is crucial in HCV replication (Elazar *et al*., 2004; Gretton *et al*., 2005) and therefore an interesting target for the development of anti-HCV therapeutics or vaccines. In addition, a nucleotide binding motif (aa 129-134) has been identified (Einav *et al*., 2004). NS4B functions have been elucidated as membrane anchoring for the replication complexes; Endoplasmic Reticulum (ER) derived membrane localisation. It serves as a scaffold for the HCV replication complex. It has a possible role in HCV carcinogenesis, impairment of ER function, and regulation of both viral and host translation (Rajyaguru *et al*., 2013).

2.2.4.9 NS5A

The NS5A protein (p56; 458 aa) is a membrane-associated phosphoprotein that appears to have multiple functions in viral replication. It is phosphorylated by different cellular protein kinases. NS5A has been found to be associated with several other cellular proteins (MacDonald *et al*., 2004) making it difficult to determine the exact functions of the protein. One important property of NS5A is that it contains a domain of 40 amino acids, the so-called IFN α sensitivity-determining region (ISDR) that plays a significant role in the response to IFN-α-based therapy (Enomoto *et al*., 1995; Enomoto *et al*., 1996). NS5A is a phosphorylated zinc-metalloprotein. It plays an important role in the regulation of cellular pathways, membrane localisation, transcriptional activation, and assembly of the replication complex (Wang *et al*., 2005a). An increasing number of

mutations within the ISDR showed a positive correlation with sustained virological response to IFN- α-based treatment (Kupfer, 2013).

2.2.4.10 NS5B

The non-structural protein 5B (p66; 591 aa) represents the RNA-dependent RNA polymerase (RdRp) of HCV (Behrens *et al*., 1996). The hydrophobic domain (21 aa) at the C- terminus of NS5B inserts into the membrane of the endoplasmic reticulum, while the active sites of the polymerase are located in the cytoplasm (Schmidt-Mende *et al*., 2001). The cytosolic domains of the viral enzyme form the typical polymerase right-handed structure with “palm”, “fingers”, and“thumb” sub domains (Ago *et al*., 1999; Bressanelli *et al*., 1999; Lesburg *et al*., 1999). In contrast to mammalian DNA and RNA polymerases the fingers and thumb sub domains are connected resulting in a fully enclosed active site for nucleotide triphosphate binding. This unique structure makes the HCV NS5B polymerase an attractive target for the development of antiviral drugs (Kupfer, 2013).

Using the genomic HCV RNA as a template, the NS5B promotes the synthesis of minus-stranded RNA that then serves as a template for the synthesis of genomic positive-stranded RNA by the polymerase. Similar to other RNA-dependent polymerases, NS5B is an error-prone enzyme that incorporates wrong ribonucleotides at a rate of approximately 10^{-3} per nucleotide per generation. Unlike cellular polymerases, the viral NS5B lacks a proof-reading mechanism leading to the conservation of misincorporated ribonucleotides. These enzyme properties together with the high rate of viral replication

promote a pronounced intra-patient as well as inter-patient HCV evolution (Kupfer, 2013).

The RdRp is another important target for the development of anti-HCV drugs (Pawlotsky and McHutchison, 2004; Di Marco *et al.,* 2005; Ma *et al.,* 2005; Pawlotsky, 2006). Interactions between NS5B and cellular components have also been reported. The C-terminus of NS5B can interact with the N-terminus of hVAP-33, and the interaction may play an important role in the formation of the HCV replication complex (Gao *et al.,* 2004; Schmidt-Mende *et al.,* 2001). NS5B was reported to bind cyclophilin B, a cellular peptidyl-prolyl *cis-trans* isomerase that apparently regulates HCV replication through modulation of the RNA binding capacity of NS5B (Watashi *et al.,* 2005).

2.2.4.11 F protein, ARFP

In addition to the ten proteins derived from the long HCV ORF, the F (frame shift) or ARF (alternate reading frame) or core+1 protein has been reported (Walewski *et al*., 2001; Xu *et al*., 2001; Varaklioti *et al*., 2002). As the designations indicate the ARFP is the result of a -2/+1 ribosomal frame shift between codons 8 and 11 of the core protein-encoding region. The ARFP length varies from 126 to 161 amino acids depending on the corresponding genotype. *In vitro* studies have shown that ARFP is a short-lived protein located in the cytoplasm (Roussel *et al*., 2003) primarily associated with the endoplasmic reticulum (Xu *et al*., 2003). Detection of anti-F protein antibodies in the serum of HCV-positive subjects indicates that the protein is expressed during infection *in vivo* (Walewski *et al*., 2001; Komurian-Pradel *et al*., 2004).

2.3 Life Cycle of HCV

The absence of a small animal model system and efficient *in vitro* HCV replication systems has been difficult to investigate the viral life cycle of HCV. The recent development of such systems has offered the opportunity to analyse in detail the different steps of viral replication (Kupfer, 2013). A model of the life cycle of Hepatitis C Virus is shown in Figure 2.3.

2.3.1 Adsorption and Viral Entry

The most likely candidate as a receptor for HCV is the tetraspanin CD81 (Pileri *et al*., 1998). CD81 is a ubiquitous 25 kD molecule expressed on the surface of a large variety of cells including hepatocytes and peripheral blood mononuclear cells (PBMCs). Experimental binding of anti-CD81 antibodies to CD81 were reported to inhibit HCV entry into Huh7 cells and primary human hepatocytes (Hsu *et al*., 2003; Bartosch *et al*., 2003a; Cormier *et al*., 2004; McKeating *et al*., 2004; Zhang *et al*., 2004; Lindenbach *et al*., 2005; Wakita *et al*., 2005). Moreover, gene silencing of CD81 using specific siRNA molecules confirmed the importance of CD81 in viral entry (Bartosch *et al*., 2003b; Cormier *et al*., 2004; Zhang *et al*., 2004; Akazawa *et al*., 2007). Finally, expression of CD81 in cell lines lacking CD81 made them permissive for HCV entry (Zhang *et al*., 2004; Lavillette *et al*., 2005; Akazawa *et al*., 2007). However, studies have shown that CD81 alone is not sufficient for HCV viral entry and that co-factors such as scavenger receptor B type I (SR-BI) are required (Scarselli *et al*., 2002; Bartosch *et al*., 2003b; Hsu *et al*., 2003; Kapadia *et al*., 2007). Moreover, it appears that CD81 is involved in a post-HCV binding step (Cormier *et al*., 2004; Koutsoudakis *et al*., 2006; Bertaud *et al*., 2006).

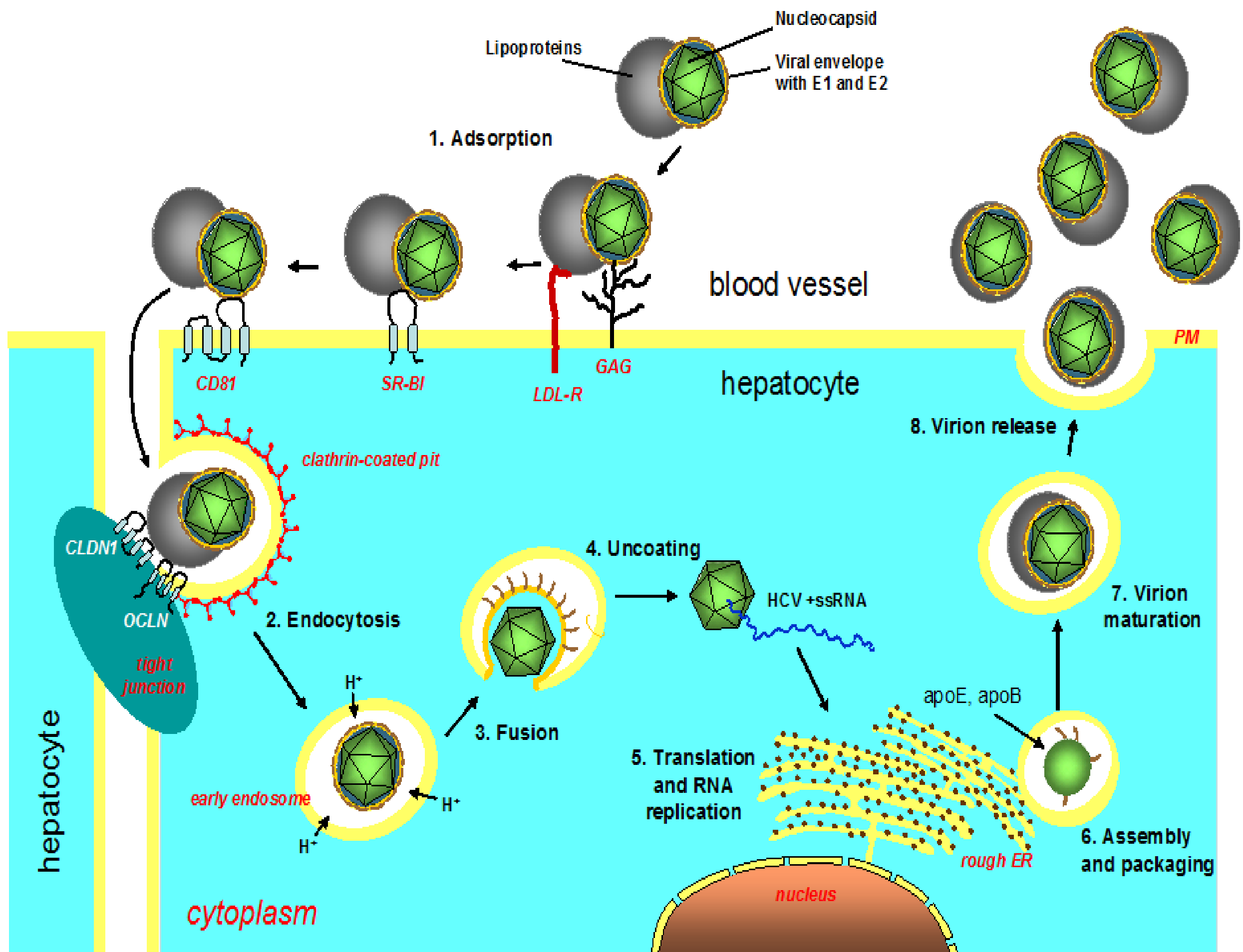

Figure 2.3: A model of the Life Cycle of Hepatitis C Virus (Kupfer, 2013)

These studies together with the identification of other host factors involved in HCV cell entry gave rise to the current model for the early steps of HCV infection (Helle and Dubuisson, 2008).

The first step to HCV viral entry is adsorption. Binding may be initiated by the interaction of the HCV E2 envelope glycoprotein and the glycosaminoglycan heparan sulphate on the surface of host cells (Germi *et al*., 2002; Barth *et al*., 2003; Basu *et al*., 2004; Heo *et al*., 2004). Several studies speculated that HCV initiates hepatocyte infection via LDL receptor binding (Agnello *et al*., 1999; Monazahian *et al*., 1999; Wünschmann *et al*., 2000; Nahmias *et al*., 2006; Molina *et al*., 2007). This process may be mediated by VLDL or LDL which is reported to be associated with HCV virions in human sera (Bradley *et al*., 1991; Thomssen *et al*., 1992; Thomssen *et al*., 1993). After the initial binding, the HCV E2 glycoprotein interacts with the SR-BI in cell culture (Scarselli *et al*., 2002). SR-BI is a protein expressed on the surface of the majority of mammalian cells. It acts as a receptor for LDL as well as HDL (Acton *et al*., 1994; Acton *et al*., 1996). The alternative splicing of the SR-BI transcript can lead to the expression of a second isoform of the receptor SR-BII (Webb *et al*., 1998), which also may take part in HCV entry into target cells (Grove *et al*., 2007). As is the case for all steps involving viral entry, the exact mechanism of the HCVE2/SR-BI interaction is unknown. Some findings reported that HCV binding to SR-BI is a prerequisite for the concomitant or subsequent interaction of the virus with CD81 (Kapadia *et al*., 2007; Zeisel *et al*., 2007).

The steps of HCV cell entry was shown to be even more complex since a cellular factor termed claudin-1 (CLDN1) has been identified as involved in this process (Evans *et al*., 2007). CLDN1 is an integral membrane protein that forms a backbone of tight junctions

and is highly expressed in the liver (Furuse *et al*., 1998). A study using inhibition assays revealed that CLDN1 involvement occurs downstream of the HCV-CD81 interaction (Evans *et al*., 2007). Some findings suggested that CLDN1 could also act as a compound enabling cell-to-cell transfer of hepatitis C virus independently of CD81 (Timpe *et al*., 2007). So also, it has been reported that two other members of the claudin family claudin-6 and claudin-9 may be involved in HCV infection (Zheng *et al*., 2007; Meertens *et al*., 2008). After the complex procedure of binding to the different host membrane factors, HCV enters the cell in a pH-dependent manner showing that the virus is internalised via clathrin-mediated endocytosis (Bartosch *et al*., 2003b; Hsu *et al*., 2003; Blanchard *et al*., 2006; Codran *et al*., 2006). The acidic environment within the endosomes may trigger HCV E1- E2 glycoprotein-mediated fusion of the viral envelope with the endosome membrane (Blanchard *et al*., 2006; Meertens *et al*., 2006; Lavillette *et al*., 2007).

2.3.2 Translation and posttranslational processes

As a result of the fusion of the viral envelope and the endosomal membrane, the genomic HCV RNA is released into the cytoplasm of the cell. The viral genomic RNA possesses a nontranslated region (NTR) at each terminus. The 5'NTR consists of four distinct domains, I-IV. Domains II-IV form an internal ribosome entry side (IRES) involved in ribosome-binding and subsequent cap-independent initiation of translation (Tsukiyama-Kohara *et al*., 1992; Wang *et al*., 1993; Fukushi, 1994; Honda *et al*., 1999). The HCV-IRES binds to the 40S ribosomal subunit complexed with eukaryotic initiation factors 2 and 3 (eIF2 and eIF3), GTP, and the initiator tRNA resulting in the 48S pre-initiation complex (Sizova *et al*., 1998; Hellen and Pestova, 1999; Spahn *et al*., 2001; Otto *et al*., 2002). Afterward, the 60S ribosomal subunit associated with that complex leading to the

formation of the translational active complex for HCV polyprotein synthesis at the endoplasmic reticulum. HCV RNA contains a large ORF encoding a polyprotein precursor. Posttranslational cleavages lead to 10 functional viral proteins Core, E1, E2, p7, NS2-NS5B. The viral F protein (or ARF protein) originates from a ribosomal frame shift within the first codons of the core-encoding genome region (Walewski *et al*., 2001; Xu *et al*., 2001; Varaklioti *et al*., 2002).

Apart from several other cellular factors that have been reported to be involved in HCV RNA translation, various viral proteins and genome regions have been shown to increase or inhibit viral protein synthesis (Zhang *et al*., 2002; Kato *et al*., 2002; Wang *et al*., 2005b; Kou *et al*., 2006; Bradrick *et al*., 2006; Song *et al*., 2006). The precursor polyprotein is processed by at least four distinct peptidases. The cellular signal peptidase (SP) cleaves the N-terminal viral proteins immature core protein, E1, E2, and p7 (Hijikata *et al*., 1991), while the cellular signal peptide peptidase (SPP) is responsible for the cleavage of the E1 signal sequence from the C-terminus of the immature core protein, resulting in the mature form of the core protein (McLauchlan *et al*., 2002). The E1 and E2 proteins remain within the lumen of the ER where they are subsequently N-glycosylated with E1 having 5 and E2 harbouring 11 putative N-glycosylation sites (Duvet *et al*., 2002).

In addition to the two cellular peptidases, HCV encodes two viral enzymes responsible for cleavage of the non-structural proteins NS2 to NS5B within the HCV polyprotein precursor. The zinc-dependent NS2-NS3 cysteine protease consisting of the NS2 protein and the N-terminal portion of NS3 autocatalytically cleaves the junction between NS2 and NS3 (Santolini *et al*., 1995), whereas the NS3 serine protease cleaves the remaining

functional proteins (Bartenschlager *et al*., 1993; Eckart *et al*., 1993; Grakoui *et al*., 1993a; Tomei *et al*., 1993). However, for its peptidase activity, NS3 needs NS4A as a cofactor (Failla *et al*., 1994; Tanji *et al*., 1995; Bartenschlager *et al*., 1995; Lin *et al*., 1995; Tomei *et al*., 1996).

2.3.3 HCV RNA replication

The complex process of HCV RNA replication is poorly understood. The key enzyme for viral RNA replication is NS5B, an RNA-dependent RNA polymerase (RdRp) of HCV (Behrens *et al*., 1996). In addition, several cellular, as well as viral factors have been reported to be part of the HCV RNA replication complex. One important viral factor for the formation of the replication complex appears to be NS4B which is able to induce an ER-derived membranous web containing most of the non-structural HCV proteins including NS5B (Egger *et al*., 2002). This web could serve as the platform for the next steps of viral RNA replication. The RdRp uses the previously released genomic positive-stranded HCV RNA as a template for the synthesis of an intermediate minus-stranded RNA. Findings has reported that the cellular peptidyl-prolyl isomerases cyclophilin A, B and C (Cyp A, Cyp B, and Cyp C) could stimulate binding of the RdRp to the viral RNA resulting in increased HCV RNA synthesis (Watashi *et al*., 2005; Nakagawa *et al*., 2005; Yang *et al*., 2008; Heck *et al*., 2009).

However, these findings are in part inconsistent and further studies are needed in order to investigate the involvement of cyclophilins in HCV RNA replication. After the viral polymerase has bound to its template the NS3 helicase is assumed to unwind putative secondary structures of the template RNA in order to facilitate the synthesis of minus-

strand RNA (Jin and Peterson, 1995; Kim *et al*., 1995). In turn, again with the assistance of the NS3 helicase, the newly synthesised antisense RNA molecule serves as the template for the synthesis of numerous plus-stranded RNA. The resulting sense RNA may be used subsequently as genomic RNA for HCV progeny as well as for polyprotein translation (Kupfer, 2013).

2.3.4 Assembly and release

After the viral proteins, glycoproteins, and the genomic HCV RNA have been synthesised these single components have to be arranged in order to produce infectious virions. As is the case for all other steps in the HCV lifecycle, the viral assembly is a multi-step procedure involving most viral components along with many cellular factors. Investigation of viral assembly and particle release is still in its infancy since the development of *in vitro* models for the production and release of infectious HCV occurred only recently. Previously, it was reported that core protein molecules were able to self-assemble *in vitro*, yielding nucleocapsid-like particles. Findings suggest that viral assembly takes place within the endoplasmic reticulum (Gastaminza *et al*., 2008) and that lipid droplets (LD) are involved in particle formation (Moradpour *et al*., 1996; Barba *et al*., 1997; Miyanari *et al*., 2007; Shavinskaya *et al*., 2007; Appel *et al*., 2008). It appears that LD-associated core protein targets viral non-structural proteins and the HCV RNA replication complex including positive and negative stranded RNA from the endoplasmic reticulum to the LD (Miyanari *et al*., 2007).

Besides the core protein, LD-associated NS5A seems to play a key role in the formation of infectious viral particles (Appel *et al*., 2008). Moreover, E2 molecules are detected in

close proximity to LD associated membranes. Spherical virus-like particles associated with membranes can be seen very close to the LD. Using specific antibodies, the virus-like particles were shown to contain core protein as well as E2 glycoprotein molecules showing that these structures may represent infectious HCV (Miyanari *et al.*, 2007). However, the precise mechanisms for the formation and release of infectious HCV particles are still unknown (Kupfer, 2013).

2.4 Clinical Symptoms and Signs

Many people with chronic hepatitis C have no symptoms of liver disease. If symptoms are present, they are usually mild, nonspecific, and intermittent. They may include fatigue, mild right-upper-quadrant discomfort or tenderness, nausea, poor appetite as well as muscle and joint pains. Similarly, the physical examination is likely to be normal or show only mild enlargement of the liver or tenderness and vascular spiders or palmer erythema. Once a patient develops cirrhosis or if the patient has severe disease, symptoms and signs of muscle weakness, poor appetite, nausea, weight loss, dark urine, fluid retention, itching and abdominal swelling are commonly observed. Physical findings of cirrhosis may include enlarged liver, enlarged spleen, jaundice, muscle wasting, excoriations, ascites and ankle swelling (Berry *et al.*, 2005).

Complications that do not involve the liver, extrahepatic manifestations develop in 1 to 2 percent of people with hepatitis C. The most common is cryoglobulinemia, which is marked by skin rashes (purpura, vasculitis, or urticaria), muscle and joint aches, kidney disease, neuropathy, rheumatoid factor, cryoglobulins and low complement levels in serum. Other complications are glomerulonephritis, porphyria cutanea tarda, seronegative

arthritis, keratoconjunctivitis sicca (Sjogren's syndrome), non-Hodgkin's type B-cell lymphomas, fibromyalgia and lichen planus (Berry *et al.*, 2005). However, there is considerable variation in the clinical manifestations of HCV, and there are no specific symptoms distinguishing one type of hepatitis from another (Lanphear *et al.*, 1994)

2.5 Epidemiology

Hepatitis C is a disease with a significant global impact. According to the World Health Organization, there are 130-170 million people infected with the hepatitis C virus (HCV), corresponding to 2-2.5% of the world's total population. There are considerable regional differences (WHO, 2011). High seroprevalence is observed in Asian and African countries, whereas the developed world including North America, northern and western Europe, and Australia have a low prevalence (Alter, 2007; Lemon *et al*., 2007). In developing countries, the seroprevalence of HCV displays a high range of variability, ranging from 0.9% in India to higher prevalence from 2.1-6.5% in many countries. Egypt has a reported seroprevalence of about 22% and is the highest in the world (Alter, 2007).

It is difficult to determine the number of new HCV infections, as most acute cases are not noticed clinically. Fewer than 25% of acute cases of hepatitis C are clinically apparent (Vogel *et al.,* 2009). In addition, the age of infection upon diagnosis is not possible to determine in most cases. Nevertheless, it has to be assumed that the number of new infections has considerably decreased over the past decades. In the US it is estimated that the number of new cases of acute HCV infection has fallen from approximately 230,000 per year in the 1980s to about 20,000 cases per year (Wasley *et al.,* 2008). This decrease is primarily associated with reduced infections in injection drug users, a probable consequence of changes in injection practices motivated by education about human

immunodeficiency virus (HIV) transmission. Transfusion-associated hepatitis C has had little impact on this decline, as the number of cases has been reduced almost to zero. The only different trend is an increase in acute hepatitis C infections in HIV-positive men who have sex with men (MSM) globally over the last decade (Boesecke and Vogel, 2011). Recent numbers from Europe show an ongoing epidemic of acute HCV especially among intravenous drug users and MSM (Rockstroh *et al.,* 2012).

In Africa and the Western Pacific, prevalence is significantly higher than in North America and Europe (CDC, 2012). It is estimated that there are 2-5 million HCV-positive persons in Europe. Certain groups are preferentially affected, like injection drug users. In Europe and the United States, chronic hepatitis C is the most common chronic liver disease. The majority of liver transplants performed in these regions are for chronic HCV. It is difficult to determine the number of new HCV infections, as most acute cases are not noticed clinically. Recent numbers from Europe still show an ongoing epidemic of acute HCV especially among IVDU and MSM (Rockstroh *et al.,* 2012).

2.5.1 Prevalence

Most descriptions of global HCV epidemiology rely heavily upon HCV seroprevalence studies. These studies are typically cross-sectional in design and are done in selected populations—e.g., blood donors or patients with chronic liver disease—which are not representative of the community or region in which they reside. Population-based studies representative of an entire community are far more useful, but this kind of study is not feasible in most parts of the world (Shepard *et al.,* 2005).

Nonetheless, for several years WHO has reported data on the worldwide prevalence of HCV infection, based on both published studies and submitted data. Although HCV is endemic worldwide, there is a large degree of geographic variability in its distribution. Countries with the highest reported prevalence rates are located in Africa and Asia; areas with lower prevalence include the industrialised nations in North America, northern and western Europe, and Australia (Shepard *et al.,* 2005).

2.5.2 Incidence and trends in HCV infection

Although HCV infection has both acute and chronic forms, most of the morbidity associated with infection is realised through the development of chronic liver disease in a subset of infected people years after initial the acquisition of the infection. Thus, a major determinant of the future burden of disease is the past and present incidence of infection (Armstrong, 2003). However, establishing the incidence of HCV infection is difficult because most infections are initially asymptomatic and available assays do not distinguish acute from chronic or resolved infection. Acute disease reporting systems can underestimate the incidence of HCV infection, even in countries with well-established surveillance systems (Hagan *et al*., 2002). Because the direct measurement of HCV infection incidence is impractical, researchers have relied upon mathematical models to infer trends in incidence. These undertakings have occurred primarily in developed countries where population-based age-specific seroprevalence data are available and rely on the assumption that current prevalence reflects the cumulative risk of acquiring infection (Shepard *et al.,* 2005).

2.6 Transmission

The primary route of transmission of HCV in the developed countries is intravenous drug use, while in the developing countries the main methods are blood transfusions and unsafe medical procedures (Maheshwari and Thuluvath, 2010). So also, the cause of transmission remains unknown in 20% of cases (Ponde, 2011).

2.6.1 Intravenous drug use (IDU)

IDU is a major risk factor for hepatitis C in many parts of the world (Xia *et al*., 2008). Of 77 countries reviewed in a study, 25 countries (including the United States) were found to have prevalences of hepatitis C in the intravenous drug user population of between 60% and 80% (Xia *et al.*, 2008; Nelson *et al*., 2011). It is believed that 10 million intravenous drug users are infected with hepatitis C; China (1.6 million), the United States (1.5 million), and Russia (1.3 million) have the highest absolute totals (Nelson *et al*., 2011). The occurrence of hepatitis C among prison inmates in the United States is 10 to 20 times that observed in the general population; this has been attributed to high risky behaviour in prisons such as IDU and tattooing amongst the inmates with nonsterile equipment (Vescio *et al*., 2008; Imperial, 2010).

Injection drug use has been the most commonly identified source of acute HCV infection. It is estimated that most newly acquired infections occur in individuals who have injected illegal drugs. The seroprevalence of anti-HCV antibodies in groups of intravenous drug users may be up to 70% with considerable variation depending on factors such as region, risk behaviour, socioeconomic status and others, underscoring the efficiency of transmission via direct blood contact (Sutton *et al.*, 2008). HCV infection also has been associated with a history of intranasal cocaine use, presumably due to blood on shared

straws or other sniffing paraphernalia. This may explain partly the recent increase in cases of acute HCV infections in HIV-positive MSM (Schmidt *et al.,* 2011; Rockstroh *et al.* 2012).

2.6.2 Blood transfusion

In the past, blood transfusion or use of other blood products was a major risk factor for transmission of HCV. In some historic cohorts, 10% or more of patients who received blood transfusions were infected with hepatitis C (Alter *et al.*, 1989). However, blood donor screening for HCV since the early 1990s has nearly eliminated this transmission route. Blood donors are screened for anti-HCV antibodies and HCV RNA – at least in developed countries. The risk is estimated to be between 1:500,000 and 1:1,000,000 units (Pomper *et al*., 2003). In cohorts of multiply transfused patients such as haemophiliacs, over 90% of patients were infected with hepatitis C in the past (Francois *et al*., 1993). With current screening methods, HCV transmission through transfusion of contaminated blood products is approximately 1 in 500,000 to 2,000,000 transfusions (Schreiber *et al*., 1996; Dodd *et al*., 2002; Roth *et al*., 2002).

Blood transfusion, transfusion of blood products, or organ transplants without HCV screening carry significant risks of infection (Wilkins *et al*., 2010). The United States instituted universal screening in 1992 (Marx, 2010) and Canada instituted universal screening in 1990 (Day *et al*., 2009). This decreased the risk from one in 200 units (Marx, 2010), to between one in 10,000 to one in 10,000,000 per unit of blood (Ponde, 2011). This low risk remains as there is a period of about 11–70 days between the potential blood donor acquiring hepatitis C and their blood testing positive depending on the

method (Ponde, 2011). Some countries do not screen for hepatitis C due to the cost (Alter, 2007).

Those who have experienced a needle stick injury from someone who was HCV positive have about a 1.8% chance of subsequently contracting the disease themselves (Wilkins *et al*., 2010). There is a risk from mucosal exposures to blood, although this risk is low and there is no risk if blood exposure occurs on intact skin. Hospital equipment has also been documented as a means of transmission of hepatitis C and these include the reuse of needles and syringes, multiple-use medication vials, infusion bags, and improperly sterilised surgical equipment, among others (Alter, 2007).

2.6.3 Organ transplantation

Transplant recipients who receive organs from HCV-positive donors have a high risk of acquiring HCV infection. Transmission rates in different cohorts vary from 30 to 80% (Pereira 1991, Roth, 1994). Therefore, most transplant organisations have developed strategies for screening and selective utilisation of organs from anti-HCV positive donors (*Boesecke and* Wasmuth, 2013).

2.6.4 Haemodialysis

Patients who participate in chronic haemodialysis programs are at increased risk for hepatitis C. The prevalence of HCV antibodies in such patients reaches 15%, although it has declined in recent years (Fissell *et al.*, 2004). A number of risk factors have been identified for HCV infection among dialysis patients. These include blood transfusions, duration of haemodialysis, the prevalence of HCV infection in the dialysis unit, and type

of dialysis. The risk is higher with in-hospital haemodialysis as opposed to peritoneal dialysis (Boesecke and Wasmuth, 2013).

2.6.5 Sexual intercourse

Whether hepatitis C can be transmitted through sexual activity is controversial (Tohme and Holmberg, 2010). While there is an association between high-risk sexual activity and hepatitis C, it is not known whether transmission of the disease is due to drug use that has not been admitted to or sex as risk factors (Wilkins *et al*., 2010). Most studies reported that there is being no risk for monogamous heterosexual couples. Sexual practices that involve higher levels of trauma to the anogenital mucosa, such as anal penetrative sex or that occur when there is a concurrent sexually transmitted infection, including HIV or genital ulceration, do present a risk (Tohme and Holmberg, 2010).

2.6.6 Body modification

Tattooing is associated with two to threefold increased risk of hepatitis C, this can be due to either improperly sterilised equipment or contamination of the dyes being used. Tattoos or piercings performed either before the mid-1980s, "underground," or nonprofessionally are of particular concern, since sterile techniques in such settings may be lacking. The risk also appears to be greater for larger tattoos. It is estimated that nearly half of prison inmates share unsterilized tattooing equipment (Jafari *et al*., 2010).

2.6.7 Vertical transmission

Vertical transmission of hepatitis C from an infected mother to her child occurs in less than 10% of pregnancies and there are no measures that alter this risk (Lam *et al*., 2010).

It is not clear when during pregnancy transmission occurs, but it may occur both during gestation and at delivery (Pondé, 2011). A long labour is associated with a greater risk of transmission (Alter, 2007). Another main risk factor identified for vertical transmission was maternal hepatitis C viremia. For mothers who tested positive for HCV- RNA, vertical transmission was significantly higher at 7.1% when compared with 0% transmission for those who tested HCV-RNA negative antenatally (Cottrell *et al.*, 2013).

2.6.8 Perinatal transmission

The risk of perinatal transmission of HCV in HCV RNA- positive mothers is estimated to be 5% or less (Ohto, 1994). In mothers coinfected with HIV this risk correlates with immunosuppression and has been described to reach up to 20%. There are no specific recommendations for prevention of perinatal transmission (Pembrey, 2005). Caesarian section has not been shown to reduce the transmission risk. There is no evidence that breastfeeding is a risk for infection among infants born to HCV-infected women. Early diagnosis of infection in newborns requires HCV RNA testing since anti-HCV antibodies are passively transferred from the mother (*Boesecke and* Wasmuth, 2013).

The baby's risk of acquiring HCV from a mother infected with HCV is not increased by mode of delivery or breast- feeding, while vaginal delivery may increase the risk of HCV transmission if the mother is co-infected with detectable HIV viral load (European Paediatric Hepatitis C Virus Network, 2001). Since there is no evidence that breastfeeding spreads HCV, however, to be cautious, an infected mother is advised to avoid breastfeeding if her nipples are cracked and bleeding (Mast, 2004) or if her viral loads are high (Ponde, 2011).

2.6.9 Other rare transmission routes

Rare sources of percutaneous transmission of HCV are contaminated equipment that are used during medical procedures, procedures involved in traditional medicine (e.g., scarification, cupping), tattooing, and body piercing (Haley, 2001). Personal care items such as toothbrushes, razors and manicuring or pedicuring equipment can be contaminated with HCV-infected blood. Sharing these items can potentially lead to exposure to HCV (Lock *et al.*, 2006; http://www.cdc.gov/hepatitis/HCV/HCVfaq.htm). Appropriate caution should be taken with respect to any medical condition that results in bleeding, such as cuts and sores. HCV is not spread through casual contacts such as hugging, kissing, or sharing eating or cooking utensils (http://www.cdc.gov/hepatitis/HCV/HCVfaq.htm). Neither can it be transmitted through food or water (Wong and Lee, 2006).

2.7 Pathogenesis and Natural History

Hepatitis C virus in 60-80% patients is able to escape innate and adaptive immune surveillance. Thus it establishes itself as an agent of chronic hepatitis. Cytotoxic lymphocytes then contribute to liver injury in an attempt to eradicate the virus. On the other hand, strong multispecific T-lymphocytes reaction against HCV proteins is associated with viral clearance. Both CD4+ and CD8+ lymphocyte functions are important to affect this outcome. In chronic infection with HCV, genetic and environmental factors play a vital role in the progression of inflammation and fibrosis in individual patients and of these factors, age, gender, race and alcohol use are the most established ones. The development of hepatocellular carcinoma is mainly restricted to patients with cirrhosis (Kohla and Bonacini, 2006).

2.7.1 Acute hepatitis C

Acute hepatitis C infection is infrequently diagnosed because the majority of acutely infected individuals are asymptomatic. In the transfusion setting, where the acute onset of HCV infection has been best documented, 70% to 80% of cases were asymptomatic (McCaughan *et al.*, 1992). Of the adults with acute HCV infection about 20% to 30% may develop clinical symptoms and the symptomatic onset ranges from 3 to 12 weeks after exposure (Alter and Seeff, 2000; Thimme *et al.*, 2001). Symptoms may include malaise, weakness, anorexia, and jaundice. Serum alanine aminotransferase (ALT) levels which signify hepatocyte necrosis begin rising 2 to 8 weeks after exposure and it often attains levels of greater than 10 times the upper limits of normal. HCV RNA can be detected in the serum samples within 1 to 2 weeks after exposure (Farci *et al.*, 1991; Thimme *et al.*, 2001).

The level of HCV RNA rises rapidly during the first few weeks, and then peaks between 10^5 to 10^7 IU/ml, shortly before the peak of serum aminotransferase levels and the onset of symptoms. In self-limited cases of acute hepatitis C, symptoms can last several weeks and subside as the ALT and HCV RNA levels decline. Acute HCV infection can be severe, while fulminant liver failure is rare (Farci *et al.*, 1996). The antibody to HCV is detected by enzyme immunoassay and it becomes positive near the onset of symptoms and about 1 to 3 months after exposure. Up to 30% of patients will test negative for antibody to HCV at the onset of their symptoms, making anti-HCV testing method unreliable in the diagnosis of acute infection (Farci *et al.*, 1991). Almost all patients will eventually develop the antibody to HCV; however, titers can be low or undetectable in

immunocompromised patients. The anti-HCV assay detects greater than 90% of HCV infections after the initial 3 months (Chen and Morgan, 2006).

2.7.2 Chronic hepatitis C

Chronic hepatitis C is characterised by the persistence of HCV RNA in the blood of an infected person for at least 6 months after onset of acute infection. HCV is self-limiting in only 15%-25% of patients in whom HCV RNA in the serum becomes undetectable and ALT levels return to normal. About 75%-85% of infected patients do not clear the virus by 6 months, and chronic hepatitis develops. The rate of chronic HCV infection is affected by many factors, including the age at the time of infection, gender, ethnicity, and the development of jaundice during the acute infection (Chen and Morgan, 2006).

2.7.3 Progression of liver fibrosis

In the setting of persistent hepatitis C viremia, the rate of progression of liver fibrosis varies widely. There have been extensive studies on the natural course of disease progression from chronic hepatitis C to cirrhosis, HCC, and death. The liver fibrosis is variable in chronic HCV infection. Fibrosis implies possible progression to cirrhosis. In mild cases, fibrosis is limited to the portal and periportal areas of the liver. More advanced changes are defined by fibrosis that extends from one portal area to another, also known as "bridging fibrosis." Cirrhosis develops in approximately 10% to 15% of individuals with chronic HCV infection (National Institutes of Health Consensus Development Conference Statement, 2002). There are external and host factors that can increase the risk of progression of liver disease. Multiple studies have shown that chronic alcohol use is a major external risk factor for the progression of chronic hepatitis C to

cirrhosis and HCC. Host risk factors include older age at time of infection, male gender, the degree of inflammation and fibrosis present on the liver biopsy, coinfection with human immunodeficiency virus (HIV) or hepatitis B virus (HBV), and comorbid conditions such as immunosuppression, insulin resistance, non-alcoholic steatohepatitis, hemochromatosis, and schistosomiasis (Chen and Morgan, 2006).

2.7.4 Extrahepatic manifestations

Chronic HCV infection has been associated with numerous extrahepatic manifestations. These manifestations can involve multiple organ systems, including renal, dermatologic, haematologic, and rheumatologic systems (Gumber and Chopra, 1995; El-Serag *et al.*, 2002). Approximately 1%-2% of HCV-infected individuals will develop extrahepatic manifestations. The most common extrahepatic condition is mixed cryoglobulinemia. Cryoglobulins are found in 50% of patients with chronic HCV infection. Cryoglobulinemia was reported to be 11 times more frequent in a veterans HCV cohort group than their respective control population (El-Serag *et al.*, 2002). Only 25%-30% of HCV patients with mixed cryoglobulinemia develop clinical symptoms which include fatigue, skin rashes, purpura, arthralgias, Raynaud's phenomenon, vasculitis, renal disease, and peripheral neuropathies (Cacoub *et al.*, 2000). The clinical manifestations are thought to be caused by immune complex deposition in several organs. Severe symptoms from cryoglobulinemia appear to respond to interferon treatment, but relapse occurs frequently once treatment is stopped (Lunel and Cacoub, 1999).

Other extrahepatic manifestations that are frequently found in patient with chronic HCV infection are membranoproliferative glomerulonephritis, porphyria cutaneous tarda,

lichen planus, and vitiligo. There is also some data that suggests an association between chronic HCV infection and non-Hodgkin's and Hodgkin's lymphoma, autoimmune thyroiditis, Sjogren's syndrome, seronegative arthritis and diabetes mellitus. It is unclear if these associated diseases are caused directly from HCV infection or from the underlying immune stimulation caused by chronic infection (Berry *et al.*, 2005; Chen and Morgan, 2006).

2.8 Laboratory Diagnosis of Hepatitis C Virus

2.8.1 Serologic assays

Diagnosis of HCV infection is mainly based on the detection of anti-HCV antibodies as a screening test with serum samples. The Architect HCV antigen assay is highly specific, sensitive, reliable, easy to perform, reproducible, cost-effective, and applicable as a screening, supplemental, and pre confirmatory test for anti-HCV assays used in laboratory procedures for the diagnosis of hepatitis C virus infection (Kesli *et al.*, 2011). Commercial Enzyme Immunoassays (EIA) uses a mixture of recombinant proteins and synthetic peptide antigens from different HCV coding regions captured on microtiter plate wells (Chevaliez and Pawlotsky, 2005).

2.8.2 Enzyme immunoassays

Three generations of EIA have been developed over time in order to improve the sensitivity and specificity of the test kits in assessing immunocompetent patients (Prats, 2005) by the introduction of new HCV proteins, increasing the reliability of the test and increasing the detection of anti- HCV at an earlier stage (Alborino *et al.*, 2011). First

generation EIAs used the c100.3 epitope of an NS4 protein (Kuo *et al.*, 1989). The sensitivity of these EIAs was low for a high prevalence population (approximately 80%) and the fraction of positive results that were false positive were as high as 70% for a low-prevalence population such as blood donors (Gretch, 1997). Second generation assays detect HCV antibodies in 20% more patients with acute non- A, non- B hepatitis and in 10% more patients with chronic infection much earlier (Alter, 1992). The second generation EIAs incorporated the c22-3 antigen derived from the core region of the HCV genome and the c33c antigen derived from the NS3 region in addition to the 5-1-1 and c100-3 antigens (Germer and Zein, 2001).

The third generation test (EIA-3) used today is more sensitive and specific than the previous ones. However, as with all enzyme immunoassays, false-positive results are occasionally a problem with an EIA-3, additional or confirmatory testing is often helpful (Berry *et al.*, 2005). Third-generation commercial EIA uses recombinant NS4 proteins (C100-3), nonstructural regions (NS3 and NS4) with antigens from the core region and NS5 (Colin *et al.*, 2001). The second and third generation assays in blood banks have dramatically reduced the incidence of post-transfusion hepatitis (Dow *et al.*, 1994).

CDC guidelines recommend a specific signal-to- cut-off (s/co) ratio for each test that would predict a true antibody-positive result ≥ 95% of the time, regardless of the characteristics of the population being tested; this is to decrease the number of the samples that need a confirmatory test (Alter *et al.*, 2003).

The limitations of HCV-Abs tests are: (1) In patients with acute HCV infection, it may remain undetectable for between 45-68 days -“the window period” (Kesli *et al.*, 2011);

(2) The high rate of false positives due to the multiple presence of circulating immunoglobulins (Chou *et al.*, 2004); (3) There may be a negative result in patients who are immunocompromised – so a negative result does not rule out exposure or infection. In the general population, and among blood donors, the specificity is lower - thus blood banks use recombinant immunoblot techniques to confirm the EIA results (Ferreira-Gonzalez and Shiffman, 2004). Automated EIAs for the detection of anti-HCV are widely-used in high-volume clinical laboratories and these instruments offer excellent precision and reliability, as well as high-speed throughput, random access and technical simplicity (Cabezas-Fernandez and Cabeza-Barrera, 2012).

2.8.3 Supplementary test (Immunoblot)

Recombinant immunoblot assays (RIBAs) are used as confirmatory tests in situations when EIA are prone to cases of repeated false positive results. Immunoblot assays use artificial HCV proteins, recombinant proteins and/or synthetic peptides that are separately coated on a nitrocellulose strip; except the Murex assay, where a mixture of 4 recombinant HCV antigens are electrophoresed on polyacrylamide gel then electroblotted to nitrocellulose. Third-generation RIBA strip assays use two recombinant antigens c33c (NS3) and NS5 and two synthetic peptides c100 (NS4) and 5-1-1 (NS4) derived from putative nonstructural regions of the virus, while the third peptide c22 corresponds to the nucleocapsid (core) viral protein (Courouce *et al.*, 1998). Assessment of the band reactivity is graded by visual calibration against immunoglobulin G control bands present on each strip. A sample is considered positive when at least two HCV bands have 1+ or greater reactivity, or indeterminate when only a single HCV band has 1+ or greater

reactivity, whilst a sample is considered negative when no HCV bands have 1+ or greater reactivity (Makuria *et al.*, 2012).

The main disadvantage of RIBA is the occurrence of indeterminate results in problematic samples, although some new improved third-generation RIBA assays have changed the positivity criteria, reducing the frequency of indeterminate results, so these are currently being carried out by molecular methods (Carreno-Garcia *et al.*, 2011).

2.8.4 Core antigen

The detection of the HCV core Ag (HCVcAg) can be used for the detection of HCV infection in the "window period" by a conventional ELISA (Bouvier-Alias *et al.*, 2002) because it appears in the serum from 1 day before the appearance of HCV RNA, and 45 days sooner than HCV antibodies (Peterson *et al.*, 2000; Leary *et al.*, 2006). It is also more stable after freezing or heating than HCV RNA (Tanaka *et al.*, 2003]. Two types of test have been developed: HCV antigens can be detected either as a stand alone antigen test or as an antigen/antibody combination test during the "window period" or during active viral replication after the development of antibodies (Muerhoff *et al.*, 2002; Laperche *et al.*, 2005; Leary *et al.*, 2006).

2.8.5 Molecular HCV assays

Molecular assays are crucial for the management of HCV- infected patients because RNA can be detected 10-14 days after infection and approximately 1 month before the appearance of antibodies (Carithers *et al.*, 2000).

Quantitative and qualitative HCV RNA assays are based on the amplification of the target region of the HCV genome [transcription-mediated amplification (TMA), classical

reverse-transcription polymerase chain reaction and real-time (RT-PCR)] or amplification of the signal (branched DNA, bDNA) (Werhren *et al.*, 2008; Le Guillou-Guillemette and Lunel-Fabiani, 2009).

2.8.5.1 Qualitative HCV RNA (RNA Detection)

Qualitative assays detect viral genomes and are used to define active infection and also to confirm EIA results (diagnosis and monitoring) as well as for the screening of blood donors (Cabezas-Fernandez and Cabeza-Barrera, 2012).

The COBAS Amplicor HCV Test v2.0 is performed on the COBAS AMPLICOR Analyser (Roche Molecular systems) for the detection of HCV RNA. The HCV RNA is isolated from virions by lysis of viral particles and alcohol precipitation. An internal control in form of an RNA script with primer binding identical regions to those of the HCV target is introduced into each sample. The test uses reverse transcription of target RNA to produce complementary DNA (cDNA), which is denatured by heating, and then the target cDNA is amplified by the Polymerase Chain Reaction and the nucleic acid is hybridised for the detection of HCV RNA in human serum or plasma. In the last phase of the reaction, a coloured complex is formed, which is measured at 660 nm (Krajden *et al.*, 2002).

A transcription-mediated amplification (TMA) is an isothermal nucleic acid amplification process that involves a more complex set of reactions with the reverse transcriptase and T7 RNA polymerase. In the Versant HCV RNA Qualitative Assay (Siemens Molecular), sample preparation, target amplification and amplicon detection are carried out in a single tube. After a lysis step, target HCV RNA is captured by magnetic particles coated with

oligonucleotides complementary to the 5' UTR of the HCV genome. An internal control is added to each sample to monitor target capture and amplification. The target RNA is then amplified with an isothermal TMA process that needs the addition of primers, reverse transcriptase, and T7 RNA polymerase. Some of the newly synthesised RNA amplification products re-enter the TMA process and serve as templates for new rounds of amplification. TMA labelled oligonucleotide probes are used to emit a chemiluminescent signal in order to detect the amplified product (Krajden *et al.*, 2002). The LLOD is 5 IU/ml and 10 IU/ml with sensitivity from 96% to 100%, respectively. It is independent of HCV genotypes (Krajden *et al.*, 2002; Hendricks *et al.*, 2003).

2.8.5.2 Quantitative HCV RNA

These methods for accurate quantification of HCV RNA levels have become key tools in the clinical management of patients under treatment and to predict the response probability to combination interferon/ribavirin therapy by assessing rates of HCV viral load decline as well as to complement the information provided by HCV genotype determination (Ghany *et al.*, 2009).

The quantification of HCV RNA can be determined by target amplification techniques (Real-Time PCR assays), or by signal amplification methods (branched DNA- bDNA Assay). Several FDA- and CE-approved standardised systems are commercially available. The Cobas Amplicor™ HCV Monitor (Roche Diagnostics) is based on a competitive PCR technique whereas the Versant™ 440 HCV RNA Assay (Siemens Healthcare Diagnostics) is based on a bDNA technique. Both assays have restricted lower limits of detection (500-615 IU/ml). The Cobas TaqMan assay and the Abbott RealTime™ HCV test, both of which are based on real-time PCR technology were

introduced not long ago and has now replaced the qualitative and quantitative methods that were in use. All commercially available HCV RNA assays are calibrated to the WHO standard based on HCV genotype 1. It has been shown that results may vary significantly between assays with different HCV genotypes despite standardisation (Chevaliez *et al.*, 2007; Vehrmeren *et al.*, 2008). The Abbott RealTime™ HCV Test provides a lower limit of detection of 12 IU/mL, a specificity of more than 99.5 % and a linear amplification range from 12 to 10,000,000 IU/mL independent of the HCV genotype (Michelin *et al.*, 2007; Sabato *et al.*, 2007).

VERSANT kPCR Molecular System Siemens Healthcare Diagnostics is also available as a real- time PCR system for quantification of HCV RNA. Rotor-Gene Q real- time PCR device and Qiagen HCV RNA kits (Qiagen GmbH, Germany) are used for quantification of HCV RNA by real- time PCR method with a specificity of 99.0 %, a lower limit of detection 34 IU/ml and capable of detecting up to 10, 000,000 IU/mL. In certain situations, HCV RNA result can be negative in persons with active HCV infection. As the titre of anti-HCV increases during acute infection, the level of circulating HCV RNA declines; intermittent HCV RNA positivity has been observed among persons with chronic HCV infection. A negative HCV RNA result can also indicate resolved infection. Follow-up HCV RNA testing is indicated only in persons with serologically confirmed anti-HCV positive results (Chevaliez and Pawlotsky, 2005; Fiebelkorn and Nolte, 2004)

2.8.5.3 Genotyping of hepatitis C virus

Knowing the infecting genotype has a direct impact on the prognosis and on the choice and duration of the treatment algorithm as well as being a statistically significant predictor of SVR to antiviral therapy (Al Olaby and Azzazy, 2011).

The reference method for HCV molecular typing is genome sequencing and subsequent phylogenetic analysis but this method cannot be used in clinical laboratories. Most of the commercial genotyping methods are based on the detection of the 5'UTR region, which is amplified easily and has enough polymorphism to distinguish between different genotypes. The NS5B region is amplified with difficulty, mainly genotype 4 (Laperche *et al.*, 2005). The serological method has lower sensitivity and specificity than molecular assays (Gault *et al.*, 2003). Firstly, in these assays, the subgenomic region is amplified, determined by polymerase chain reaction in real time (RT-PCR), after which the different types are discriminated by direct sequencing (Simmonds *et al.*, 1993) –"TRUGENE HCV 5'NC region genotyping assay; Visible Genetics, Canada", followed by reamplification with genotype-specific primers or subtype (Okamoto *et al.*, 1992); the reverse hybridization of amplicons with immobilized membrane (INNO-LiPA HCV I/II/ VERSANT HCV genotype assay 2.0; Innogenetics, N.V./Siemens Medical Solutions Healthcare) with either genotype-specific probes or subtype (Stuyver *et al.*, 1996); Restriction fragment length polymorphism DNA (RFLP) (Nakao *et al.*, 1991); heteroduplex mobility analysis using capillary electrophoresis gradient temperature (White *et al.*, 2000) or dissociation curve analysis using fluorescent probes- fluorescence energy transfer resonance (FRET) technology (Schroter *et al.*, 2002).

Direct Nucleic Acid Sequencing: The product amplified in the TRUGENE assay is neutralised, purified and then subsequently sequenced in both directions by a capillary method (ClipTM). The sequence obtained is aligned with prototypical sequences of different genotypes and subtypes and then a computer program performs a phylogenetic analysis to determine genotype and subtype (Halfon *et al.*, 2001).

DNA Hybridization: The INNOLIPA HCV II technique is based on reverse hybridization, and it uses PCR products from the 5´UTR, after they are being denatured, it is then subjected to hybridization with multiple probes and two control targets which are then aligned and fixed to the nitrocellulose membrane by means of respective poly-(T) queues. The hybrids formed are evidenced by the addition of a conjugate (streptavidin-labelled alkaline phosphatase) followed by chromogenic substrate. The genotype is determined after alignment with the card strip test reference. LiPA II includes 22 probes of different genotypes and subtypes (Bouchardeau *et al.*, 2007). The Invader HCV Genotyping Assay (Third Wave Technologies, Inc., Madison, Wisconsin) is based on the analysis of the 5'UTR, using DNA cleavage technology and FRET. The amplicons may be generated from different commercial methods. It can be completed in 1.5 h (after amplification) and is precise with genotypes yet imprecise with subtypes (Germer *et al.*, 2006).

Restriction Fragment-Length Polymorphism (RFLP): These use universal primers that are subjected to nested PCR followed by digestion of the amplicon with restriction enzymes at the genotype-specific site (Rho *et al.*, 2008). Other commercial assays are based on the analysis of subgenomic regions: such as the core region (GEN-ETI-K DEIA kit, Sorin, Italy) or the NS5B region (NS5B Genotyping Assay TRUGENE, Siemens Medical Solutions) or 5'UTR and core regions (VERSANT 2.0, Siemens). These assays have improved the discrimination between HCV subtypes or the Abbott Real- Time HCV Genotype® (Abbott Molecular Diagnostics), where the RNA is purified from plasma and then amplified using specific primers of the NS5B regions, 5'UTR and a recombinant thermostable polymerase with transcriptase and DNA polymerase activity. The cycles of

amplification are realised on the ABI PRISM® 7000 and the data obtained are analysed using the Sequence Genotyping Software Program 2.0 (Cook *et al.*, 2006).

Primer-Specific & Mispair Extension Analysis (PSMEA): The PSMEA HCV genotyping test detects genotype- specific sequence differences in the 5´UTR and is considered to be a high throughput rapid genotyping assay that can reliably identify mixed HCV infections (Antonishyn *et al.*, 2005). Liquid microarrays are used for HCV genotyping and are based on the analysis of the 5´UTR and NS5B regions. The xMap Technology (Luminex Corp, Austin, Texas) is based on short pieces of DNA attached to tiny plastic beads, or microspheres, floating in a sample. It is similar to a gene chip, where many nucleic acid sequences can be detected simultaneously with an LLOD of 50 copies/reaction (Duarte *et al.*, 2010).

2.8.5.4 Nanoparticles

Nanoparticles have been proposed as promising tools to develop the next generation of assays. The most common nanoparticles used are quantum dots and gold nanoparticles (AuNPS). AuNPS are spheres with a typical diameter of 2-50 nm and have the property known as “surface Plasmon resonance”: when the particles are distributed evenly throughout a liquid, they reflect light in a way that makes them appear red; when they clump together, they look blue (Shawky *et al.*, 2010; Deweerdt, 2011). The RNA is extracted from the virion and then short pieces of DNA complementary to the HCV RNA and the gold nanoparticles are added to this solution. In the absence of HCV RNA, the primers stick to the gold nanoparticles and separate them, thus the solution appears red. If the virus is present, the primers pair with the viral RNA instead and the gold

nanoparticles aggregate, turning the solution blue. The test is performed in a tube and takes only 30 minutes (Shawky *et al.*, 2010).

2.8.5.5 Liver Biopsy

A liver biopsy is helpful in the grading of inflammation and staging the degree of fibrosis. Biopsy has prognostic value since all patients with initial periportal fibrosis are likely to develop cirrhosis after 2 decades of untreated infection (Yano, 1996). In patients with the less severe histologic disease who may never develop cirrhosis, careful clinical monitoring is an alternative to antiviral therapy. In addition, a liver biopsy may be repeated in 5 years to assess progression rate (Strader *et al*., 2004).

There are three primary reasons for performing a liver biopsy, it provides helpful information on the current status of the liver injury, it identifies features useful in the decision to embark on therapy, and it may reveal advanced fibrosis or cirrhosis that necessitates surveillance for hepatocellular carcinoma (HCC) and/or screening for varices. The biopsy is assessed for grade and stage of the liver injury but also provides information on other histological features that might have a bearing on liver disease progression (Kleiner, 2005).

The liver biopsy has been widely regarded as the “gold standard” for defining the liver disease status, but it has drawbacks that have prompted questions about its value (Reiss and Keeffe, 2005; Crockett *et al*., 2006).The procedure is not without risks such as pains, bleeding and perforation of other organs (Cadranel *et al*., 2000; Dienstag, 2002). Fatal haemorrhage may occur in up to 0.11% of all liver biopsies, with higher prevalence in malignant liver disease (McGill *et al.*, 1990; Bravo *et al*., 2001).It is subject to sampling

error (Regev *et al*., 2002) it requires special expertise for interpreting the histopathology, it adds cost to medical care, and it is anxiety-provoking for the implicated person. Thus, efforts are underway to seek alternative means of establishing information on the extent of fibrosis by focusing on noninvasive blood marker panels (Rockey and Bissell, 2006).

2.8.5.6 Other markers for the diagnosis of HCV

In chronic hepatitis C, increases in the alanine and aspartate aminotransferases range from 1 to 20 times (but usually less than 5 times) the upper limit of normal. Alanine aminotransferase (ALT) levels are usually higher than aspartate aminotransferase (AST) levels, but that finding may be reversed in patients who have cirrhosis. Alkaline phosphatase and gamma glutamyl transpeptidase are usually normal. If elevated, they may indicate cirrhosis (Berry *et al*., 2005).

Rheumatoid factor, low platelet and white blood cell counts are frequent in patients with severe liver fibrosis or cirrhosis, providing clues to the presence of advanced disease. The enzymes lactate dehydrogenase and creatine kinase are usually normal. Albumin levels and prothrombin time are normal until late-stage disease. Iron and ferritin levels may be slightly elevated. Acute hepatitis C is diagnosed on the basis of symptoms such as jaundice, fatigue and nausea, along with a marked increase in serum ALT and presence of anti-HCV or de novo development of anti-HCV (Berry *et al*., 2005).

2.9 Prevention

Measures to avoid transmission of HCV include: (1) HCV-infected persons should be counselled to avoid sharing toothbrushes and dental or shaving equipment, and be cautioned to cover any bleeding wound in order to prevent contact of their blood with

others; (2) Persons should be counselled to stop using illicit drugs. Those who continue to inject drugs should be counselled to avoid reusing or sharing syringes, needles, water, cotton or other paraphernalia to clean the injection site with a new alcohol swab and to dispose syringes and needles after one use in a safe, puncture-proof container; (3) HCV-infected persons should be advised not to donate blood, body organs, other tissue or semen; and (4) HCV-infected persons should be counselled that the risk of sexual transmission is low and that the infection itself is not a reason to change sexual practices i.e., those in long-term relationships need not start using barrier precautions and others should always practice “safer” sex (CDC, 1998).

There is no vaccine or effective immune globulin (IG) products available for the prevention HCV infection. Blood banks should not use donor’s blood units with elevated liver enzyme levels (ALT and/or AST) even after the test for anti-HCV has been established as negative (Lemon *et al*., 1995). The usefulness of prophylactic IG is still not clear (Hsu and Greenberg, 1994; Purcell, 1994). Post-exposure prophylaxis with IG is not effective in preventing infection. A small fraction of potentially infectious donors who escape detection because they lack detectable antibodies still exists. For the prevention of post- transfusion hepatitis, it is, therefore important that blood transfusions should be given only when absolutely necessary (Hsu and Greenberg, 1995). Needle-exchange programs for injecting drug users may help to limit the spread of HCV infection as well as of HIV and HBV (Hsu and Greenberg, 1994; EASL, 1999). For couples in a stable relationship, the risk posed to sexual partners of HCV-infected patients is not sufficiently high to support recommendations against specific sexual practices (Hsu and Greenberg, 1994).

HCV carriers should be strongly discouraged from drinking alcohol because there is evidence that HCV acts as a cofactor in developing more severe liver injury in alcoholics (Hsu and Greenberg, 1994; Houghton, 1996; CDC, 1998; Degos, 1999; Walker, 1999). As there is no vaccine against hepatitis C available, the only means of protection are the implementation universal precautions and safe injection practices. Screening and treatment of blood products are the only way to prevent transfusion-associated cases (Viral Hepatitis Prevention Board, 1995; van der Poel, 1999).

CDC (1998) and WHO (1999) states a comprehensive strategy on the prevention and control of hepatitis C virus (HCV) infection and HCV-related disease to include:

(1) Primary prevention activities such as I. Screening and testing of blood, plasma, organ, tissue, and semen donors; II. Virus inactivation of plasma-derived products; III. Adequate sterilisation of reusable material such as surgical or dental instruments; IV. Risk-reduction counselling and services; V. Implementation and maintenance of infection-control practices and VI. Needle and syringe exchange programs;

(2) Secondary prevention activities such as I. Identification, counselling, and testing of persons at risk and II. Medical management of infected persons;

(3) Professional and public education and

(4) Surveillance and research to monitor disease trends and the effectiveness of prevention activities and to develop improved prevention methods.

2.10 Vaccines

There is no vaccine against HCV. There are major challenges to the future development of a hepatitis C vaccine. The primary infection of chimpanzees with the virus does not protect them against subsequent challenge by either the identical viral strain or a heterologous strain. Protective or neutralising antibodies have not been found (Hsu and Greenberg, 1994; Lemon and Brown, 1995). An additional major obstacle to vaccine development is the extensive genetic variation between different strains and genotypes, and even the significant antigenic variation among virus quasispecies within individual patients (Hsu and Greenberg, 1994; Lemon and Brown, 1995; Abrignani *et al.*, 1999; Walker, 1999).

The absence of a clearly defined protective immune response after natural infection complicates the prospects of ultimately developing a vaccine against HCV infection (Lemon and Brown, 1995; WHO, 1997). Although an ideal vaccine should give immunity to infection, in the case of HCV, where acute HCV infection is a limited health problem and infection can only be assessed by PCR, a more realistic goal might be to find a vaccine that protects from chronic infection (Abrignani *et al.*, 1999). Preliminary results with chimpanzees show that a vaccine made of recombinant envelope proteins may prevent chronic infection in caused by different HCV genotypes. Although the scientific and clinical challenges that need to be addressed are still substantial, advances in recombinant protein technology, novel adjuvants, and DNA vaccines, will be key in developing strategies to generate immunity against chronic HCV infection (Abrignani *et al.*, 1999). In the absence of a vaccine, all precautions to prevent infection by other means must be taken (WHO, 1997).

2.11 Treatment of Hepatitis C Virus

2.11.1 Goal of antiviral therapy

The goal of antiviral therapy is to cure hepatitis C via a sustained elimination of the virus. A sustained elimination of HCV is achieved if the HCV RNA remains negative six months after the end of treatment (sustained virological response, SVR). Follow-up studies document that more than 99% of patients who achieve an SVR remain HCV RNA negative 4-5 years after the end of treatment and no signs of hepatitis have been documented (Manns, 2008; Swain, 2010). Importantly, long-term benefits of SVR are the reduction of HCV-related hepatocellular carcinoma and overall mortality (Veldt *et al.,* 2007; Backus *et al.,* 2011; van der Meer *et al.,* 2013). In 2011, the FDA accepted SVR-12 as an endpoint for future trials because HCV relapse usually occurs within the first 12 weeks after the end of treatment. However, virologic relapses at later time points may appear in rare cases (Lawitz *et al.,* 2012).

2.11.2 Basic therapeutic concepts and medication

Before the identification of HCV as the infectious agent for non- A, non-B hepatitis (Choo *et al.,* 1989), interferon α (IFN) led to a normalisation of transaminases and an improvement of liver histology in some patients (Hoofnagle *et al.,* 1986). After the identification of HCV, it became possible to measure the success of therapy as the long-lasting disappearance of HCV RNA from serum, the SVR. Since then, SVR rates have increased from 5-20% with IFN monotherapy, up to 40-50% with the combination of IFN + ribavirin (RBV) (McHutchison *et al.,* 1998; Poynard *et al.,* 1998). Different HCV genotypes (HCV GT) show different SVR rates. Patients with the most frequent HCV

GT1 require longer treatment duration and still get a lower SVR compared to HCV GT2 and HCV GT3. The development of pegylated interferon α (PEG-IFN) improved the pharmacokinetics of IFN, allowing more convenient dosing intervals and resulting in higher SVR, especially for HCV GT1. Two PEG-IFNs are available: PEG-IFN α-2a and PEG-IFN α-2b (Cornberg *et al.*, 2013).

In 2011, the first direct antiviral agents (DAA) were approved for patients with HCV GT1. Two inhibitors of the HCV protease (PI), boceprevir and telaprevir improve SVR rates up to 75% in naïve HCV GT1 patients (Jacobson *et al.,* 2011; Poordad *et al.,* 2011) and 29-88% in treatment-experienced HCV GT1 (Bacon *et al.,* 2011; Zeuzem *et al.,* 2011). However, both PIs require combination with PEG-IFN + RBV because monotherapy results in a rapid emergence of drug resistance. Both boceprevir (BOC) and telaprevir (TLV) can be combined with PEG-IFN α-2a or PEGIFN α-2b (Sarrazin *et al.,* 2012).

2.11.3 Antiviral resistance

The development of direct antiviral agents leads to the emerging problem of drug resistance due to so-called resistant-associated amino acid variants (RAVs) of the virus. Patients who received

monotherapy with BOC or TLV developed resistance within a few days (Sarrazin *et al.,* 2007). RAVs are associated with resistance to BOC and TLV. Due to their overlapping resistance profiles, one PI cannot substitute the other in the case of a viral breakthrough. Also, a combination of the two PIs does not make sense. As mentioned above, a combination with PEG-IFN/RBV is mandatory for the usage of BOC or TLV and RAVs

to BOC and TLV have not been associated with less sensitivity to PEGIFN/ RBV (Kieffer *et al.,* 2007). Importantly, if patients have a decreased PEG-IFN/RBV response, the risk of developing significant RAVs is higher. Measures for the prevention of drug resistance are adherence to the dose of the medications (most importantly to the PI) and compliance with the futility rules. If RAVs emerge, it is not completely known for how long they will persist and if this has any significant consequences for future therapies. Some studies suggest that the majority of resistant variants revert to wild type within 1-2 years after the end of therapy (Sarrazin *et al.,* 2007, Sherman *et al.,* 2011).

2.12 Immunology of Hepatitis C Virus

The outcome of HCV infection is under influence of many factors such as genetics, host defence, environment, and the virus (Ray and Thomas, 2010). Both autoimmunity (Nelson, 2001) and cryoglobulinemia (Pawlotsky *et al*., 1995) are frequently associated with HCV infection. Investigators have documented that cell-mediated immunity (CD8+) plays a significant role in the pathology of HCV infection as well as for the killing of HCV- infected hepatic cells (Nelson, 2001). HCV- associated extrahepatic manifestations such as porphyria cutanea tarda, lichen planus, vitiligo, cryoglobulinemia, membranoproliferative glomerulonephritis, lymphoproliferative disorders, a Sjogren-like syndrome, ischemic retinopathy, Systemic Lupus Erythematosus, and autoimmune thrombocytopenia are strong evidence for immune participation in the pathogenesis of HCV infection (Pawlotsky *et al*., 1995; Mowla and Hajiani, 2008; Mignogna *et al*., 2002).

2.12.1 Humoral immunity

Only 15% of HCV-infected individuals; spontaneously clear hepatitis C infection, these phenomena are frequently associated with the development of specific antibodies directed against HCV. The fact that HCV infection persists in the face of the antibody response indicates that, in the chronically infected patients, the antibody is insufficient to clear the infection. With respect to HCV infection, humoral immunity can assist in the direct neutralisation of cell-free virions but can only play an extremely limited role in eradicating HCV inside cells (Ray and Thomas, 2010).

2.12.2 Cellular immunity

Both helper and cytotoxicity responses play important roles in host defence against HCV infection. Previous studies demonstrate that T-helper and T-cytotoxic responses are both associated with resolution of HCV infection (Chang *et al*., 2001; Eckels *et al*., 2000). Studies have shown that neutralising antibodies are produced during HCV infection, but they do not appear to be protective against reinfection in humans or in chimpanzees (Ferrari, 1999). The more critical determinant of the outcome of HCV infection seems to be the cell-mediated immune response or the T-cell (CD4) response. People who are able to spontaneously clear HCV from their body have evidence of strong T-cell responses. Conversely, people who are chronically infected with HCV do not appear to either mount a strong T-cell response early after HCV infection or maintain an initial strong T-cell response to HCV antigens (Gerlach *et al*., 1999).

The final outcome of infections by viruses that cause chronic diseases is believed to depend mostly upon the rate of replication of the infecting virus and the capacity of the

immune system to mount rapid, multispecific and efficient virus-specific responses to inhibit infection before the virus can devise strategies to evade immune surveillance (Ferrari *et al.,* 1999).

The possible strategies for a virus to escape immune elimination include: Decrease its visibility to the immune system; Decrease the effectiveness of antiviral cytokines; Increase the resistance of infected cells to CTL-mediated killing; Infect immunologically privileged sites; Induce immunologic tolerance and Immunologic evasion (Cerny and Chisari, 1999). People can be re-infected with HCV after they have had the initial HCV infection eradicated; there is no life-long HCV immunity (Farci *et al.*, 1992).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in the North Central Nigeria comprises of six states and Abuja the capital of Nigeria. However, this study covered three states which comprised of Plateau State, Nasarawa State and Benue State, the old Benue Plateau State. Plateau State of Nigeria has an area of 26,890 sq kms. It enjoys a relatively temperate climate, with average temperatures between 18°C and 22°C. Based on the 2006 population census figures, Plateau state had an estimated population of 3,178,712 people with a population density of 103 per sq km. Nasarawa state has a population of 1,863,275 people according to the 2006 census. Nasarawa State has a total land area of 27,137.8 sq km and it lies within the Guinea Savannah region and has a tropical climate with moderate rainfall (annual mean rainfall of 1311.75cm). Benue State has a population of 4,219,244 (2006 census), occupying a land mass of 32,518sqkm (http://www.plateaustate.gov.ng/page/at-aglancePlateauState; http://www.cometonigeria.com/ search-by-region/north-central/benue-state; http://www.cometonigeria.com/search-by-region /north-central/benue-state; http://www.nigeriamasterweb.com/Nigeria06CensusFigs.html.). The three States under study have a diverse range of indigenous ethnic groups. The main occupation in the rural areas is farming, while those in urban areas are civil servants, traders and students. The states have several tourist sites, institutions, hospitals, markets, hotels and brothels cut across the states. All these attract people from different parts of the country or the world thus creating avenues for various social interactions which can

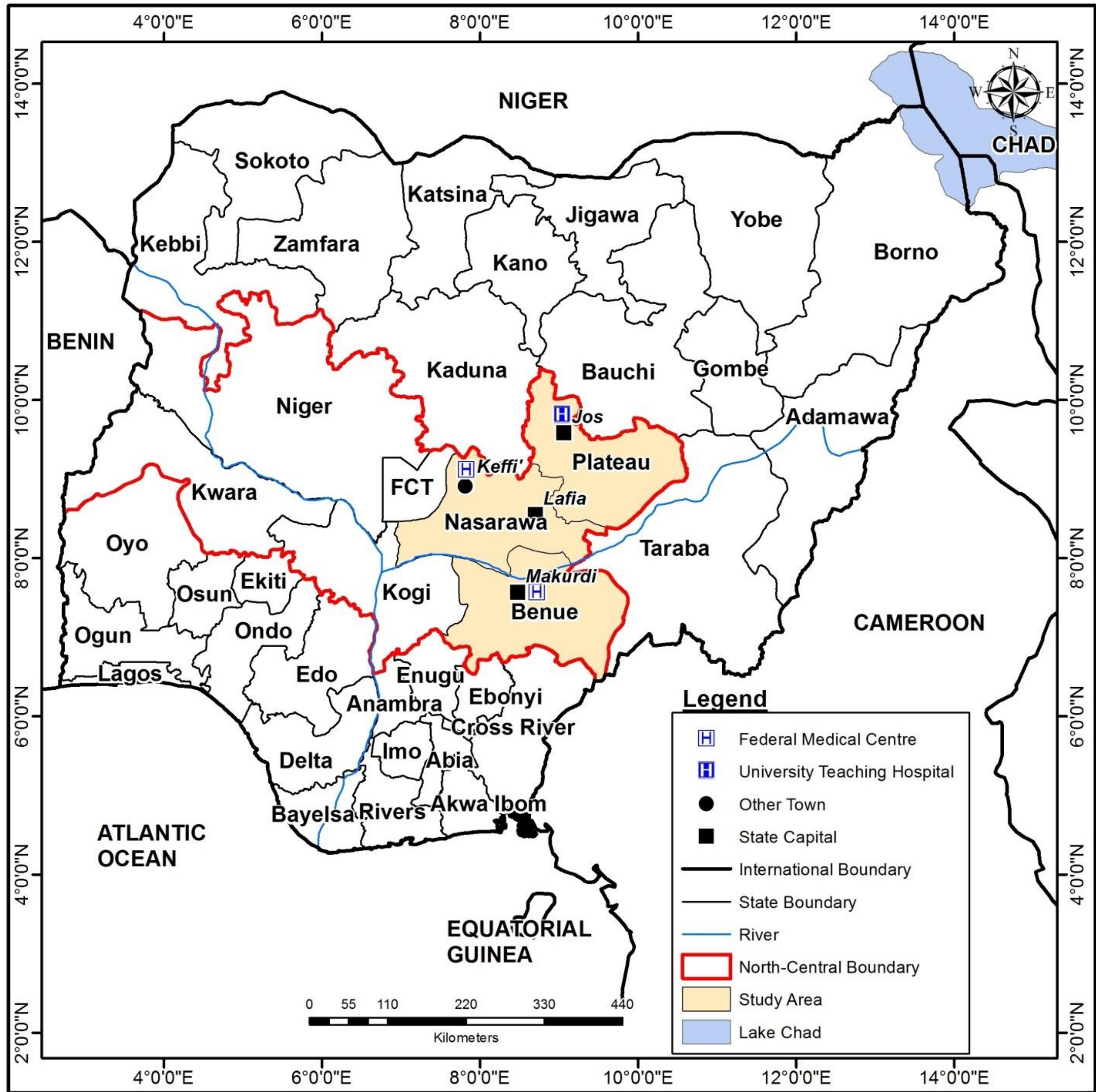

Figure 3.1: Map of Nigeria showing the Study Area

Source: Adapted and modified from the administrative map of Nigeria.

contribute to the spread of the infection. The hospitals used for sampling were the major tertiary health facilities in the study area which also serve as a referral centres to the other primary and secondary health care facilities for patients in the 3 selected states.

3.2 Ethical Consideration

Ethical approvals were obtained from the Health Research and Ethics Committees of the major tertiary health facilities (Jos University Teaching Hospital (JUTH) Jos, Plateau State, Federal Medical Centre (FMC) Keffi, Nasarawa State and Federal Medical Centre (FMC) Makurdi, Benue State) in the study area before the commencement of the work.

3.3 Study Design

The study was a hospital based cross-sectional study. Samples were collected randomly from patients from the study populations and examined for the presence of the disease with regards to the demographic data captured in the structured questionnaire.

3.4 Study Population

The study focused on two population groups which were male and female blood donors and pregnant women attending antenatal clinics in the selected areas. The patients were recruited amongst intended blood donors in Department of Haematology/Blood Banks and the Antenatal Clinics and Laboratory Departments of the selected health facilities.

3.5 Sampling Method and Questionnaire Administration

A structured questionnaire and consent forms (Appendices II and III) were administered randomly to subjects who gave their consent in other to obtain some demographic data. Samples were collected in the major tertiary health facilities in the three States.

3.6 Inclusion Criteria

All those that consented and were HIV negative. For blood donors: all those within the age group 16- 65 years, weigh at least 50 kg and have not donated blood within the previous 56 days while for pregnant women, all pregnant women within age group 15 and above 40 years.

3.7 Exclusion Criteria

All those who declined their consent, those not within the age groups, those infected with Human Immunodeficiency Virus (HIV) and those that did not meet the criteria for blood donation.

3.8 Determination of Sample Size

The sample size was determined in accordance to Lameshow *et al*. (1991) as shown below:

$$n = \frac{[Z_1 - \alpha\sqrt{2P(1-P)} + Z1 - \beta\sqrt{P_1(1-P_1) + P_2(1-P_2)}]^2}{P_1 - P_2}$$

n = Sample size

$Z_1- \alpha$ = Confidence interval at 99% (2.57)

$Z_1 - \beta$ = Power of study at 90 % (1.282)

P_1 = Known prevalence of the disease, 5.4% among blood donors in Makurdi (Alao *et al*., 2009)

P_2 = Known prevalence of the disease, 0.5% among antenatal women in Bayelsa (Buseri *et al*., 2010).

$$P = \frac{(P_1 + P_2)}{2}$$

$$n = \frac{[2.57\sqrt{0.059(0.9705)} + 1.282\sqrt{0.054(0.946) + 0.005(0.995)}]^2}{(0.054 - 0.005)^2}$$

$n = 345$

Three hundred and forty- five (345) samples was the minimum sample size for the research. The minimum sample size were collected in each States given an overall total of One Thousand and Thirty Five (1035) blood samples collected from the three States in the ratio 1: 1: 1 respectively, but 32% of the total samples collected was added, given 1511 samples collected for the study, this is to increase the power of the study.

3.9 Sample Collection

Five (5) ml of blood was collected in an anti-coagulated tube. The plasma was separated and stored in a freezer at -20 ^{o}C until ready for use.

3.10 Detection of HCV Antibodies

For the detection of antibodies to HCV the following kits were used: a third generation ELISA Kit (Autobio Diagnostics, China) and two HCV rapid test kits from two different companies, Global Diagnostic Products, USA and Wondfo Biotech Diagnostic, China).

3.10.1 Anti-HCV antibody ELISA technique

Principle of the Assay:

This assay is based upon the two-step indirect method. In the first step, sample and recombinant HCV coated microwells are combined. During the incubation, the Anti-HCV present in sample binds to the antigen coated wells. After the washing in the second

step, enzyme conjugate is added to the reaction mixture. During the incubation, the Anti-HCV within the sample and the mouse Anti-human IgG in the enzyme conjugate by immunological reactions. After a second washing, substrate A and substrate B are added and catalysed by this complex, resulting in a chromogenic reaction. The resulting chromogenic reaction is measured as absorbance. The colour intensity is proportional to the amount of Anti-HCV in the sample.

Assay procedure:

For the detection of antibodies to HCV, ELISA kit (Autobio Diagnostics, China) was used in accordance with the manufacturer's instructions. The ELISA Kit for Antibody to Human Hepatitis C Virus (all reagents), and samples were brought to room temperature before use (approximately 30 minutes).

Concentrated wash buffer was diluted 1:20 with distilled water (1 volume of wash solution concentrate to 19 volumes of distilled water) and mixed well with a magnetic stirrer. For each micro titre plate, a well was left for blank, three wells for negative controls and two wells for positive controls. One hundred microlitres (100 µl) positive and negative control serum were added to positive and negative control wells respectively without sample diluents and 100 µl of sample diluents were then added into each test wells, followed by 10 µl test serum into test wells and then mix thoroughly for 30 seconds.

The wells were covered with paper seal and then incubated at 37 ^{o}C for 30 minutes. The liquid in all wells were discarded and the wells were then filled with wash solution (350µl per well) and allowed to lay aside for 15 seconds. After which the liquid in all wells were

discarded and then the wells filled with wash solution. The washing was repeated 5 times, the wells were dried after the last wash and 100 µl enzyme conjugate was then added to the wells except the blank well. The wells were covered with seal paper and then incubated for 20 minutes at 37 ^{o}C.

The plate was washed 5 times as described above and 50 µl of substrate A and B were then added to each well, mixed gently for 15 seconds and then it was incubated in the dark at 37 ^{o}C for 15 minutes. The reaction was stopped by the addition of 50 µl of stop solution to each well including the blank well. The absorbance was measured at 450 nm/630 nm within 20 minutes.

Interpretation of results

Colorimetric Method: Cut Off Value calculation (COV) = 0.1 + the average OD of negative controls, (where the average OD of negative controls was below or equal to 0.05, the value 0.05 were used).

Positive and negative controls were used to verify assay performance. The result was valid if the following criteria for the controls were both met: (1.) Negative control – mean absorbance lower than 0.1; (2.) Positive control – mean absorbance of positive control equal to or higher than 0.6.

Nonreactive – samples that gave an absorbance less than the cut-off value were considered nonreactive.

Reactive – samples that gave an absorbance equal to or greater than the cut-off value were considered reactive i.e. **Negative** OD450 of sample $<$ COV; **Positive** OD450 of sample $\geq$ COV.

3.10.2 Immunochromatographic anti-HCV techniques

Two rapid immunochromatographic kits (Global Diagnostic Product, USA and Wondfo Biotech Diagnostic Product, China) were used in screening 500 samples out of the samples earlier analysed for HCV antibodies using ELISA kit.

3.10.2.1 Global laboratory product (a one step hepatitis C virus test strip)

For the detection of antibodies to HCV, Global Diagnostic product (a one step Hepatitis C virus Test Strip, USA, Lot: HCV1120005) was used in accordance with the manufacturer's instructions.

Principle of the Assay:

The HCV one Step Hepatitis C Virus Test Strip is a qualitative, membrane based immunoassay for the detection of antibody to HCV in serum or plasma. The membrane is coated with recombinant HCV antigen on the test line region of the strip. During testing, the serum or plasma reacts with the protein coated particles. The mixture migrates upward on the membrane by capillary action to react with the recombinant HCV antigen on the membrane and produce a coloured line. The presence of this coloured line indicates a positive result, while its absence indicates a negative result. To serve as a

procedural control, a coloured line will always appear at the control line region indicating that proper volume of specimen has been added.

Assay procedure:

The device and specimen were brought to room temperature prior to use. The test strip was removed from the foil pouch by tearing at the notch and placed on a level surface. The strip was then immersed vertically into the specimen and was taken out after at least 15 seconds and then the strip was laid flat on a clean, dry, non-absorbent surface. The result was read in 10 minutes.

Results:

Positive – Two distinct red lines appeared. One line in the control region (C) and the other in the test region (T).

Negative – One red line appeared on the control region (C). No apparent or red line in the test region (T).

3.10.2.2 Wondfo Biotech Diagnostic product (a one step hepatitis C virus test strip)

For the detection of antibodies to HCV, Wondfo Biotech Co. Ltd Product (a one step Hepatitis C virus Test Strip, China, Lot: W00520403-1) was used in accordance with the manufacturer's instructions.

Principle of the Assay:

This HCV rapid test kit adopts double antigen sandwich method.When the test device is immersed into the specimen, the specimen is absorbed into the device by capillary action,

mixes with the antigen-dye conjugate, and flows across the pre-coated membrane. When the HCV antibody levels are at or above the target cutoff (the detection limit of the test), HCV antibodies in the specimen bind to the antigen-dye conjugate and are captured by antigen immobilized in the test region (T) of the device. This produces a coloured test band and indicates a positive result. When the HCV antibody levels are zero or below the target cutoff, there is no visible coloured band in the test region (T) of the device.This indicates a negative result. To serve as a procedure control, a coloured line will appear at the the control region (C), if the test has been performed properly.

Assay procedure:

The pouch was brought to room temperature before used. The test strip was removed from the sealed pouch by tearing at the notch and was placed on a level surface and used as soon as possible. With arrows pointed toward the serum or plasma specimen, the strip was immersed vertically into the serum for at least 10 seconds. The maximum line on the test strip was noted when the strip was immersed so that the line was not passed. The test strip was then placed on a non- absorbent flat surface. The result read at 10 minutes.

Interpretation of results:

Positive: Where rose-pink bands were visible in both the control region and the test region. It indicated a positive result for HCV antibodies in the specimen.

Negative: A rose-pink band was visible in the control region. No coloured band appeared in the test region. It indicated that the concentration of the HCV antibodies in the specimen was zero or below the detection limit of the test.

Invalid: No visible band at all or there was a visible band only in the test region but not in the control region.

3.11 Qualitative Detection of HCV RNA

3.11.1 Extraction of HCV RNA

The extraction of HCV RNA was performed using ZR Viral RNA Kit (Zymo Research Corporation, USA). The protocol was carried out in accordance with the manufacturer's instructions.

Buffer preparation

Five hundred microlitres (500 µl) of beta-mercaptoethanol was added to 100ml ZR Viral RNA Buffer and mixed well. Twenty four milliltres (24 ml) of absolute ethanol was added to 6ml RNA Wash Buffer concentrate and vortexed.

Procedure

In a 1.5ml microcentrifuge tube, 300 µl of ZR Viral RNA lysis buffer was added to each 100 µl of each blood sample. The mixture was then transferred to a Zymo-Spin™ IC Column in a collection tube. It was centrifuged at 12,000× g for 2 minutes and the flow-through from the collection tube was discarded.

Three hundred microlitres (300 µl) of RNA Wash Buffer was added to the column and centrifuged at 12,000 × g for 30 seconds. The flow-through was discarded and the Zymo-Spin™ IC Column was placed back into the collection tube. This step was repeated.

The Zymo- Spin™ IC Column was then transferred into an empty collection tube to ensure complete removal of wash buffer and centrifuged at 12,000× g for 2 minutes. The Zymo- Spin™ IC Column was then placed into a sterile 1.5ml microcentrifuge Nuclease-free tube and 10 µl of Nuclease– Free Water was added and allowed to stand at room temperature for 1 minute. The Zymo- Spin™ IC Column was centrifuged at 12,000 × g for 1 minute to elute the RNA and then it was used immediately for synthesis of cDNA.

3.11.2 Synthesis of the first strand HCV cDNA

The synthesis of the HCV cDNA was performed using Maxima H Minus First Strand cDNA synthesis kit (Thermo Scientific, USA). The protocol was in accordance with the manufacturer's instructions.

Procedure for cDNA sythesis

Ten microlitres (10 µl) of the RNA template was added into a sterile DNase/RNase free-PCR tube, followed by 1 µl of Oligo (dt) 18 primer, 1µl of 10 mM of dNTP Mix, 3 µl nuclease-free water, 4 µl of 5X RT Buffer and 1 µl Maxima H Minus Enzyme respectively, then mixed gently, centrifuged and incubated at 50 ^{o}C for 30 minutes. The reaction was terminated by heating at 85 ^{o}C for 5 minutes. The reaction product of the first strand cDNA was then stored at -20 ^{o}C until used.

3.11.3 Polymerase Chain Reaction (PCR) amplification and detection

The PCR amplification was carried out using Dream Taq Green PCR Master Mix (Thermo Scientific, USA). The primers used in this study were based on the 5' UTR and NS5B regions. All the primers sequences used (Table 3.1) were synthesised at Inqaba

Table 3.1: PCR primers used in 5'UTR and NS5B regions amplification/sequencing

Primer name	Sequence	Product	Source
NS5B			
NS5B2F	5'-TTCACGGAGGCTATGACYAGG-3'	688 bp	
NS5B-2R	5'-CGGGCATGMGACASGCTGTGA-3'		Nakatani *et al*., 2011
DM100 Antisense	5'-TACCTVGTCATAGCCTCCGTGAA-3'	389 bp	Murphy *et al*., 2007
DM101 Sense	5'-TTCTCRTATGAYACCCGCTGYTTTGA-3'		
DM106 Antisense	5'-GGNGCYGAGTAYCTGGTCATGGC-3'	407 bp	Murphy *et al*., 2007
DM107 Sense	5'-CCHATGGGGTTYTCCTAYGACACCAG-3'		
5' UTR	HCVS1(F): GCCATGGCGTTAGTATGAGT		
	HCVAS1(R): GTGCACGGTCTACGAGACCT		Ahmad *et al*., 2007
	HCVS2(F): GTGCAGCCTCCAGGACCC	70-80bp	Anwar *et al*., 2013
	HCVAS2(R): GGCACTCGCAAGCACCCTAT		

Biotec Laboratory, Pretoria, South Africa. The Semi-nested PCR and conventional PCR were performed as previously described (Ahmad *et al*., 2007).

3.11.4 Semi-nested PCR amplification of '5 UTR (untranslated region) of HCV

First round PCR reaction

A thin-walled PCR tube was placed on ice and the following components were added for each 50 µl reaction: 25 µl Dream Taq Green PCR Master Mix(x2); 0.5 mM each of Forward primer and Reverse primer for 5'UTR; 4 µl of cDNA; made up to 50 µl with Nuclease-free water.

The samples were then vortexed gently, spun down and loaded inside the Thermal Cycler (GenAmp PCR Systems 9700 Applied Biosystems, USA). The PCR was performed using the following thermal cycling conditions: Initial denaturation at 95 ^{o}C for 5 minutes, followed by 35 cycles with each cycle consisting of - denaturation at 94 ^{o}C for 30 seconds, annealing at 59 ^{o}C for 30 seconds and extension at 72 ^{o}C for 30 seconds, followed by final extension at 72 ^{o}C for 7 minutes and a final hold at 4 ^{o}C.

Two microlitres (2 µl) of the first round PCR amplified product was loaded directly on 1.5% Agarose Gel and was allowed to run on an Electrophoresis Tank containing x1 TAE buffer for 40 minutes at 80 V. The amplified PCR product electrophoresed pattern was captured using a Molecular Image Gel Doc XR+ Imaging System, BIORAD, USA).

Second round PCR reaction

A thin-walled PCR tube was placed on ice and the following components were added for each 50 µl reactions: 25 µl Dream Taq Green PCR Master Mix(x2); 0.5 mM each of Forward primer and Reverse primer; 2 µl of 1st round PCR product the preparation was made up to 50 µl with Nuclease-free water.

The samples were then vortexed gently, spun down and loaded inside the Thermal Cycler (GenAmp PCR Systems 9700 Applied Biosystems, USA). The temperature conditions were the same as in the first round of PCR except the primers used were complementary to the PCR product of the first round PCR.

Two microliters (2 µl) of the 2nd round PCR product was loaded directly on 1.5% Agarose Gel and was allowed to run on an Electrophoresis Tank containing x1 TAE buffer for 40 minutes at 80 V. The RT-PCR electrophoresed pattern was captured using a Molecular Image Gel Doc XR+ Imaging System, BIORAD, USA.

3.11.5 PCR amplification of NS5B

A thin-walled PCR tube was placed on ice and the following components added for each 50 µl reaction: 25 µl Dream Taq Green PCR Master Mix (x2); 0.5mM each of Forward primer and Reverse primer; 4 µl of cDNA; made up to 50 µl with Nuclease-free water.

The samples were then votexed gently, spun down and loaded inside the Thermal Cycler (GenAmp PCR Systems 9700 Applied Biosystems, USA). The PCR was performed using the following thermal cycling conditions: Initial denaturation at 95 ^{o}C for 5 minutes,

Denaturation at 94 ^{o}C for 30 seconds, Annealing at 60.5 ^{o}C for 30 seconds, Extension at 72 ^{o}C for 30 seconds, Final extension at 72 ^{o}C for 7 minutes and a final hold at 4 ^{o}C.

Two microlitres (2 µl) of the PCR product was loaded directly on 1.5% Agarose Gel and was allowed to run on an Electrophoresis Tank containing x1 TAE buffer for 40 minutes at 80 V. The RT-PCR electrophoresed pattern was captured using a Molecular Image Gel Doc XR+ Imaging System, BIORAD, USA.

3.12 Detection of HCV Genotypes and Subtypes

Sequence and phylogenetic analysis were carried out on the PCR amplified products for the dertermination of the HCV genotypes/ sub-types in circulation in the study area.

3.12.1 Sequence analysis and phylogenetic analysis

Nine 5'UTR HCV Amplicons were studied for the diversity and sequence variations. The sequencing was carried out using Sanger Sequencer (Big Dye terminator). The DNA sequencing was carried out using the PCR products with the specific primers.

3.12.2 DNA sequencing clean-up/ sequencing

ZR-96 DNA Sequencing Clean-up Kit™ (Zymo Research Corporation, USA) was used. The manufacturer's instruction was followed. The ZR-96 DNA Sequencing Clean-up Kit™ provides a simple method for the rapid removal of post-cycle sequencing reaction contaminants (i.e., unincorporated fluorescent dyes, residual salts, dNTPs, primers, and enzymes) from DNA extension products. These contaminants often interfere with the quality and signal strength of sequencing data.

Procedure

Two hundred and forty microliters (240 µl) of Sequencing Binding Buffer was added to 20 µl sequencing reaction and the mixture was transferred to a Zymo-Spin™ IB-96 Plate mounted onto a collection Plate and centrifuged at 3,000 x *g* for 2 minutes.

Three hundred microliters (300 µl) of Sequencing Wash Buffer was added to each well of the plate and then centrifuged at 3,000 x *g* for 5 minutes and 15-20 µl Nuclease- free water was added directly to the column matrix of the filter plate.

The Zymo-Spin™ IB-96 Plate was then placed on top of the supplied 96-Well PCR Plate and the assembly mounted onto the Collection Plate and then centrifuge at 3,000 x *g* for 2 minutes to elute the DNA.

Sequencing reactions were performed using the Big Dye Version 3.1 Chemistry kit (Applied Biosystems, USA) and the products were detected using an automated sequencer ABI 3500 XL Genetic Analyzer (Applied Biosystems, USA) and ABI 2720 Thermal Cycler were used.

3.13 Comparison of the Efficiency of Diagnostic Tools used

This was determined using the results of the different anti-HCV screening methods used. The sensitivity, specificity, efficiency, positive predictive value (PPV), and negative predictive value (NPV) for the strip methods were calculated in relation to the third generation ELISA kit which was used as the gold standard in this study using the following formula:

Percentage Sensitivity = $a/ (a+c) \times 100\%$

Percentage Specificity = $d/(b+d) \times 100\%$

Efficiency = $(a+d) / (a+b+c+d) \times 100\%$

Percentage Positive Predictive Value = $a/(a+b) \times 100\%$

Percentage Negative Predictive Value = $d/(c+d) \times 100\%$

Where

a = number of true positives

b = number of false positives

c = number of false negatives

d = number of true negatives

Performance of kits for each lot was evaluated in terms of sensitivity, specificity, positive predictive value, negative predictive value and efficiency which can be defined as follows:

Sensitivity = It is the ability of an assay kit to detect truly infected individuals and very small amounts of analyte.

Specificity = It is the ability of an assay kit to correctly identify all the uninfected individuals and there should be no false positives.

Positive Predictive Value (PPV) = It is the ability of a test to identify actually infected

individuals among all persons giving a positive result with the kit being used.

Negative Predictive Value (NPV) = It is the ability of a test to identify correctly the real

non- infected individuals among all persons giving a negative result with the kit being used.

Efficiency = It is the overall ability of a test to correctly identify all positives results as positive

and all negatives results as negative. This is also referred to as 'accuracy' (Maity *et al*., 2012).

So also, the Likelihood Ratios were determined as follows:

Likelihood Ratio Positive (LR+) = Probability of positive test in those with disease / Probability of positive test in those without disease

LR+=Sensitivity/ (1-Specificity)

Likelihood Ratio Negative (LR-) =Probability of negative test in those with Disease / Probability of negative test in those without disease

LR- = (1-Sensitivity)/Specificity.

3.14 Data Analysis/ Statistical Method

The sequence analysis and phylogenetic analysis were carried out using CLC MAIN workbench 7.5.1. The chromatograms generated from the sequencing instrument were annotated and aligned to the reference sequences of GenBank data base (NCBI) and were

used for multiple alignments and phylogeny construction. The NCBI BLAST Sequence similarity searching tool was used to compare the sequences of the samples generated with their closest relatives in the GenBank.

Phylogenetic analysis was performed using the neighbour-joining method. Phylogenetic trees were inferred using the neighbour- joining method. Robustness of the tree branches was tested using bootstrap analysis (1000 replicates).

The data obtained from the study were analysed by Chi-square using SPSS 23.0 software. Statistical significance was accepted at P values < 0.05(95% confidence level). Results were presented in tables, figures and charts. The statistical tool chosen compared the effective relationship between two or more variables.

CHAPTER FOUR

4.0 RESULTS

The seroprevalence of HCV among the blood donors and pregnant women in the study population based on location is presented in Table 4.1. The result indicates that Nasarawa State recorded the highest seroprevalence of 6.9% (33), followed by Plateau State with 4.9% (25) and Benue State with the least seroprevalence of 4.0% (21). The study reveals that there was no significant difference in the prevalence in respect to location within the study area (p= 0.108). The overall seroprevalence of 5.2% (1511) was recorded in this study.

The seroprevalence of HCV among blood donors in the study area based on location reveals that Nasarawa State had the highest prevalence of 11.0% (24), followed by Plateau State with 5.4% (13) and Benue State with the least prevalence of 5.2% (13). The seroprevalence of HCV among blood donors in the area was 7.0% (50). The study reveals that there was a significant difference in the seroprevalence in respect to location within the study area (p= 0.023).

The seroprevalence of HCV among pregnant women in the study area based on location shows that Plateau State recorded the highest seroprevalence of 4.4% (12), followed by Nasarawa State with 3.5% (9) while Benue State had the least seroprevalence of 2.9% (8). The seroprevalence of HCV among pregnant women in the study area was 3.6% (29). The study reveals that there was no significant difference in the seroprevalence in relation to location within the study area (p= 0.644).

Table 4.1: Seroprevalence of HCV among blood donors and pregnant women based on location

	Blood donor		Pregnant women		Total	
Location	No. Examined	No. Positive (%)	No. Examined	No. Positive (%)	No. Examined	No. Positive (%)
Plateau	242	13(5.4)	271	12(4.4)	513	25(4.9)
Benue	250	13(5.2)	272	8(2.9)	522	21(4.0)
Nasarawa	218	24(11.0)	258	9(3.5)	476	33(6.9)
Total	710	50(7.0)	801	29(3.6)	1511	79(5.2)
χ^2	7.568		0.879		4.452	
p-value	0.023		0.644		0.108	

Of the 710 samples examined from blood donors in the study as presented in Table 4.2, males of age group 41-50 years had the highest seroprevalence of 11.9% (7), followed by age group 21-30 years with a seroprevalence of 7.5% (24), age group ≤ 20 years had 6.7% (3), age group 31-40 years had 5.4% (10), while the age groups 51 - ≥ 61 had the least with no detectable antibodies in their samples. The result showed no statistically significant difference in the seroprevalence of HCV in relation to age and sex (p= 0.583).

Females of the age group 41-50 years had the highest seroprevalence of 10.9% (7), followed by age group ≤ 20 with 8.8% (5), age group 21-30 with 7.7% (28), while the age groups ≥ 51 years had no detectable HCV antibodies (0.0%). The result showed no statistically significant difference in the seroprevalence of HCV among female blood donors in relation to age and sex (p= 0.326) as presented in Table 4.3.

Of the 801 samples examined from pregnant women in the study as presented in Table 4.3, age group ≤ 20 years had the highest seroprevalence of 6.3% (5), followed by age group 31-40 years with a seroprevalence of 3.8% (8), age group 21-30 years had 3.2% (16), while the age groups 41 -50 years had the least with no detectable antibodies in their samples. The result showed no significant difference in the seroprevalence of HCV in relation to age (p= 0.528).

Table 4.2: Seroprevalence of HCV among blood donors in relation to Age and Sex

	Male		Female		Total	
Age group (Years)	No. Examined	No. Positive(%)	No. Examined	No. Positive (%)	No. Examined	No. Positive (%)
≤20	45	3(6.7)	12	2(16.7)	57	5(8.8)
21-30	320	24(7.5)	45	4(8.9)	365	28(7.7)
31-40	185	10(5.4)	27	0(0.0)	212	10(4.7)
41-50	59	7(11.9)	5	0(0.0)	64	7(10.9)
51-60	10	0(0.0)	1	0(0.0)	11	0(0.0)
≥61	1	0(0.0)	0	0(0.0)	1	0(0.0)
Total	620	44(7.1)	90	6(6.7)	710	50 (7.0)
χ^2	3.769		4.643		4.625	
p-value	0.583		0.326		0.463	

Table 4.3: Seroprevalence of HCV among pregnant women in relation to Age

Age group (Years)	No. Examined	No. Positive (%)
≤20	80	5(6.3)
21-30	499	16(3.2)
31-40	212	8(3.8)
41-50	10	0(0.0)
Total	801	29(3.6)
χ^2	2.220	
p-value	0.528	

The seroprevalence of HCV among blood donors in relation to marital status as presented in Table 4.4 shows that the separated had the highest with 33.3% (2), followed by the singles who had 7.2% (24), the married had 6.7% (24) while the divorced and widowed who showed no detectable evidence of HCV antibodies (0.0%) in their samples. However, there was no significant difference between the seroprevalence and marital status (p= 0.123).

The seroprevalence of HCV among pregnant women in relation to marital status as presented in Table 4.5 shows that the married had the highest with 8.9% (4), followed by the singles with 5.3% (1), while the divorced, widowed and separated showed no detectable evidence of HCV antibodies (0.0%) in their samples. There was no significant difference between the seroprevalence and marital status (p= 0.930).

The seroprevalence of HCV among blood donors in relation to educational status as presented in Table 4.6 shows that those with tertiary educational status had the highest with 8.4% (28), followed by those with secondary educational status with 6.7% (19), those with non-formal educational status had 3.3% (1) and the least was among those with primary educational status with 3.2% (2). There was no significant difference (p= 0.392).

Table 4.4: Seroprevalence of HCV among blood donors in relation to marital status

Marital status	No. Examined	No. Positive (%)
Singles	333	24(7.2)
Married	360	24(6.7)
Divorced	4	0(0.0)
Widowed	7	0(0.0)
Separated	6	2(33.3)
Total	710	50(7.0)
χ^2	7.260	
p-value	0.123	

Table 4.5: Seroprevalence of HCV among pregnant women in relation to marital status

Marital status	No. Examined		No. Positive (%)
Singles	19		1(5.3)
Married	45		4(8.9)
Divorced	3		0(0.0)
Widowed	5		0(0.0)
Separated	0		0(0.0)
Total	801		29(0.0)
χ^2		0.447	
p-value		0.930	

Table 4.6: Seroprevalence of HCV among blood donors in relation to educational status

Educational status	No. Examined	No. Positive (%)
Non formal	30	1(3.3)
Primary	63	2(3.2)
Secondary	282	19(6.7)
Tertiary	335	28(8.4)
Total	710	50(7.0)
χ^2	2.996	
p-value	0.392	

The seroprevalence of HCV among pregnant women in relation to educational status as presented in Table 4.7 shows that those with non-formal education had the highest with 4.9% (3), followed by those with secondary educational status who had 4.1% (14), those with tertiary educational status had 3.2% (10), while those with primary educational status had the least with 2.4% (2). However, there was no significant difference (p= 0.789).

The seroprevalence of HCV among blood donors in relation to occupational status shows that the unemployed had the highest with 8.5% (21), health workers, civil servants, business people, and farmers with 8.3% (2), 8.0% (13), 6.0% (14) and 0.0% (0) respectively. However, there was no statistically significant difference (p = 0.301) as presented in Table 4.8.

The seroprevalence of HCV among pregnant women in relation to occupational status as presented in Table 4.9 shows that the farmers had the highest with 6.8% (3), the unemployed, civil servants, business people, and health workers, with 4.7% (8), 3.4% (5), 3.1% (13) and 0.0% (0) respectively. The result showed that there was no statistical significant difference (p = 0.580).

Table 4.7: Seroprevalence of HCV among pregnant women in relation to educational status

Educational status	No. Examined	No. Positive (%)
Non formal	61	3(4.9)
Primary	85	2(2.4)
Secondary	343	14(4.1)
Tertiary	312	10(3.2)
Total	801	29(3.6)
χ^2	1.049	
p-value	0.789	

Table 4.8: Seroprevalence of HCV among blood donors in relation to occupational status

Occupational status	No. Examined	No. Positive (%)
Farmer	44	0(0.0)
Civil servant	162	13(8.0)
Business	234	14(6.0)
Unemployed	246	21(8.5)
Health worker	24	2(8.3)
Total	710	50(7.0)
χ^2	4.874	
p-value	0.301	

Table 4.9: Seroprevalence of HCV among pregnant women in relation to occupational status

Occupational status	No. Examined	No. Positive (%)
Farmer	44	3(6.8)
Civil servant	145	5(3.4)
Business	420	13(3.1)
Unemployed	172	8(4.7)
Health worker	19	0(0.0)
Total	800	29(3.6)
χ^2	2.868	
p-value	0.580	

The seroprevalence of HCV among blood donors in relation to some habits/ behavioural characteristics (Sociodemographic factors) is represented in Table 4.10. Of 172 samples examined for HCV antibodies from married blood donors in relation to their types of marriage, those who practice polygamy had the higher seroprevalence of 12.5% (1) while those who practice monogamy had 4.9% (8). However, the result showed no significant difference (p = 0.344).

The seroprevalence of HCV among those involved in multiple sex activities in relation to use of condoms as presented in Table 4.10 shows that those who did not use condoms during sexual intercourse with their multiple partners had the higher seroprevalence of 7.8% (9), while those who used condoms before sexual activities with their multiple partners had 4.1% (4). However, there was no significant difference (p = 0.4054).

The seroprevalence of HCV among the alcoholics and non-alcoholics among blood donors as presented in Table 4.10 shows that those who were non- alcoholics had the higher seroprevalence of 7.5% (42) while the alcoholics had the lowest with 5.4% (8). However, there was no l significant difference (p = 0.382).

The seroprevalence of HCV among the smokers and non-smokers among the blood donors as presented in Table 4.10 reveals that the non- cigarette smokers had the higher seroprevalence of 7.3% (48) while the smokers had 3.6% (2). This showed no significant difference (p = 0.290).

Table 4.10: Seroprevalence of HCV among blood donors in relation to some habits / behavioural characteristics (sociodemographic factors).

Habit	No. Examined	No. Positive (%)	χ^2	p-value
Types of Marriage				
Polygamy	8	1(12.5)	0.894	0.344
Monogamy	164	8(4.9)		
Sub-Total	172	9(5.2)		
Use of condoms				
Yes	97	4(4.1)	0.6924	0.4054
No	115	9(7.8)		
Sub-Total	212	13(6.1)		
Alcohol consumption				
Yes	148	8(5.4)	0.765	0.382
No	562	42(7.5)		
Sub-Total	710	50(7.0)		
Cigarette smoking				
Yes	56	2(3.6)	1.119	0.290
No	654	48(7.3)		
Sub-Total	710	50(7.0)		

The seroprevalence of HCV among pregnant women in relation to some habits/ behavioural characteristics (sociodemographic factors) is presented in Table 4.11. Of 398 samples examined for HCV antibodies from married pregnant women in relation to their types of marriage, those who practice polygamy had the higher seroprevalence of 3.4% (1) while those who practice monogamy had 3.0% (11). However, the result showed no significant difference ($p = 0.887$).

The seroprevalence of HCV among those involved in multiple sex activities in relation to use of condoms as presented in Table 4.11 reveals that those who use condoms at all times had a higher seroprevalence of 4.1% (2), while those who did not use condoms had 0.7% (1). However, there was no significant difference ($p = 0.3191$).

The seroprevalence of HCV among the alcoholics and non-alcoholics among pregnant women as presented in Table 4.11 shows that those who were non- alcoholics had the higher seroprevalence of 3.8% (28) while the alcoholics had the lowest seroprevalence of 1.4% (1). This showed no significant difference ($p = 0.296$).

The seroprevalence of HCV among the smokers and non-smokers among pregnant women as presented in Table 4.11 reveals that the non- cigarette smokers had the higher seroprevalence of 3.6% (29) while the smokers were all seronegative for HCV, 0.0% (0). However, there was no significant difference ($p = 0.634$).

Table 4.11: Seroprevalence of HCV among pregnant women in relation to some habits / behavioural characteristics (sociodemographic factors).

Habit	No. Examined	No. Positive (%)	χ^2	p-value
Types of Marriage				
Polygamy	29	1(3.4)	0.020	0.887
Monogamy	369	11(3.0)		
Sub-Total	398	12(3.0)		
Use of condoms				
Yes	49	2(4.1)	1.002	0.3191
No	146	1(0.7)		
Sub-Total	195	3(1.5)		
Alcohol consumption				
Yes	71	1(1.4)	1.092	0.296
No	730	28(3.8)		
Sub-Total	801	29(3.6)		
Cigarette smoking				
Yes	6	0(0.0)	0.227	0.634
No	765	29(3.6)		
Sub-Total	801	29(3.6)		

The seroprevalence of HCV among blood donors in relation to risk factors as presented in Table 4.12 showed that those without a history of blood transfusion had a seroprevalence of 6.0% (25) while those with a history of transfusion had 4.5% (1). The result showed no statistical association with the seroprevalence of HCV (p= 0.779; OR= 0.747). Those with a history of surgery had the higher seroprevalence of 15.6% (5), while those without a history of surgery had the lowest with 5.2% (21). The result showed a significant association (p= 0.016, OR= 3.404). Those without a history of multiple sexual partners had the higher seroprevalence of 7.1% (14), while those with a history of multiple sexual partners had 5.4% (3).This showed no signification association with the seroprevalence of HCV (p= 0.651; OR= 0.744). Those with a history of Dilation and Curettage (D & C) and those without a history of D & C did not show the presence of HCV antibodies in their blood samples, this also shows that there was no significant association between this group and the seroprevalence. Those with a history of unsafe injections had 50% (1), while those without a history of unsafe injections had 5.9% (25). This showed a significant association with the seroprevalence (p= 0.009; OR= 15.960). Those with a history of sustaining an injury from sharp instruments had the higher seroprevalence of 10% (3), while those without a history of sustaining an injury from sharp instruments had 5.6% (22). This showed no significant association with seroprevalence (p= 0.318; OR= 1.889). Those without a history of sharing toothbrush had 5.9% (24), while those with a history of sharing toothbrush had 0.0% (0). This showed no significant association with the seroprevalence (p= 0.277; OR= 1.063). Those without a history of tribal/ body marks had the higher seroprevalence of 6.0% (24), while those with a history of tribal/ body marks had the least with 3.6% (1). This showed no significant association with the

seroprevalence (p= 0.593; OR= 0.577). Those with a history of attending a dental care had the higher seroprevalence of 6.5% (2), while those without a history of dental care had 5.8% (23). This showed no significant association with the seroprevalence (p= 0.889; OR= 1.112). Those without a history of tattooing had the higher seroprevalence of 6.0% (25), while those with a history of tattooing had 0.0% (0). This showed no significant association with seroprevalence (p= 0.537; OR= 1.064). Those without a history of ear piercing had the higher seroprevalence of 6.2% (23), while those with a history of ear piercing had 0.0% (0). This showed no significant association with the seroprevalence (p= 0.239; OR= 1.066). Those with a history of Sexually Transmissible Infection (STI) had the higher seroprevalence of 10.0% (1), while those without a history of STI had the lowest with 5.8% (24). This showed no significant association (p= 0.572; OR= 1.818).

Table 4.12: Seroprevalence of HCV among blood donors in relation to some risk factors

Risk factor	No. Examined	No. Positive (%)	χ^2	p-value	OR	95% CI
Blood transfusion						
Yes	22	1(4.5)	0.079	0.779	0.747	0.096-5.779
No	417	25(6.0)				
Surgery						
Yes	32	5(15.6)	5.832	0.016	3.404	1.191- 9.731
No	407	21(5.2)				
Multiple sex partners						
Yes	56	3(5.4)	0.205	0.651	0.744	0.206-2.686
No	198	14(7.1)				
D and C						
Yes	2	0(0.0)	-	-	-	-
No	32	0(0.0)				
Unsafe injections						
Yes	2	1(50.0)	6.759	0.009	15.960	0.969-262.747
No	424	25(5.9)				
Sharing of sharp instruments						
Yes	30	3(10.0)	0.997	0.318	1.889	0.532-6.712
No	396	22(5.6)				
Toothbrushes						
Yes	19	0(0.0)	1.184	0.277	1.063	1.037-1.089
No	408	24(5.9)				
Tribal/body piercing						
Yes	28	1(3.6)	0.286	0.593	0.577	0.075-4.431
No	398	24(6.0)				
Dental care						
Yes	31	2(6.5)	0.020	0.889	1.112	0.250-4.953
No	394	23(5.8)				
Tattooing						
Yes	6	0(0.0)	0.381	0.537	1.064	1.038-1.090
No	418	25(6.0)				
Ear piercing						
Yes	21	0(0.0)	1.384	0.239	1.066	1.040-1.093
No	403	25(6.2)				
STI*						
Yes	10	1(10.0)	0.319	0.572	1.818	0.221-14.958
No	417	24(5.8)				

STI*= Sexually Transmitted Infections; OR= Odd Ratio; CI= Confidence Interval.

The seroprevalence of HCV among pregnant women in relation to risk factors as presented in Table 4.13 shows that those with a history of blood transfusion had the higher seroprevalence of 8.3% (5) while those without a history of transfusion had 3.2% (24). This showed significant relationship with the seroprevalence of HCV (p= 0.042; OR= 2.716). Those without a history of surgery had 3.6% (23), while those with a history of surgery had the least with 3.5% (23). However, there was no significant association (p= 0.943, OR= 0.967). Those with a history of multiple sexual partners had seroprevalence of 1.9% (2), while those without a history of multiple sexual partners also had 1.9% (2). However, there was no significant association with the seroprevalence of HCV (p= 0.977; OR= 0.972). Those with a history of D& C had a seroprevalence of 3.8% (8), while those without a history of D& C had 3.6% (21). There was no significant association (p= 0.893; OR= 1.058). Those without a history of unsafe injections had 3.7% (29), while those with a history of unsafe injections had 0.0% (0). However, there was no significant association with the seroprevalence (p= 0.464; OR= 1.038). Those with a history of sustaining an injury from sharp instruments had the higher seroprevalence of 4.7% (10), while those without a history of sustaining an injury from sharp instruments had 3.2% (19). However, there was no significant association with seroprevalence (p= 0.329; OR= 1.473). Those without a history of sharing toothbrush had 3.8% (27), while those with a history of sharing toothbrush had 2.2% (2). There was no significant association with the seroprevalence (p= 0.461; OR= 0.582). Those with a history of tribal/ body marks had the higher seroprevalence of 3.7% (6), while those without a history of tribal/ body marks had the least with 3.6% (23). There was no significant association with the seroprevalence (p= 0.979; OR= 1.012). Those without a history of

attending a dental care had the higher seroprevalence of 3.7% (25), while those with a history of dental care had 3.1% (4). However, there was no significant association with the seroprevalence (p= 0.741; OR= 0.835). Those without a history of tattooing had the higher seroprevalence of 3.7% (29), while those with a history of tattooing had 0.0% (0). This indicated no significant association with seroprevalence (p= 0.448; OR= 1.024). Those with a history of ear piercing had the higher seroprevalence of 3.7% (28), while those without a history of ear piercing had 2.9% (1). However, there was no significant association with the seroprevalence (p= 0.805; OR= 1.290). Those with a history of Sexually Transmissible Infection (STI) had the higher seroprevalence of 6.3% (5), while those without a history of STI had the least with 3.3% (24). There was no significant association (p= 0.188; OR= 1.925).

Table 4.13: Seroprevalence of HCV among pregnant women in relation to some risk factors

Risk factor	No. Examined	No. Positive (%)	χ^2	p-value	OR	95% CI
Blood transfusion						
Yes	6	5(8.3)	4.128	0.042	2.716	0.997-7.396
No	741	24(3.2)				
Surgery						
Yes	170	6(3.5)	0.005	0.943	0.967	0.387- 2.414
No	631	23(3.6)				
Multiple sex partners						
Yes	108	2(1.9)	0.001	0.977	0.972	0.134-7.028
No	105	2(1.9)				
D and C						
Yes	211	8(3.8)	0.018	0.893	1.058	0.462-2.427
No	585	21(3.6)				
Unsafe injections						
Yes	14	0(0.0)	0.536	0.464	1.038	1.024- 1.053
No	786	29(3.7)				
Sharing of sharps						
Yes	213	10(4.7)	0.951	0.329	1.473	0.674- 3.220
No	587	19(3.2)				
Toothbrushes						
Yes	89	2(2.2)	0.544	0.461	0.582	0.136- 2.492
No	711	27(3.8)				
Tribal/body piercing						
Yes	164	6(3.7)	0.001	0.979	1.012	0.405- 2.528
No	634	23(3.6)				
Dental care						
Yes	128	4(3.1)	0.109	0.741	0.835	0.286- 2.441
No	672	25(3.7)				
Tattooing						
Yes	15	0(0.0)	0.576	0.448	1.024	1.024- 1.053
No	784	29(3.7)				
Ear piercing						
Yes	766	28(3.7)	0.061	0.805	1.290	0.170- 9.764
No	35	1(2.9)				
STI*						
Yes	80	5(6.3)	1.729	0.188	1.925	0.713- 5.194
No	717	24(3.3)				

STI*= Sexually Transmitted Infections; OR= Odd Ratio; CI= Confidence Interval.

The comparison of ELISA (Reference) kit with the two commercially available chromatographic rapid Kits for the detection of anti-HCV revealed that out of 79 anti-HCV positive and 421 anti-HCV negative by ELISA, Wondfo Kit had a sensitivity of 75.0%, specificity of 99.0%, overall accuracy of 95.2%, positive predictive value of 93.6%, negative predictive value of 95.4%, positive likelihood ratio of 75.0, negative likelihood ratio of 0.25 and Kappa value of 0.803,while Global Kit had a sensitivity of 57.0%, specificity of 100.0%, overall accuracy of 93.2%, positive predictive value of 100%, negative predictive value of 92.5% , positive likelihood ratio of 0.57, negative likelihood ratio of 0.43 and Kappa value of 0.672 (Table 4.14).

Plate I: shows the amplicons generated from transcription mediated amplification of HCV RNA from the samples studied. The region amplified using a nested PCR was the ‘5 UTR of the HCV genome with 70bp. Attempts to amplify the NS5B region with three different primers failed.

Out of the 1511 blood donors and pregnant women examined for antibodies to HCV, only the seventy- nine (79) that were HCV antibodies positive were used for the qualitative detection of HCV RNA. The reason for this choice was based on the consideration of the cost, time and the large samples involved in this study. The result showed a higher HCV RNA distribution among participants from Plateau State with 40.00% (n= 10), followed by Nasarawa State with 12.12% (n= 4) and the least was from Benue State with 4.76% (n= 1) as shown in Table 4.15.

Table 4.14: Comparative Sensitivity and specificity of ELISA and two Rapid immunochromatographic Kits for detection of HCV antibodies in the study population

N=500

Test Kit	No. Positive (%)	No. Negative (%)	TP	FP	TN	FN	S	SP	OA	PPV	NPV	LR+	LR-	Kappa
ELISA	**79(15.8)**	**421(84.2)**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
WONDFO	**59(11.8)**	**441(88.2)**	**59**	**4**	**417**	**20**	**75.0%**	**99.0%**	**95.2%**	**93.6%**	**95.4%**	**75.0**	**0.25**	**0.803**
GLOBAL	**45(9.0)**	**445(91.0)**	**45**	**0**	**421**	**34**	**57.0%**	**100%**	**93.2%**	**100%**	**92.5%**	**0.57**	**0.43**	**0.672**

Key: N=Total number examined; TP = True Positive; TN = True Negative; FP = False Positive; FN = False Negative; S = Sensitivity; SP = Specificity; OA = Overall Accuracy; PPV = Positive Predictive Value; NPV = Negative Predictive Value; LR+=Positive Likelihood Ratio; LR-=Negative Likelihood Ratio.

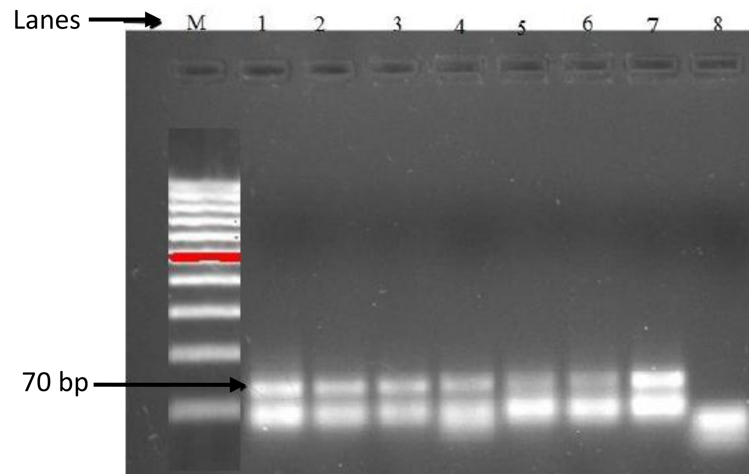

Plate I: Amplicons of 5’ UTR of Hepatitis C Virus on Agarose Gel Electrophoresis.

Lane M: 50bp DNA ladder; Lane 1-6 PCR products; Lane 7 Positive control and Lane 8 Negative control.

Table 4.15: Distribution of HCV RNA in HCV antibodies positive participants in the study area

Location	No. Examined	No. positive (%)
Benue	21	1(4.76)
Nasarawa	33	4(12.12)
Plateau	25	10(40.00)
Total	79	15(18.98)

χ^2= 10.950 df= 2 p = 0.004

Out of the 15 samples amplified at the 5'UTR, only 9 were successfully sequenced. Table 4.16 shows the BLAST information obtained from our queried sequences and the closest corresponding sequences from the GenBank. The result revealed that samples 2, 3, 4, 5, 6 and 9 corresponded with genotype 1b, sample 8 corresponded with genotype 1a, sample 7 corresponded with genotype 2a/2f, while sample1 corresponded with genotype 4a.

The phylogenetic relatedness of the HCV isolates in the study with those in the GenBank as shown in Figure 4.1 revealed a common ancestral origin. Samples 2, 3,4,5,6 and 9 were closely related to reference AB828700 (1b) Japan, sample 8 was closely related to EU255988 (1a) USA, sample 1 was closely related to AB550028 (4a) Egypt and sample 7 was closely related to both KC844043 (2a) China and KC844042 (2f) China.

The results obtained from the sequence analysis and the alignment trees as shown in Table 4.17 indicates that of the nine (9) samples sequenced, Nasarawa State had Genotypes/subtypes 1b and 4a, Plateau State had Genotypes 1a, 1b and 2a/f, while Benue had Genotype 1b. Genotypes 1a, b and 2 a/f were recorded among Blood Donors, while Pregnant Women had Genotypes 1b and 4a. The sub-types detected in genotype 2 in the study were inconclusive. Genotype 1b had the highest distribution with 66.70%, while genotypes 1a, 2a/f and 4a had 11.10% prevalence each.

Table 4.16: Closest affiliates of HCV sequences obtained compared with those from the GenBank

No.	Accession No.	Source	Length	Score	Identities %	E-Value	Inference *
1.	AB550019	HCV Subtype 4a, 5'UTR	259	154	99.4	9.8E-80	Subtype 4a
2.	Z77102	HCV Subtype 1b 5'UTR	197	147	98.7	3.6E-75	Subtype 1b
3.	Z77102	HCV Subtype 1b 5'UTR	197	158	100.0	9.7E-82	Subtype 1b
4.	Z77102	HCV Subtype 1b 5'UTR	197	158	100.0	9.7E-82	Subtype 1b
5.	Z77102	HCV Subtype 1b 5'UTR	197	152	98.1	2.6E-79	Subtype 1b
6.	Z77101	HCV Subtype 1b 5'UTR	197	146	98.7	1.4E-74	Subtype 1b
7.	L38336	HCV Type 2a	1195	153	99.4	3.9E-79	Subtype 2a
	L38329	HCV Type 2c	1188	153	99.4	3.9E-79	Subtype 2c
8.	EU482831	HCV Subtype 1a	9283	153	98.7	3.9E-79	Subtype 1a
9.	Z77102	HCV Subtype 1b 5'UTR	197	158	100.0	9.7E-82	Subtype 1b

*The query sequences covers 100%.

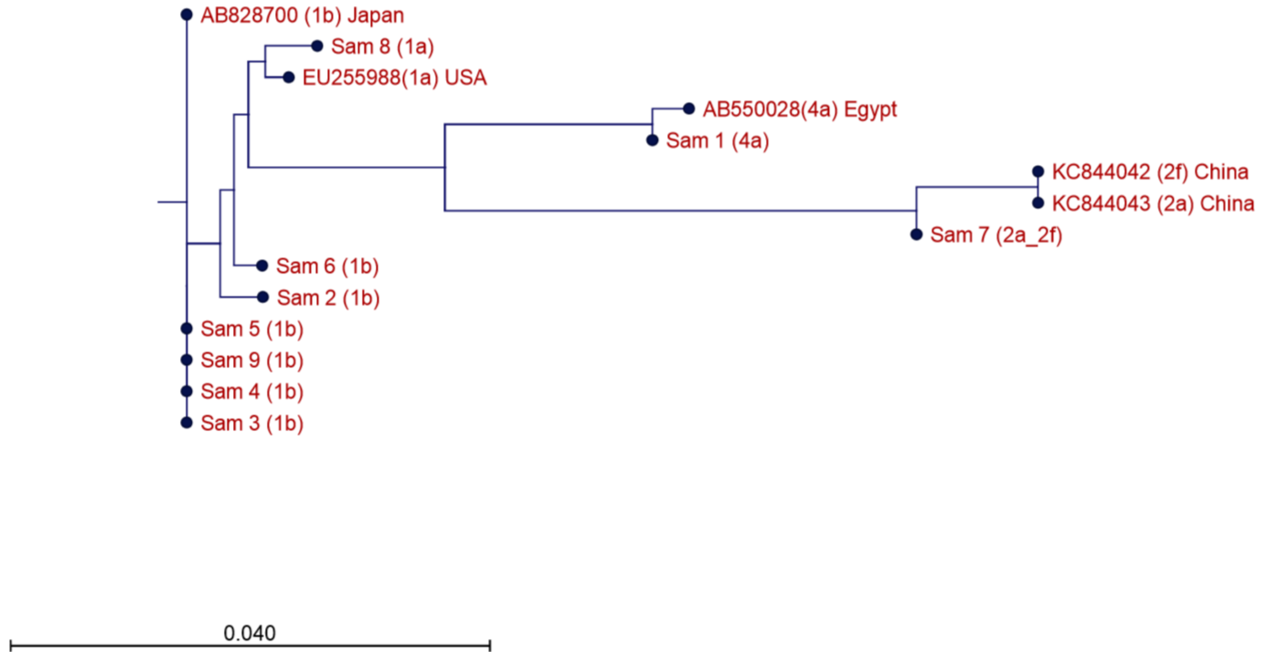

Figure 4.1: Phylogenetic analysis of partial 5'UTR sequences of 9 HCV samples. HCV prototype sequences from GenBank were included. The evolutionary history was inferred using the Neighbour-Joining (NJ) method.

Table 4.17: Distribution of HCV Genotypes and Subtypes in the study population based on location

	Location			
	Benue	Nasarawa	Plateau	Total (%)
Genotypes/Subtypes				
1a	0	0	1	1 (11.10)
1b	1	3	2	6 (66.70)
2a/f	0	0	1	1 (11.10)
4a	0	1	0	1 (11.10)
Total	1	4	4	9 (100.00)

CHAPTER FIVE

5.0 DISCUSSION

5.1 Discussion

Of 1511 plasma samples examined in the study (Table 4.1), 5.2% had evidence of HCV antibodies. This finding is comparable to the reports of similar studies across the country. Ogunro *et al*. (2007) reported a prevalence of 9.2% in Ladoke Akintola University of Technology Teaching Hospital (LTH), Osogbo, Osun State, Southwestern Nigeria; Ugbebor *et al*. (2011) reported 3.6% in Benin, Southeastern, Nigeria and Sheyin *et al*. (2012b) reported a seroprevalence of 4.5% in Kaduna State, Northwestern, Nigeria.

The seroprevalence of HCV among pregnant women in the study area based on location (Table 4.1) revealed that those from Plateau State had the highest rate of 4.4% (12), while Benue State had the least with 2.9% (8). The study revealed that there was no significant relationship in the seroprevalence with respect to location within the study area (p= 0.644). Sheyin *et al*. (2012b) in a similar study in Kaduna State reported that there was no significant association in the seroprevalence of the infection in relation to location (3 Senatorial Districts). This may be attributed to the increased awareness of the people on the preventive measures for the transmission of blood borne pathogens, sensitivity and specificity of the detection kits used, improved health care services and the rate of the viral transmission in the area.

Also, of the 801 pregnant women examined (Table 4.2), 3.6% (29) were seropositive for HCV this finding agreed or showed some variation with similar studies carried out in the

country and outside the country such as Sheyin *et al*. (2012b) in a study in Kaduna State, Northwestern Nigeria reported a seroprevalence of 4.5%, Mboto *et al*. (2010) reported a lower seroprevalence of 0.4% in Calabar, Southeastern Nigeria, Ogunro *et al*. (2007) reported a much higher rate of 9.2% for anti-HCV in Southwestern Nigeria while Shaikh *et al*. (2009) reported 3.4% in Pakistan and Kumar *et al*. (2007) reported a prevalence of 1.03% in a similar study in north India. These differences may be attributed to differences in the sensitivity and specificity of the detection kits used, cultural practices, beliefs, awareness of the preventive measures of the infection, health care services, the incidence rate and also the sample sizes in the various studies.

Of the 710 blood donors examined in the study, 7.0 % (50) were sero-positive for HCV antibodies. This finding is in agreement with the report of Chukwurah *et al*. (2005) who reported a prevalence of 7.6% among blood donors at University Teaching Hospital, Enugu, Southeastern Nigeria; Sheyin *et al*. (2012a) reported a seroprevalence of 5.8% among blood donors in Kaduna State, Northwestern Nigeria, however, Afolabi *et al*. (2012) recorded a much lower seroprevalence of 1.4% among blood donors in Ibadan, Southwestern Nigeria. This may be attributed to the differences in the performances of the detection kits used, the rate of exposure of the virus among the blood donors and habits/activities that can predispose them to the infection.

The seroprevalence of HCV among blood donors in the study area (Table 4.1) based on location shows that participants from Nasarawa State had the highest seroprevalence of 11.0% (24), while those from Benue State had the least prevalence of 5.2% (13). The study revealed that there was a significant association in the seroprevalence with respect to location within the study area ($p= 0.023$). This may be due to exposure of the blood

donors to contaminated blood or blood products through accidental injuries, unprotected sex, medical procedures or other risk factors within the three States. Blood donors comprised of voluntary and commercial donors. Voluntary Donors usually come from family members or friends, while commercial donors come from the wider community. Commercial donors who are involved in risky behaviours can pose a serious threat in the transmission of blood infections in this part of the country where the use of the molecular diagnostic test is yet to be put in place for the screening of blood before transfusion. Damulak *et al*. (2013) reported a prevalence of 6.1% for HCV antibody among voluntary blood donors and family replacement donors in Jos.

Gender-related seroprevalence among blood donors in this study (Table 4.2) showed that the seroprevalence was higher among males with 7.1% (44) than among females with 6.7% (6). This is in agreement with the findings of Vardas *et al*. (1999) in a similar study in Namibia who reported that males had higher prevalence of 1.6% (13) than females who had 0.4% (5); Udoze *et al*. (2009) in Ibadan, Southwestern Nigeria also reported that males had a higher seroprevalence of 8.2% compared to 6.3% in females. Olokoba *et al*. (2009) in a study in Yola Northeastern Nigeria reported that males had a higher seroprevalence of 96.0% while females had 4.0%. The result of this finding may be attributed to the fact that most blood donors in Nigeria are males, only a few females do donate blood for transfusion mostly in situations where their family male members are not disposed. The blood lost during child delivery and monthly menstrual cycle among women of reproductive age may also contribute in low female blood donors.

The seroprevalence of Hepatitia C Virus in relation to age and gender among blood donors as shown in Table 4.2 revealed that the entire trend of the seropositivity decreases

with increase in age. In the case of the males, the seroprevalence was within the age groups ≤20 – 50 years with the age group 40- 50 years recorded the highest with 11.9% (7), this may be attributed to the low sample size of those recruited within that age group or chances. However, no statistically significant difference (p= 0.583) was recorded in relation to age and sex. While among the females, the seroprevalence decreases with increase in age between the age group between ≤20 – 30 years, with no statistically significant difference established (p= 0.326). Earlier findings recorded a higher seroprevalence within age groups below 40 years which coincided with the most productive years (Olokoba *et al*., 2009; Udoze *et al*., 2009; Afolabi *et al*., 2012; Sheyin *et al*., 2012b).

The seroprevalence of HCV among pregnant women in relation to age as shown in Table 4.3 revealed that the age group ≤ 20 years had the highest seroprevalence of 6.3% (5) and it decreases with increase in age. However, there was no statistically significant difference between age and seroprevalence (p= 0.528). Earlier studies showed that the highest seropositivity of HCV antibodies occurred within the most productive age group (Marranconi *et al*., 1994; Centers for Disease Control and Prevention, 1998). Seropositivity has been found to increase up to the age of 40 years and then declines (Wasley and Alter, 2000). In our study, the seroprevalence in younger women was found to be higher up to the age of 35 years, similar to the other studies (Stevens *et al*., 1990; Shaikh *et al*., 2009). The result of this study may be due to the exposure of these women to the risk factors such as transfusion with contaminated blood during child delivery or contact with contaminated instruments during medical care.

The seroprevalence of HCV among blood donors in relation to marital status (Table 4.4) in this study showed that the separated had the highest with 33.3% (2), and the least were recorded among the divorced and widowed had 0.0% (0). Statistically, the result showed no significant differences (p= 0.528). While in the seroprevalence of HCV among pregnant women in relation to marital status (Table 4.5), HCV were detected in only two groups, the married and the singles. Afolabi *et al*. (2012) reported that the unmarried had the highest with 2.1% and the married with 1.0%. Nwannadi *et al*. (2012) in a similar study on seroprevalence of HCV reported that the singles had a higher prevalence of HCV antibodies (60%; n= 6) than the married patients (40%; n= 4). Sangha *et al*. (2009) in a study in Egypt reported the highest prevalence among those once married (16.8%), followed by the married (12.8%) and the least observed among the singles (3.0%). The differences recorded in these studies may be due to variation in certain risky activities engaged in by the different marital groups such as unprotected sex, use of contaminated sharp instruments for body piercing, unsafe injections and the sample size used in the different studies or chances.

This study as shown in Table 4.6 revealed that blood donors with a tertiary educational status had the highest prevalence of 8.4% (28), while those with primary educational status had the least with 3.2% (2). However, the result showed no significant association (p= 0.392). Also, the seroprevalence of HCV among pregnant women in this study (Table 4.7) revealed that those with non-formal education had the highest with 4.9% (3) while those with primary education had the least with 2.4% (2). However, there was no statistically significant difference (p= 0.789). Afolabi *et al*. (2012) in a study among blood donors at Ibadan, Southwestern Nigeria reported that subjects with tertiary

educational status had the highest seroprevalence with 2.0%, followed by those with secondary education (1.1%) and the least recorded among the illiterate/ Quranic education with 0.0% each. The higher seroprevalence recorded among those with tertiary and secondary education in this study may be due to exposure of these groups to certain risk factors or practices that can predispose them to the infection such as religious beliefs, use of contaminated tools for body/ ear piercings, cultural differences and involvement in gangs or cults. Kumar *et al*. (2007) reported in a similar study in north India that literacy status had no significant effect on the prevalence of anti-HCV. On the contrary, Rajesh and Sadiq (2012) in a study among the general Population in Central Region of Yemen showed the highest seroprevalence of HCV antibodies among illiterate subjects (0.59%; n= 14) and lowest among postgraduate subjects (0.00%; n= 0). The difference was found to be statistically significant ($p < 0.001$).

The seroprevalence of HCV antibodies in relation to occupation (Table 4.8) among blood donors revealed that the unemployed had the highest seroprevalence of 8.5% (21) while farmers had the least with 0.0% (0). However, the result showed that there was no significant difference between the seroprevalence and occupation (p = 0.301). Also, the seroprevalence of HCV among pregnant women in relation to educational status revealed that farmers had the highest rate of 6.8% (3), while health workers had 0.0% (0). However, there was no significant difference (p= 580). Sangha *et al*. (2009) in a survey among adults in Egypt recorded the highest prevalence among the poorest (12.4%) while richest had the least prevalence (6.9%). This showed that HCV infection risks were significantly lower for those in the highest wealth quintiles. Nwannadi *et al*. (2012) in a seroprevalence on HCV reported the highest prevalence amongst the employed (36.4%),

followed by students (33.7%) and the least amongst the unemployed (29.7%). The differences in the seroprevalence in this study may be attributed to the differences in the sample sizes of the individual groups, exposure to the infection through involvement in risky behaviours in an attempt to meet both ends financially or emotionally, chances and through contaminated medical devices during healthcare services.

The seroprevalence of HCV in relation to types of marriage in this study revealed that those who practice polygamy had a higher seroprevalence of 12.5% (1) while those who practice monogamy had 4.9% (8). However, the result showed no significant difference (p = 0.344). Also, the seroprevalence of HCV among pregnant women (Table 4.11) showed a higher seroprevalence among the polygamous subjects with 3.4% (1) while the monogamous had 3.0% (11). However, there was no statistically significant difference (p= 0.887). This finding may be attributed to the sample size between the two groups, or marital unfaithfulness among the spouses. This is in disagreement with the findings of Mboto *et al*. (2010) who reported that those in polygamous marriage had 0.0% (0) of HCV antibodies while those into monogamous marriage had 0.004% seroprevalence of HCV. In most studies, sexual transmission of HCV is considered to be rare, and some studies have shown that the risk of HCV transmission in heterosexual or monogamous relationships is rare or even null (Gordon *et al*., 1992; Vandelli *et al*., 2004; Karmochkine *et al*., 2006).

The seroprevalence of HCV in blood donors among those involved in sexual activities with multiple partners in relation to use of condoms showed that those who did not use condoms had the higher seroprevalence of 7.8%, while those who use condoms had 4.1% (p = 0.4054). Also, the seroprevalence of HCV among those involved in multiple

sex activities in relation to use of condoms revealed that those who used condoms at all times had a higher seroprevalence of 4.1%, while those who did not use condoms had 0.7% (p = 0.3191). The use of condoms for subjects involved in sex with multiple sexual partners in the study did not show any significant association with the seroprevalence this may be attributed to improper usage of the condoms or accidental failure of the condoms.

CDC (1998) reported that both heterosexuals and homosexuals with greater numbers of sex partners, a history of prior STDs, and failure to use the condom were among specific factors associated with HCV antibodies positivity. Although the findings of this study did not show any significant relationship, there is still need for more enlightenment campaigns on abstinence from sexual activity with a person(s) other than one's spouse and where it is impracticable, proper usage of condoms should be considered to minimise transmission of HCV and other blood-borne transmissible infections.

Seroprevalence of HCV antibodies among blood donors in the alcoholics and non-alcoholics in the study (Table 4.10) revealed that the non- alcoholics had the higher seroprevalence of 7.5% (42) while the alcoholics had 5.4% (8). This showed no statistically significant association (p= 0.382). Also, the seroprevalence among the pregnant women (Table 4.11) showed that the non-alcoholics had a higher seroprevalence of 3.8% (28) while the alcoholics had the lowest with 1.4% (1). However, there was no statistically significant difference (p= 0.296). This may be attributed to the sample size between the two groups, this study did not target population groups with high intake of alcohol. There is a similarity in this finding with a study conducted among prison inmates in Nasarawa State, North Central Nigeria by Adoga *et al*. (2009) who reported a higher prevalence of HCV antibodies (12.5%) among non-alcoholics than the alcoholics

(12.2%). CDC (1998) recommended that treatment of patients who are drinking excessive amounts of alcohol should be delayed until this behaviour is discontinued for ≥ 6 months. Smith *et al*. (2011) reported that complications in health outcomes among HCV-infected persons that were born during the period 1945–1965 included the use of alcohol. Furthermore, of all anti-HCV positive persons in the 1945–1965 birth cohorts who self-reported alcohol use, 57.8% reported consuming an average of two or more alcoholic drinks per day.

For persons identified with HCV infection, CDC recommends that they receive appropriate care, including HCV-directed clinical preventive services (e.g., screening for alcohol use, hepatitis A and hepatitis B vaccination as appropriate, and medical monitoring of disease). Recommendations are available to guide treatment decisions (Ghany *et al*., 2011). Treatment decisions should be made by the patient and provider after several factors are considered, including the stage of disease, hepatitis C genotype, comorbidities, therapy-related adverse events, and benefits of treatment (CDC, 2012).

The seroprevalence of HCV antibodies in this study among blood donors in cigarette smokers and non-smokers in this study revealed that non- cigarette smokers had the higher seroprevalence of 7.3% (48), while the smokers had the lowest seroprevalence of 3.6% (2). However, this showed no significant difference (p= 0.290). Also, the seroprevalence among pregnant women revealed that the non-alcoholics had the higher seroprevalence of 3.6% (29), while the alcoholics had 0.0% (0). However, showed no statistically significant difference (p= 0.634). This may be attributed to the sample size in this study as most of the subjects recruited in this study were non-alcoholics. This finding is comparable with the findings of Bala *et al*. (2012) in a study among blood donors in

Kano, Nigeria who reported a prevalence of 3.4% among non- cigarette smokers and no evidence of HCV antibodies (0.0%) among cigarette smokers (p= 0.566). Although there was no significant association between cigarette smoking and the seroprevalence of HCV in this study, there is still need for the enlightenment of the general public on the danger of cigarette smoking in disease progression in people with hepatocellular carcinoma (HCC). Chuang *et al*. (2010) reported a significant interaction between HCV infection and cigarette smoking. Fujita *et al*. (2006) evaluated the interaction of Hepatitis C Virus and cigarette smoking on death from hepatocellular cancer in the Japan collaborative cohort study found out that the odd ratio of death from HCC for smoking was 9.60(1.50-61.35) and 1.71(0.58-5.08) among anti-HCV positive and negative individuals, respectively.

The seroprevalence of HCV among blood donors in relation to some possible risk factors in this study (Table 4.12) revealed that of all the risk factors examined only those examined with a history of surgery and unsafe injections showed a significant association. Those with a history of surgery had a higher seroprevalence of 15.6% (5), while those without a history of surgery had 5.2% (21), this showed a significant association (p= 0.016; OR= 3.404). Likewise, those with a history of unsafe injections had a higher seroprevalence of 50% (1), while those without a history of unsafe injections had 5.9% (25). However this showed a significant association (p= 0.009; OR= 15.960).

Also, the seroprevalence of HCV among pregnant women in relation to some possible risk factors (Table 4.13) showed that only those with a history of blood transfusion showed a significant association (p= 0.042; OR= 2.716). Most of the risk factors examined in this study showed no significant association with the seroprevalence of HCV

($p > 0.05$; Odd Ratios below significant levels). This may be attributed to the improved health practices in our health facilities, increased awareness of the risks associated with unsterile or blood-contaminated objects or sharp instruments and unprotected sexual activities.

Earlier studies have found an association between the prevalence of HCV infection and the known risk factors of this infection *i.e*., blood transfusion, intravenous drug abuse, multiple sexual partners, and homosexuality, history of STI (Bohman *et al*., 1992; Silverman *et al*., 1993; Conte *et al*., 2000; Shustov *et al*., 2005; Cristianne *et al*., 2007). In a study from northern Italy, the principal risk factors were the history of intravenous drug abuse with 32% and exposure to blood products with 24% (Conte *et al*., 2000). A similar study in London reported a higher proportion of the HCV-positive women who had used illicit drugs and a higher proportion of infected women had undergone ear piercing (Ward *et al*., 2000). Most patients had a history of surgery; nevertheless, they were also exposed to other risk factors. Recent studies have described low or no association to HCV for patients who had previously undergone surgery, general anaesthetic or dental treatment (Shustov *et al*., 2005; Leao *et al*., 2006). On the other hand, an African study showed a rate of 50% of HCV positivity among patients who were given injections without proper safety measures or with non-sterile needles and syringes, therefore contributing to the HCV dissemination in that continent (Madhava *et al*., 2002). This study disagreed with Obienu *et al*. (2011) in a similar study among Nigerian patients revealed that all the risk factors considered in that study showed no significant association.

The comparison of ELISA technique which served as the goal standard in this study with the two commercially available immunochromatographic rapid Kits for the detection of HCV antibodies as shown in Table 4.14 reveals that out of 79 anti-HCV positives and 421 anti-HCV negative by ELISA samples, the Wondfo Kit had a sensitivity of 75.0%, specificity of 99.0%, overall accuracy of 95.2%, positive predictive value of 93.6%, negative predictive value of 95.4%, positive likelihood ratio of 75.0, negative likelihood ratio of 0.25 and Kappa value of 0.803. Although this result indicated that the test kit showed a comparable result with the ELISA, the kit should just be used for a routine check -ups or screenings but not for transfusion purposes in order to minimise chances of transfusing infected blood to uninfected individuals thus increasing the disease burden among the general population.

The Global Kit had a sensitivity of 57.0%, specificity of 100.0%, overall accuracy of 93.2%, positive predictive value of 100%, negative predictive value of 92.5%, positive likelihood ratio of 0.57, negative likelihood ratio of 0.43 and Kappa value of 0.672 (Table 4.14). This finding is comparable with a similar work carried out by Muhibi *et al*. (2013) who reported that the Global kit gave 68.8% sensitivity, 100% specificity, while the positive and negative predictive values were 100% and 97.42% respectively. The overall performance of this kit was lower than that of the Wondfo kit when compared with the ELISA. The kit has an excellent specificity but the major setback of the kit is its sensitivity.

Based on the data obtained in this study (Table 4.14) wondfo kit proved to be more efficient than the Global kit. This was supported by the Kappa statistic test. Kappa has a range from 0-1.00, with larger values signifying better reliability. Generally, a Kappa of >

0.70 is considered satisfactory. The efficiency of rapid immunochromatographic kits was compared with the ELISA kit to assess their reliability with the use of Cohen's Kappa test (Fleiss *et al*., 2004). A level of agreement above 0.75 was considered to be excellent (Fleiss *et al*.,2004; Talebkhan *et al*., 2009); in this study Kappa agreement value of 0.803 was recorded for Wondfo kits, which falls within the acceptable range ,while the Global kit was 0.672 which was below the acceptable range (Feuerman and Miller,2008).

The range of sensitivity in this study was lower than those stated in the manufacturer's manuals (both Global kit and Wondfo had 99.0%). The sensitivity differences noted could be due to different immunochromatographic flow characteristics, antigen composition, concentration, or deposition or strength of colorimetric indication due to antibody binding. Conformational epitopes have been demonstrated to be more immunoreactive than linear recombinant proteins (Lin *et al*., 2005).

The study suggested that apart from likely defects on the products from the manufacturers of the kits, poor handling or storage from the manufacturer to the users of the kits might have resulted in the significant decrease in the sensitivity for the rapid immunochromatographic kits compared to the ELISA. Possible explanations for heat-related decrease include the impact on protein antigens, instability of reagents, and/or damage to the lateral flow matrix resulting in inhibition of lateral flow chromatography (O'Connell *et al*., 2013). The specificity reported in the manufacturer's manuals were 98.6% for Global kit while 99.8% for Wondfo kit. The specificity in this study was not significantly affected and this agreed with an earlier report (O'Connell *et al*., 2013).

The study indicated that the ELISA Kit had a better specificity and sensitivity over the two rapid immunochromatographic techniques. This is in consonance with the findings of Khan *et al*. (2010); Hussain *et al*. (2011); Muhibi *et al*. (2013)) who reported that ELISA proved to be more sensitive than the rapid immunochromatographic techniques.

Although the rapid screening method is the commonly used method adopted by diagnostic laboratories in the study area because it is cheaper and less expensive than ELISA technique and considering the superiority of ELISA over the rapid diagnostic techniques, there is need to adopt ELISA technique for screening anti-HCV especially for transfusion purposes to minimize chances of transfusing the virus to uninfected individuals.

The results as shown in Table 4.15 indicated that ELISA technique detected a much higher positive cases (79) than the PCR which detected only 15(18.98%) cases. Mohamoud *et al*. (2013) in a study in Egypt reported an overall average RNA prevalence among those that were HCV-antibody positive of about 60%. Some earlier findings reported a higher RNA prevalence than the HCV antibodies seroprevalence using ELISA (Itshad *et al.*, 2007; Swellam *et al.*, 2011).The disparity in these findings and the results obtained in this study may be attributed to several reasons which may include: false positive results from resolved infections, the detection limit of the kits, specificity, sensitivity and reliability of the kits used and the storage of the samples and kits used.

The sequence/phylogenetic analysis in this study (Table 4.17) showed the distribution of the genotypes and subgenotypes in the study area using the amplicon products from the 5'UTR region of the HCV genome. Failure of amplification of the NS5B region using

conventional PCR may be due to the presence of PCR inhibitors in the samples; PCR primers and/or probes design may not be optimal, inaccurate sample and reagent pipetting and poor storage temperature due to unstable power supply.

The result of this study as showed in Figure 4.1 agreed with earlier findings that substantial regional differences exist in the distribution of HCV genotypes in the world. Genotypes 1, 2, and 3 have a worldwide distribution and HCV subtypes 1a and 1b are the most common genotypes prevalent (Zein, 2000). Genotypes 3, 5 and 6 were not found in this study, this further supports the fact that there are substantial regional differences in the global distribution of the types of HCV genotypes.

Isolates 3, 4, 5 and 9 as shown in Figure 4.1 were more closely related with the reference isolate AB828700 from Japan which was identified as genotype 1b (Ando *et al*., 2013) than isolates 2 and 6. The higher homology of isolates 3, 4, 5 and 9 with the reference isolate than isolates 2 and 6 shows that their origin may be Japan, while that of isolates 2 and 6 may be from a different country. Isolate 8 showed a high homology with reference isolate EU255988 which is identified as genotype 1a of USA sequence (Kuntzen *et al*., 2008). The phylogenetic tree showed that they are clustered into one branch. The origin of this isolate is closely related to the USA strain.

Likewise, isolate 1 showed a high relatedness with reference AB550028 which is identified as genotype 4a of Egypt (Elkady *et al*., 2010) this signified that the isolate may be of Egyptian origin. Isolate 7 showed a high identity with reference isolates KC844042 which is identified as genotype 2f from China and also KC844043 which is identified as

genotype 2a also from China (Xu *et al*., 2013) these showed that the isolate identified may either be genotype 2a or 2f. The isolate is closely related to the Chinese strain.

The genotypes detected in this study as shown in Table 4.17 were genotypes 1, 2 and 4. Genotype 1b was the predominant (66.70%) HCV genotype in circulation in the study area. These findings agreed with earlier findings such as Oni and Harrison (1996) in a pilot survey of hepatitis C virus (HCV) infection in Southwestern Nigeria carried out on healthy adult blood donors and children of preschool age reported genotypes 1 and 4 in circulation in the area. Sheyin *et al*. (2012a) in a study in Kaduna State, reported the presence of only genotype 1b, while Forbi *et al*. (2012) in a study on the endemic history of hepatitis C virus infection in two remote communities in Nasarawa State, Nigeria, reported the presence of genotypes 1 and 2.

The phylogenetic sequencing results for the '5 UTR of HCV in this study differentiated well the different genotypes in the study area, but the subtypes for genotype 2 were poorly differentiated. This agreed with the findings of Hraber *et al.* (2006) who reported that the highly conserved 5' untranslated region (UTR) yields phylogenetic trees with topologies that differ from the HCV polyprotein and complete genome phylogenies. The 5' UTR contains insufficient variation to resolve HCV classifications to the level of viral subtype.

Knowing the infecting genotype has a direct impact on the prognosis and on the choice and duration of the treatment algorithm as well as being a statistically significant predictor of sustained virological response to antiviral therapy (Al Olaby and Azzazy, 2011). Based on the result of the HCV genotypes obtained in this study, clinicians and

health workers in the study area can find the data useful in managing patients who are infected with the infection and also in creating awareness on the prevention and control of the infection to the general public, blood donors, pregnant women on antenatal care and other patients coming for health care in their facilities.

Although the HCV subtype afflicting a patient is not currently used to make clinical treatment decisions, knowing the viral subtype is important for studies of its origin, transmission, and evolution (Fried *et al*., 2002; Hadziyannis *et al*., 2004; Simmonds, 2004; Weck, 2005). For example, new emerging variants can be characterised better when they can be assigned an unequivocal subtype classification (Simmonds *et al*., 2005). Molecular epidemiology analyses rely on information about sequence variation at the subtype level (Weck, 2005; Simmonds *et al*., 2005).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The study covered three states from North Central Nigeria and it aimed at the detection and genotyping of Hepatitis C Virus in the area. Questionnaires were used to capture vital demographic data from the subjects and 1511 subjects were recruited for the study (801 pregnant women and 710 blood donors). ELISA technique was used to analyse all the samples, while two immunochromatographic test kits (Global, USA, and Wondfo, China) were used to compare 500 samples analysed with an ELISA kit (Autobio, China). Polymerase Chain Reaction was carried out on all the anti-HCV positive samples and sequencing/phylogenetic analysis was carried out on the PCR positive amplicons.

Of 1511 participants examined in the study population which comprised of the pregnant women and blood donors, 79 (5.2%) had evidence of HCV antibodies. The seroprevalence of HCV among the Blood donors and pregnant women in the study revealed that there was no significant difference in the prevalence in respect to a location within the study area ($p = 0.108$). Of the 801 pregnant women examined in the study area, 3.6% (29) were seropositive for HCV. So also, of the 710 blood donors screened in the study area 7.0% (50) were seropositive for HCV.

Of the 710 blood donors examined, 7.0 % were seropositive for HCV. Nasarawa State had the highest seroprevalence of 7.0%, followed by Plateau State with 5.4%, and Benue State with 5.2% ($p = 0.023$). Of the pregnant women examined, Plateau State had the

highest seroprevalence of 4.4%, followed by Nasarawa State with 3.5% and Benue State had the least with 2.9% ($p = 0.644$).

The seroprevalence of HCV among blood donors in relation to gender showed that males had the higher seroprevalence of 7.1% (44), while females had 6.7% (6). The result of the study indicated that the male blood donors of age group 41 – 50 years had the highest prevalence of 11.9%, while the age groups 51- ≥ 61 years had the least seroprevalence with 0.0% ($p = 0.583$). The female blood donors of the age group 41- 50 years had the highest seroprevalence of 10.9%, while the age groups ≥ 51 years had 0.0% ($p = 0.326$). Pregnant women of the age group ≤ 20 had the highest seroprevalence of 6.3% (5) while the age group 21- 30 had the least with 3.2% (5). However, this showed no statistical significance difference ($p = 0.528$).

The seroprevalence of HCV among blood donors in relation to marital status showed that the separated had the highest with 33.3%, followed by the singles who had 7.2%, the married had 6.7% while the divorced and widowed had (0.0%) in their samples ($p = 0.123$).

The seroprevalence of HCV among pregnant women in relation to marital status showed that the married had the highest with 8.9%, followed by the singles with 5.3%, while the divorced, widowed and separated had (0.0%) in their samples ($p = 0.930$).

The seroprevalence of HCV among blood donors in relation to educational status showed that those with tertiary educational status had the highest with 8.4%, followed by those with secondary educational status with 6.7%, those with non-formal educational status had 3.3% and the least was among those with primary educational status with 3.2% (p=

0.392). Also, the seroprevalence of HCV among pregnant women in relation to educational status showed that those with non-formal education had the highest with 4.9%, followed by those with secondary educational status who had 4.1%, those with tertiary educational status had 3.2%, while those with primary educational status had the least with 2.4% (p= 0.789).

The seroprevalence of HCV among blood donors in relation to occupational status showed that the unemployed had the highest with 8.5%, health workers, civil servants, business people, and farmers had 8.3%, 8.0%, 6.0% and 0.0% (p = 0.301). The seroprevalence of HCV among pregnant women in relation to occupational status showed that the farmers had the highest with 6.8%, the unemployed, civil servants, business people, and health workers, with 4.7%, 3.4%, 3.1% and 0.0% respectively. The result showed that there was no statistical significant difference (p = 0.580).

The seroprevalence of HCV among blood donors in relation to types of marriage revealed that those who practice polygamy had the higher seroprevalence of 12.5% (1) while those who practice monogamy had 4.9% (8). However, the result showed no significant difference (p = 0.344). Also, the seroprevalence of HCV among pregnant women revealed that those who practice polygamy had the higher seroprevalence of 3.4% while those who practice monogamy had 3.0% (p = 0.887).

The seroprevalence of HCV in blood donors among those involved in sexual activities with multiple partners in relation to use of condoms showed that those who did not use condoms had the higher seroprevalence of 7.8%, while those who used condoms had 4.1% (p = 0.4054). Also, the seroprevalence of HCV in pregnant women among those

involved in multiple sex activities in relation to use of condoms revealed that those who use condoms at all times had a higher seroprevalence of 4.1%, while those who did not use condoms had 0.7% (p = 0.3191).

The seroprevalence of HCV among the alcoholics and non-alcoholics among blood donors showed that those who were non- alcoholics had the higher seroprevalence of 7.5% while the alcoholics had the lowest with 5.4% (p = 0.382). The seroprevalence of HCV in the alcoholics and non-alcoholics among pregnant showed that those who were non- alcoholics had the higher seroprevalence of 3.8% while the alcoholics had the lowest seroprevalence of 1.4% (p = 0.296).

The seroprevalence of HCV among the smokers and non-smokers among the blood donors revealed that the non- cigarette smokers had the higher seroprevalence of 7.3% while the smokers had 3.6% (p = 0.290). Also, the seroprevalence of HCV among the smokers and non-smokers among pregnant women revealed that the non- cigarette smokers had the higher seroprevalence of 3.6% while the smokers had 0.0% (p = 0.634).

The seroprevalence of HCV among blood donors in relation to some possible risk factors in this study revealed that of all the risk factors examined, only those examined with a history of surgery and unsafe injections showed a significant association. Those with a history of surgery had a higher seroprevalence of 15.6%, while those without a history of surgery had 5.2% (p= 0.016; OR= 3.404). Likewise, those with a history of unsafe injections had a higher seroprevalence of 50%, while those without a history of unsafe injections had 5.9% (p= 0.009; OR= 15.960).

Also, the seroprevalence of HCV among pregnant women in relation to some possible risk factors showed that only those with a history of blood transfusion showed a significant association (p= 0.042; OR= 2.716).

The comparison of ELISA technique with the two commercially available chromatographic rapid Kits for the detection of anti-HCV revealed that ELISA remains the gold standard for anti-HCV detection, the overall performance of Wondfo Kit performed better than Global Kit. This study suggested the use of more than two rapid kits with very high sensitivity and specificity in low in-come countries where the use of ELISA/PCR is unaffordable to minimise transfusion of HCV.

Of the 1511 subjects screened for HCV antibodies, only the seventy- nine (79) samples that were HCV antibodies positive were used for the qualitative detection of HCV RNA. The result showed that 15 (18.98%) samples were HCV RNA positive. Nucleic Acid Technology remains the gold standard in determining viremia.

Of the nine (9) samples sequenced the results obtained from the sequence analysis and the alignment trees showed that seven samples were genotype 1, one sample was genotype 2 and another one sample was genotype 4. This indicated that the predominant HCV genotype in the study area was genotype 1. The study showed that Nasarawa State had Genotypes 1 and 4; Plateau State had Genotypes 1 and 2, while Benue had Genotype 1. Genotypes 1 and 2 were detected among blood donors in this study, while pregnant women recorded Genotypes 1 and 4. For the characterisation of the genotype and subtypes, one sample had genotype 1a, six samples were genotype 1b, one sample was genotyped 2a/f and the last sample was genotype 4a. The sample that was detected

genotypes 2a/f indicated an inconclusive result. All the genotypes determined were from the same ancestral HCV strain. There were high homogeneities between the genotypes determined in this study with earlier strains from Japan, United States of America, Egypt and China.

6.2 Conclusion

This study showed an overall seroprevalence of 5.2% HCV antibodies amongst the blood donors and pregnant women in the study area. The blood donors had a seroprevalence of 7.0%, while the pregnant women had a seroprevalence of 3.6%. Males recorded the highest seroprevalence of 7.1% and the females had 3.9%. The study revealed that the highest seroprevalence occurred within the most productive age groups. The seroprevalence of HCV in relation to other sociodemographic variables such as educational status, occupational status, types of marriage, use of condoms, alcoholic intake and cigarette smoking did not show any statistical significance. Of the demographic data considered in this study, only location of blood donors within the study area showed significant associations (p= 0.023). Of all the risk factors considered among blood donors, only those with a history of surgery (p= 0.016; OR= 3.404) and a history of unsafe injections (p= 0.009; OR= 15.960) showed a significant association with seroprevalence. Also, of all the risk factors considered for the pregnant women, only those with history of blood transfusion showed a significant association (p= 0.042; OR= 2.716).

The two rapid immunochromatographic kits, when compared with ELISA kit, further proved the superiority of ELISA over the two kits. The study showed that of the two

rapid kits compared, Wondfo kit performed better than the Global kits. Considering the consequences of transfusing an infected blood to an uninfected patient, the use of ELISA technique for screening blood before transfusion and where it can not be affordable, the use of two to three rapid immunochromatographic kits with high sensitivity and specificity can be adopted to minimise cases of transfusing positive samples.

PCR still remains the gold standard for establishing viremia in a patient. Out of the 79 HCV antibodies positive samples recorded in the study, only 15 tested positive for HCV RNA. The use of the nucleic acid technology if adopted in our Blood Banks will go a long way in reducing the transmission of HCV and other blood-borne pathogens through the blood transfusion to the bare rest minimum level. The HCV genotypes detected in this study by sequence and phylogenetic analysis were genotypes 1, 2 and 4, while the genotypes with subtypes were 1a, 1b, 2a/f and 4a. Genotype 1b was the predominant (66.70 %) in this study. The sequences/phylogenetic analysis failed to differentiate the subtypes for genotype 2 due to the limitation that can be associated with the target region of the HCV genome that was successfully used in this study as such the result for genotype 2 i.e. 2a/f is inconclusive. All the genotypes determined were from the same ancestral HCV strain. There were high homogeneities between the genotypes determined in this study with earlier strains from Japan, United States of America, Egypt and China. The result of the genotypes is essential in the management of people infected with the infection.

6.3 Recommendations

In order to curtail the spread of HCV in the study area and the whole country, the following recommendations are necessary:

1. There is a need for wider HCV serosurveys to capture the general population and risk groups all over the nation, this can be helpful to policymakers in the fight against the infection.
2. Screening of blood donors and confirmation at all levels should be ensured by the practitioners, patients and health regulatory bodies.
3. Screening for anti-HCV should be included among the screening for all ante-natal patients and those confirmed to be positive by HCV molecular detection should be treated free through support from the government and non-governmental agencies.
4. The Federal Government should strength the National Transfusion Board Centre through increased funding, manpower and infrastructural development toward having accessible transfusion centres in all our local government areas to service all our health facilities in the country.
5. There should be a wider study in the study area on the genotypes/subtypes targeting the NS5B or all the various regions of the HCV genome.
6. Since there is no vaccine currently for the treatment of HCV there is a need for more awareness campaigns on the prevention and control of the infection among every group of Nigerians.
7. There should be more political will and commitment at the different levels of the government on the fight against the infection such as the establishment of

standard diagnostic and research laboratories, provision of stable power, subsidising or provision of free treatment for all patients with viral Hepatitis, funding of research works on HCV and the implementation of the findings.

8. There is a need for global strategies toward the eradication of HCV especially in the area of vaccines development that can prevent or cure infections of all the genotypes in circulation and preventive measures to reduce cases of transferring all the genotypes globally considering the world now being a global village.

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APPENDICES

Appendix Ia: Ethical clearance from Jos University Teaching Hospital

JOS UNIVERSITY TEACHING HOSPITAL
JOS, NIGERIA

Phone: 073-450226 - 9
E-mail:juth@infoweb.abs.net

Cables & Telegram: JUTH
P.M.B. 2076
Jos.

Ref: JUTH/DCS/ADM/127/XIX/5103

Date: 2nd July, 2012.

Bigwan Emmanuel Isa
Department of Microbiology
Ahmadu Bello University Zaria,
Zaria, Nigeria.

RE: ETHICAL CLEARANCE/APPROVAL

I am directed to refer to your application dated, 3rd April, 2012 on the research proposal titled:

"Detection and Genotyping of Hepatitis C Virus among Blood Donors and Pregnant Women in Plateau, Benue and Nasarawa States, North Central Nigeria" and your appearance before the Ethical Committee on 1st June, 2012.

Following recommendation from the Institutional Health Research Ethical Committee, I am to inform you that Management has given approval for you to proceed on your research topic as indicated.

You are however required to obtain a separate approval for use of patients and facilities from the department(s) you intend to use for your research.

The Principal Investigator is required to send a progress report to the Ethical Committee at the expiration of three (3) months after ethical clearance to enable the Committee carry out its oversight function.

Submission of final research work should be made to the Institutional Health Research Ethical Committee through the **Secretary in Room 14, Administration Department,** please.

On behalf of the Management of this Hospital, I wish you a successful research outing.

Hajia R. Danfillo
For: Chairman, MAC

Appendix Ib: Ethical clearance from Federal Medical Centre Keffi

FEDERAL MEDICAL CENTRE

P.M.B. 004
KEFFI NASARAWA STATE,
HEALTH RESEARCH ETHICS COMMITTEE
047-620020, 620095, 080339700028

Website: http:www.fmckeffi.net
E-mail: fmc.keffi@gmail.com, info@fmckeffi.net

31st May, 2012

Bigwan Emmanuel Isa
Department of Microbiology,
Ahmadu Bello University,
Zaria.

RE: ETHICAL CLEARANCE TO CONDUCT RESEARCH

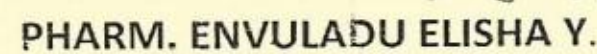
The above subject matter refers.

2. I write to convey ethical approval to you to embark on the said research project **Titled: Detection and Genotyping of Hepatitis C virus amongst Blood donors and pregnant Women in some parts of North Central Nigeria.** This follows successful defend of your research proposals during the Screening interview by the Health Research Ethical Committee (HREC) on the 22nd May, 2012.

3. You are however expected to adhere strictly to internationally acceptable ethical standard governing the conduct of research please. Accordingly, all rights and privileges of the volunteering participants must be respected.

4. Extracts of your findings should be forwarded to our Institution's Review Board /HREC for filing and possible action at the end of your project.

Thank you.


31/5/12

PHARM. ENVULADU ELISHA Y.
Secretary HREC.

Appendix Ic: Ethical clearance from Federal Medical Centre, Makurdi

FEDERAL REPUBLIC OF NIGERIA

FEDERAL MEDICAL CENTRE MAKURDI

HOSPITAL ROAD, MAKURDI, BENUE STATE

P.M.B. 102004

Telephone/fax: 044/533-133

Ref: No. FMH/FMC/MED.108/VOL.I/112

Date: 5th July, 2012.

Mr. Bigwan Emmanuel Isa,
Department of Microbiology,
Ahmadu Bello University,
Zaria,
Kaduna State.

ETHICAL LETTER OF APPROVAL

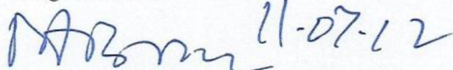
On the directives of the Management of this hospital, the committee on Ethical Review Committee hereby conveys approval to your study "Detection and Genotyping of Hepatitis C Virus amongst blood donors and pregnant women in some parts of North Central Nigeria".

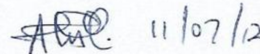
The committee has not seen any ethical problem arising from your methodology.

You are hereby permitted to go on with the study. However, you are advised to stick strictly to your design and work within the time limit.

Equally note that a copy of your final work must be submitted to this committee on completion of your work.

Congratulations.





DR. INUNDUH, M. P., *MBBS, FWACS*
Head of Clinical Services
Chairman Ethical Review Committee

MRS. ALOCHA R. I.
Secretary Ethical Review Committee

Appendix II: Questionnaire

QUESTIONNAIRE ON DETECTION AND GENOTYPING OF HEPATITIS C VIRUS AMONGST BLOOD DONORS AND PREGNANT WOMEN IN SOME PARTS CENTRAL NIGERIA

1. Participant's initial……………………………………………………
2. Identification number…………
3. Address:…………………………………………………………….

4. Age…………

5. Sex: Male/ Female

6. Marital Status: (a) Single (b) Married (c) Divorced (d) Widowed (e) Separated

7. Education level (a) Non Formal (b) primary (c) Secondary (d) Tertiary

8. Occupational status (a) farmer (b) Civil servant (c) Business (d) Unemployed

   (f) Health worker (g) Others ……………………….

9. Have you ever received blood transfusion? Yes/No

10. Have you ever been operated upon? Yes/No

11. Have you been tested for HIV? Yes/No. If yes, what was the result?

    (a) Positive (b) negative

12. Do you have multiple sexual partners? Yes/No

13. Do you use condoms when having sex with any person other than your spouse? Yes/No

14. Have you ever undergone D&C for abortion? Yes/No
15. Have you been sharing injecting or sharp equipments with others? Yes/No

16. Do you smoke cigarette? Yes/No

17. Do you drink alcoholic drinks? Yes/No

18. Any history of tribal mark/tattooing? Yes/No

Appendix III: Consent Form

CONSENT FORM

I Bigwan Emmanuel Isa, a post graduate student of Ahmadu Bello University Zaria in the Department of Microbiology will be undertaking a research work on the topic: Detection and Genotyping of HCV among pregnant women and blood donors in some parts of Central Nigeria.

The purpose of the research is to determine the prevalence of Hepatitis C virus infection, the likely genotypes/sub-types, risks factors associated with the infection and evaluating some of the commonly used diagnostic kits in the area. This will provide information to aid Medical Practitioners in the management of the infection, base line data for future researches and recommendations that will be helpful to the public on the prevention and control of the infection.

I will appreciate your participation in this study.

The information obtained will be kept secret and use only for the purpose of the research.

Participation is voluntary. Non participation will not hinder you from getting full medical services from the hospital. All investigations will be free.

Kindly fill the space below if you have your consent to participate.

I …………………………..give my consent to participate in this study. I have been duly informed of the process and have given my free will and not under pressure.

Signature/Thumbprint of subject Date………………………………….
……………………………

Signature of witness Date………………………………….
………………………………….

Signature of the investigator Date…………………………………..
………………………………….

Appendix IV: Multiple sequence alignment of the HCV strains in circulation and the reference strains in the NCBI data base

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                                              20                                      40
                                               |                                       |
AB828700 (1b) Japan  GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
       Sam 3 (1b)    GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
       Sam 4 (1b)    GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
       Sam 9 (1b)    GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
       Sam 2 (1b)    GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
       Sam 6 (1b)    GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
  EU255988(1a) USA   GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
       Sam 8 (1a)    GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
       Sam 5 (1b)    GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCYATAGT  GGTCTGCGGA  50
       Sam 1 (4a)    GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
AB550028(4a) Egypt   GTGCAGCCTC  CAGGACCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
KC844043 (2a) China  GTACAGCCTC  CAGGCCCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
KC844042 (2f) China  GTACAGCCTC  CAGGCCCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
    Sam 7 (2a_2f)    GTGCAGCCTC  CAGGMCCCCC  CCTCCCGGGA  GAGCCATAGT  GGTCTGCGGA  50
                                  60                                      80                                     100
                                   |                                       |                                       |
AB828700 (1b) Japan  ACCGGTGAGT  ACACCGGAAT  TGCCAGGACG  ACCGGGTCCT  TTCTTGGATC  100
       Sam 3 (1b)    ACCGGTGAGT  ACACCGGAAT  TGCCAGGACG  ACCGGGTCCT  TTCTTGGATC  100
       Sam 4 (1b)    ACCGGTGAGT  ACACCGGAAT  TGCCAGGACG  ACCGGGTCCT  TTCTTGGATC  100
       Sam 9 (1b)    ACCGGTGAGT  ACACCGGAAT  TGCCAGGACG  ACCGGGTCCT  TTCTTGGATC  100
       Sam 2 (1b)    ACCGGTGAGT  ACACCGGAAT  TGCCAGGACG  ACCGGGTCCT  TTCTTGGATC  100
       Sam 6 (1b)    ACCGGTGAGT  ACACCGGAAT  TGCCAGGACG  ACCGGGTCCT  TTCTTGGATC  100
  EU255988(1a) USA   ACCGGTGAGT  ACACCGGAAT  TGCCAGGACG  ACCGGGTCCT  TTCTTGGATC  100
       Sam 8 (1a)    ACCGGTGAGT  ACACCGGAAT  TGCCAGGACG  ACCGGGTCCT  TTCTTGGATC  100
       Sam 5 (1b)    ACCGGTGAGT  ACACCGGAAT  TGCCAGGACG  ACCGGGTCCT  TTCTTGGATC  100
       Sam 1 (4a)    ACCGGTGAGT  ACACCGGAAT  CGCCGGGATG  ACCGGGTCCT  TTCTTGGATY  100
AB550028(4a) Egypt   ACCGGTGAGT  ACACCGGAAT  CGCCGGGATG  ACCGGGTCCT  TTCTTGGATT  100
KC844043 (2a) China  ACCGGTGAGT  ACACCGGAAT  TGCCGGGAAG  ACTGGGTCCT  TTCTTGGAT-  99
KC844042 (2f) China  ACCGGTGAGT  ACACCGGAAT  TGCCGGGAAG  ACTGGGTCCT  TTCTTGGAT-  99
    Sam 7 (2a_2f)    ACCGGTGAGT  ACACCGGAAT  TGCCGGGAAG  ACTGGGTCCT  TTCTTGGAT-  99
                                              120                                     140
                                               |                                       |
AB828700 (1b) Japan  AA-CCCGCTC  AATGCCTGG-  AGATTTGGGC  GTGCCCCCGC  GAGACTGCTA  148
       Sam 3 (1b)    AA-CCCGCTC  AATGCCTGG-  AGATTTGGGC  GTGCCCCCGC  GAGACTGCTA  148
       Sam 4 (1b)    AA-CCCGCTC  AATGCCTGG-  AGATTTGGGC  GTGCCCCCGC  GAGACTGCTA  148
       Sam 9 (1b)    AA-CCCGCTC  AATGCCTGG-  AGATTTGGGC  GTGCCCCCGC  GAGACTGCTA  148
       Sam 2 (1b)    AA-CCCGCTC  TATGCCTGGT  AGATTTGGGC  GTGCCCCCGC  GAGACTGCTA  149
       Sam 6 (1b)    AA-CCCGCTC  TATGCC--GG  AGATTTGGGC  GTGCCCCCGC  GAGACTGCTA  147
  EU255988(1a) USA   AAACCCGCTC  AATGCCTGG-  AGATTTGGGC  GTGCCCCCGC  AAGACTGCTA  149
       Sam 8 (1a)    AAACCCGCTY  TATGCCTGG-  AGATTTGGGC  GTGCCCCCGC  AAGACTGCTA  149
       Sam 5 (1b)    AA-CCCGCTC  AATGCCTGG-  AGATTTGGGC  GTGCCYCCGC  GAGACTGCTA  148
       Sam 1 (4a)    AAACCCGCTC  AATGCCCGG-  AAATTTGGGC  GTGCCCCCGC  GAGACTGCTA  149
AB550028(4a) Egypt   AA-CCCGCTC  AATGCCCGG-  AAATTTGGGC  GTGCCCCCGC  GAGACTGCTA  148
KC844043 (2a) China  AAACCCACTC  TATGCCCGGC  C-ATTTGGGC  GTGCCCCCGC  AAGACTGCTA  148
KC844042 (2f) China  AAACCCACTC  TATGCCCGGC  C-ATTTGGGC  GTGCCCCCGC  AAGACTGCTA  148
    Sam 7 (2a_2f)    AAACCCACTC  TATGCCCGGC  C-ATTTGGGC  GTGCCCCCGC  AAGACTGCTA  148
                                  160
                                   |
AB828700 (1b) Japan  GCCGAGTAGT  158
       Sam 3 (1b)    GCCGAGTAGT  158
       Sam 4 (1b)    GCCGAGTAGT  158
       Sam 9 (1b)    GCCGAGTAGT  158
       Sam 2 (1b)    GCCGAGTAGT  159
       Sam 6 (1b)    GCCGAGTAGT  157
  EU255988(1a) USA   GCCGAGTAGT  159
       Sam 8 (1a)    GCCGAGTAGT  159
       Sam 5 (1b)    GCCKAGTAGT  158
       Sam 1 (4a)    GCCGAGTAGT  159
AB550028(4a) Egypt   GCCGAGTAGT  158
KC844043 (2a) China  GCCGAGTAGC  158
KC844042 (2f) China  GCCGAGTAGC  158
    Sam 7 (2a_2f)    GCCGAGTAGC  158
```